



Establishing reference values for age-related spermatogonial quantity in prepubertal human testes: a systematic review and meta-analysis

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Objective: To collect published data on spermatogonial quantity in the testes of healthy children and calculate the reference values of spermatogonial quantities throughout prepuberty.

Design: Systematic literature search in PubMed and EMBASE focusing on the number of spermatogonia per transverse tubular cross section (S/T) and spermatogonial density per cubic centimeter (cm³) of testicular volume (S/V) throughout prepuberty.

Setting: None.

Patient(s): None.

Intervention(s): None.

Main Outcome Measure(s): Polynomial meta-regression analyses of S/T and S/V of healthy boys from the ages of 0 to 14 years.

Result(s): We found six papers describing original quantitative data on S/T and S/V of healthy boys (total n = 334 and 62, respectively) that were suitable for meta-analysis. Polynomial meta-regression analyses of S/T and S/V demonstrated a clear pattern of spermatogonial quantity throughout prepubertal life. This consisted of a decline during the first 3 years of life, a gradual increase until the ages of 6 to 7 years, a plateau until the age of 11 years, and a sharp incline reaching pubertal numbers at 13 to 14 years of age. The association between S/T and S/V allowed us to perform S/T to S/V extrapolation, creating reference S/V (rS/V) values throughout prepubertal life from a cohort of 372 boys.

Conclusion(s): Spermatogonial quantity varies during testicular development toward puberty. The values found in this study may serve as a baseline clinical reference to study the impact of diseases and adverse effects of gonadotoxic treatments on spermatogonial quantity in prepubertal testes. Spermatogonial quantity reference values may also help to evaluate the quality of testicular biopsy samples acquired for fertility preservation of prepubertal boys. (Fertil Steril® 2016;106:1652–7. Copyright ©2016 The Authors. Published by Elsevier Inc. on behalf of the American Society for Reproductive Medicine. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)).

Key Words: Healthy boys, prepuberty, spermatogonial density, spermatogonial quantity, testicular development

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Spermatogonia are male germ cells located at the basal membrane of seminiferous tubules that give rise to functional spermatozoa. These early germ cells are present from birth onward, and spermatozoa are produced af-

ter puberty. The number of spermatogonia in prepubertal testes is influenced by the rate of proliferation, apoptosis, and differentiation into more advanced germ cells (1–3) as well as the growth rate of Sertoli and peritubular cells that determines the tubular length and total volume of the testis (1, 4–6). These physiologic processes can be disturbed by Klinefelter syndrome, cryptorchidism, genetic or endocrine disorders, and medical interventions like chemotherapy or irradiation and lead to partial or complete depletion of

Received May 18, 2016; revised August 18, 2016; accepted September 2, 2016; published online October 4, 2016.

I.M. has nothing to disclose. J.M.H. has nothing to disclose. K.J. has nothing to disclose. J.-B.S. has nothing to disclose. S.R. has nothing to disclose. F.v.d.V. has nothing to disclose. M.v.W. has nothing to disclose. A.M.M.v.P. has nothing to disclose.

I.M. and J.M.H. should be considered similar in author order

Supported by EU-FP7-PEOPLE-2013-ITN 603568: "Growsperm."

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Fertility and Sterility® Vol. 106, No. 7, December 2016 0015-0282

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<http://dx.doi.org/10.1016/j.fertnstert.2016.09.002>

spermatogonia including spermatogonial stem cells during childhood (7–13).

To assess the effects of these conditions and interventions on spermatogonial counts, reference values of spermatogonial quantity throughout prepubertal life of healthy boys need to be established. To date, only a few small cohort studies have reported data on spermatogonial count per age group throughout prepuberty. Although some studies did not describe any age-related changes in spermatogonial numbers, other studies did. Therefore, we pooled data on spermatogonial quantity in human prepubertal testes by a systematic literature search and polynomial meta-regression analyses to estimate spermatogonial density throughout childhood and provide baseline clinical reference values.

MATERIALS AND METHODS

Literature Search Strategy

We used PubMed and EMBASE electronic databases to search for articles on spermatogonial number in testes of healthy prepubertal boys (final update on April 8, 2015). In PubMed, we used queries for relevant keywords and medical subject headings (MeSH) to generate three subsets of references, where the first comprised “seminiferous tubules/anatomy and histology” AND “germ cells” OR “spermatogonia” AND “germ cells/cytology” OR “cell count” OR “sperm count,” the second of “apoptosis” AND “spermatozoa” OR “germ cells” OR “spermatogonia,” and the third of “cell proliferation” OR “proliferation” OR “cell division” AND “spermatozoa” OR “germ cells” OR “spermatogonia,” limiting all outputs by “species: humans,” “sex: male,” and “age: child from birth to 18 years.” Similarly, we searched for terms “testicular activity” OR “testis development” OR “ontogeny” OR “prepuberty” AND “germ cell” OR “spermatogonia” OR “spermatocyte” AND “proliferation” OR “apoptosis” in EMBASE using filters “human,” “male,” “child.” We used review papers and original research reports from this search to trace references of relevant primary data missing from the electronic search.

Study Selection and Data Extraction

We screened abstracts of the electronic search results to select developmental and quantitative reports on spermatogonia (comprising gonocytes, type A spermatogonia, and type B spermatogonia) in healthy prepubertal boys, including cases where data were reported for a control group, and excluded reports describing only spermatogonial counts of boys with (testicular) tumors, cryptorchidism, varicocele, or other health problems that might influence spermatogenesis. We summarized reported prepubertal spermatogonial cell counts per seminiferous tubular cross section (S/T) and spermatogonial numerical density per testicular tissue volume of 1 cm^3 (S/V) calculated using a stereometric counting grid by extracting data from quantitative studies. To estimate the common trend of S/T and S/V as a function of age, we pooled data from studies that specified a cohort size (n) and a range or standard deviation (SD) per each age group of their results and performed polynomial meta-regression analyses.

We excluded reports that did not specify the method of spermatogonial counting or that used correction factors to adjust germ cell counts for shrinkage, tubular diameter, or tubular shape. The study selection and the data extraction strategy are summarized in the PRISMA pipeline (Fig. 1) as described previously elsewhere (14).

Statistical Analysis

We used either smoothed fractional polynomial or least square fractional polynomial without smoothing together with a random effects model as appropriate to perform the meta-regression analysis (95% confidence interval [CI]) (15). To measure the heterogeneity between values reported in the studies, we performed I^2 statistics for both S/T and S/V (16). To build reference S/V (rS/V) values, we extrapolated S/T to S/V values by using correction factors 11 (for age group 0 to 4 years) and 16 (for age group 4 to 14 years). This strategy was chosen based on the previously described constant volume density of the tubular compartment and constant testicular volume in the respective age groups, as well as individual values within each of these age groups and the described S/T to S/V correlation (6, 17). As the polynomial meta-regression data represent an estimate of the number of spermatogonia throughout prepubertal development, we considered further statistical analyses between various ages within this developmental period to be inaccurate, so we presented these as a trend. Finally, to establish reference values for age-related spermatogonial quantity in prepuberty, we extracted regression fit and 95% CI boundary values from the S/T meta-analysis, as well as boundary values for the rS/V polynomial regression. The data analysis was performed using Stata/IC 14.0 (2015; StataCorp).

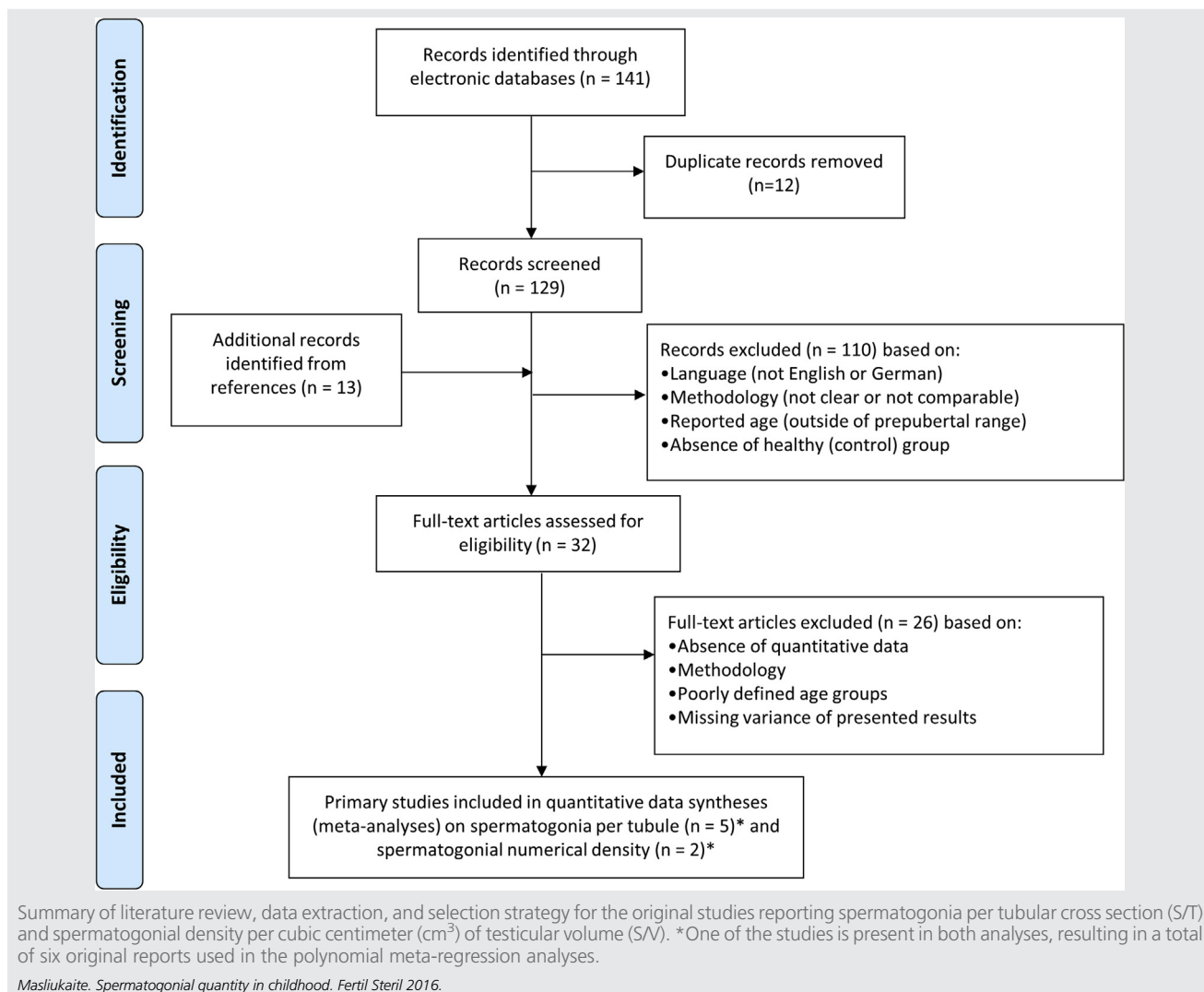
RESULTS

From a total of 141 abstracts, we screened 129 full, relevant, original studies and reviews with an additional 13 articles located from references (Fig. 1). After applying the inclusion criteria, 32 full-text articles were processed. We found nine studies describing original quantitative data (Supplemental Table 1, available online), six of which satisfied the inclusion criteria and were used for S/T (5, 17–20) and S/V (6, 17) polynomial meta-regression analyses, and three (21–23) that did not satisfy the inclusion criteria. In all the selected studies, the tissue processing methodologies included histology for S/T and stereology using counting grid for S/V assessment without correction for tissue shrinking.

Changes in Spermatogonia per Tubular Cross Section (S/T) during Prepuberty

In the five studies describing S/T of healthy prepubertal boys, we identified three patterns as a function of age (Supplemental Fig. 1, available online). [1] The first pattern showed a decline in S/T numbers during the first 2 to 4 years of life followed by a gradual increase toward puberty. [2] The second pattern described S/T as a plateau from the ages of 3 to 12 years, with a sharp increase during puberty. [3] The third pattern depicted a decrease in numbers during the first 3 years

FIGURE 1



of life followed by a gradual rise toward puberty with a light drop around the age of 8 years (5, 17–20).

The polynomial meta-regression analysis of the pooled S/T data from these studies (n = 334, I² = 12%) showed that S/T tends to decrease over the first 3 years of life from 2.5 to 1.2 (Fig. 2A), followed by a twofold increase until the peak of 2.6 at the age of 6 to 7 years is reached. Thereafter, there is a small decline in S/T numbers and a plateauing phase of 2.5 that lasts until the age of 11 years, which is followed by an accelerated increase reaching values of 7, marking the onset of puberty (Table 1).

Changes of Spermatogonial Density per Testicular Volume (S/V) during Prepuberty

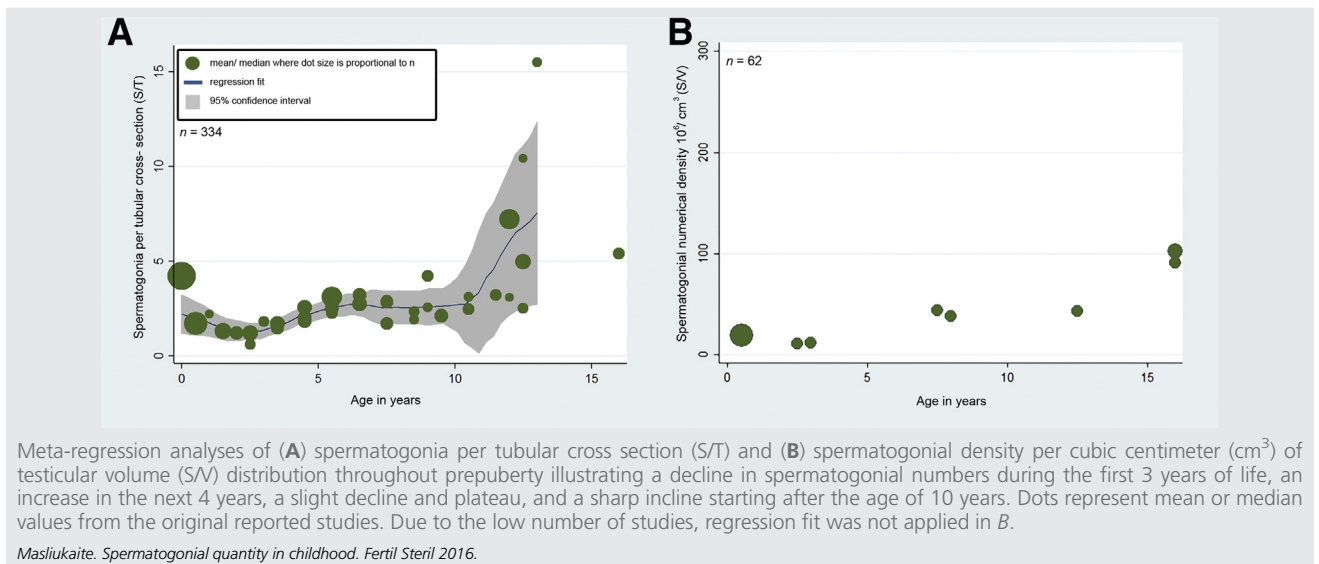
The two studies describing S/V of healthy boys showed a pattern of decrease in spermatogonial density in the first 3 years of life followed by an increase in numbers toward puberty (6, 17). The polynomial meta-regression analysis of the

pooled S/V data (n = 62) showed a twofold drop in spermatogonial density at the age of 3 to 4 years, an increase in values at the age of 7 years, and a sharp incline toward puberty starting at 11 to 12 years (see Fig. 2B).

The Reference S/V (rS/V) Values

Spermatogonial quantity in S/T and S/V revealed similar patterns throughout prepubertal life, as shown in Figure 2A and B. This allowed us to extrapolate S/T to S/V numbers (n = 310 boys). Together with the original S/V values, these extrapolated S/V values comprised a reference S/V (rS/V) data set with a cohort size of 372 boys. The rS/V trend corresponded to S/V polynomial meta-regression analysis results and showed a pattern of decline during the first 3 years of life, with rS/V from approximately 30×10^6 to 19×10^6 (Fig. 3), an increase until the peak of 48×10^6 at the age of 7, a phase of plateau of 45×10^6 until 11 years of age, and

FIGURE 2



a sharp incline reaching pubertal values of approximately 100×10^6 around the age of 13 to 14 (Table 1).

DISCUSSION

In this study we combined the literature data on S/T and S/V of healthy prepubertal boys in polynomial meta-regression analyses and performed S/T extrapolation, creating a reference S/V cohort (rS/V) of 372 boys. The analysis showed that both S/T and rS/V values had a trend of decrease over the first 3 years of life (2.5 to 1.2 and 30 to $19 \times 10^6/\text{cm}^3$, respectively), an increase until the peak at ages 6 to 7 years (2.6 and 48×10^6 , respectively), a plateau until the age of 11 years, and a sharp incline marking the onset of puberty (7 and approximately 100×10^6 , respectively).

Our study is the first to combine the available quantitative data on the number of spermatogonia in various age groups of healthy prepubertal boys. Using polynomial meta-regression analysis we provide the common reference S/T and S/V trends of a large cohort throughout prepubertal life.

A limitation of this study is the reliability of data obtained from a small number of old studies. Although we could not compare the S/T and rS/V values with spermatogonial quantities of freshly collected, healthy prepubertal testicular tissue for ethical reasons, we applied stringent screening of the published studies to ensure comparable high-quality spermatogonial quantification methods. The tissue-processing methodologies referenced in the selected studies included histologic (S/T) and stereologic (S/V) spermatogonial quantity assessments without correction for tissue shrinking, all methods still routinely used in clinical and research settings (24–26). The number of evaluated tubular cross sections in the selected studies was in a recommended range to achieve result validity (21). Furthermore, even though age-grouping strategies could not be unified, the measured heterogeneity implied that age-divided spermatogonial quantities in

individual studies could be confidently combined to provide a reliable reference estimate throughout prepuberty.

Another limitation of this study is that due to the low number of studies reporting S/V values we could not apply a regression fit for S/V meta-analysis (27). However, based on a previously described statistically significant ($P < .001$) positive correlation (17), we extrapolated our S/T values to rS/V from a total of 372 boys, allowing us to provide the regression fit with a wide confidence interval of 95%.

The pattern of spermatogonial quantity throughout prepuberty that we found can be explained by testicular growth that is influenced by endocrine activity. More specifically, the pattern

TABLE 1

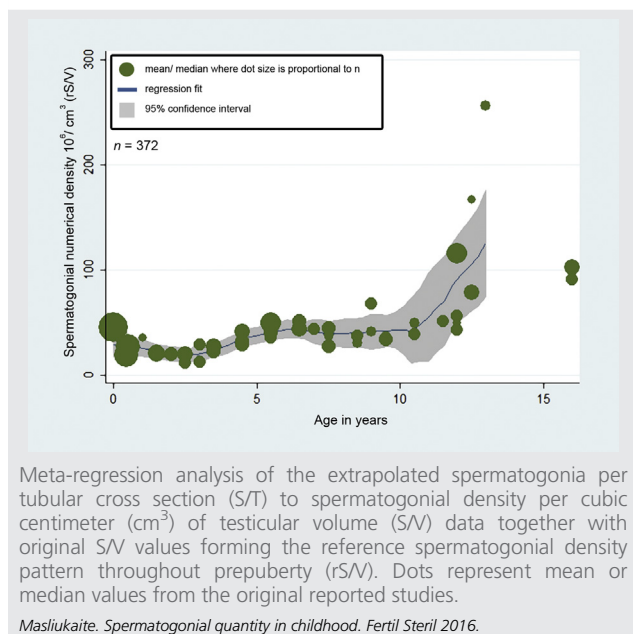
Clinical reference values to evaluate spermatogonial quantity in prepubertal testes.

Age (y)	S/T Regression fit (95% CI boundary)	rS/V (95% CI estimated boundary)
0	2.5 (2.3–3)	(20–40)
1	2.2 (1.8–2.6)	(15–35)
2	1.2 (1.0–1.4)	(15–25)
3	1.2 (0.9–1.4)	(14–24)
4	2.0 (1.7–2.3)	(20–35)
5	2.5 (2.1–2.9)	(38–50)
6	2.6 (2.2–3.0)	(40–54)
7	2.6 (2.2–3.1)	(40–56)
8	2.5 (1.9–3.1)	(35–55)
9	2.4 (1.8–3.1)	(35–55)
10	2.5 (1.9–3.1)	(35–55)
11	3.3 (1.0–5.6)	(30–100)
12	5 (2–8)	(45–120)
13	7 (2.5–11)	(80–180)

Note: Spermatogonial quantification performed by histology (S/T) or stereology (S/V) without correction for tissue shrinkage when standard fixatives (glutaraldehyde, Cleland's, Bouin's, or Stevie's) are used for tissue processing. CI = confidence interval in polynomial meta-regression analysis; rS/V = spermatogonial numerical density $10^6/\text{cm}^3$ testicular tissue; S/T = spermatogonia per tubular cross section.

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FIGURE 3



of a decrease in spermatogonial numbers during the first 3 years of life could be addressed by the fact that spermatogonial numbers consist of A spermatogonia and gonocytes, which are difficult to distinguish from each other (17, 28). The differentiation from gonocytes to spermatogonia during this period is influenced by the postnatal gonadotropin surge (29), where elevated luteinizing hormone (LH) stimulates Leydig cells to increase total plasma testosterone (30–34). Once LH levels drop, gonocytes that failed to reach the basal membrane degenerate, resulting in reduced numbers of spermatogonia (5, 34, 35) (Supplemental Fig. 2, available online). In addition, follicle-stimulating hormone (FSH) fuels rapid proliferation of immature Sertoli cells, causing the dispersion of spermatogonia across the elongating seminiferous tubules (4–6, 17, 29, 36–38).

After that, around the age of 4 years, the increase in the spermatogonial quantity up to the age of 6 to 7 years could be explained by higher spermatogonial proliferation and differentiation into B spermatogonia, primary spermatocytes (5% to 25% of tubules), and very occasional spermatids (2, 5, 18, 19, 23, 28, 32, 40). This proliferation and differentiation is induced by elevated FSH and LH, as well as increased inhibin B and testosterone secretion (32, 40, 41, 42) during that period. Although LH and FSH levels continue to rise between the ages of 7 and 10 years, immature Sertoli cells cannot support the differentiation of spermatogonia to functional spermatozoa, resulting in the plateau in spermatogonial numbers (5, 28, 32, 40, 41). We showed that S/T and rS/V display a progressive pattern of increase from the age of 11 years. This rapid spermatogonial proliferation is associated with increasing levels of gonadotropins (5, 32), the last wave of Sertoli cell proliferation followed by maturation (6, 18, 23, 35, 36), and enhanced germ cell differentiation, resulting in complete spermatogenesis (5, 35).

In conclusion, our study provides age-related reference values for spermatogonial quantity in the testes of healthy prepubertal boys. This can serve as a clinical tool to evaluate the effect of diseases and gonadotoxic therapies on the quality of prepubertal testicular tissue acquired for fertility preservation or pathologic analysis.

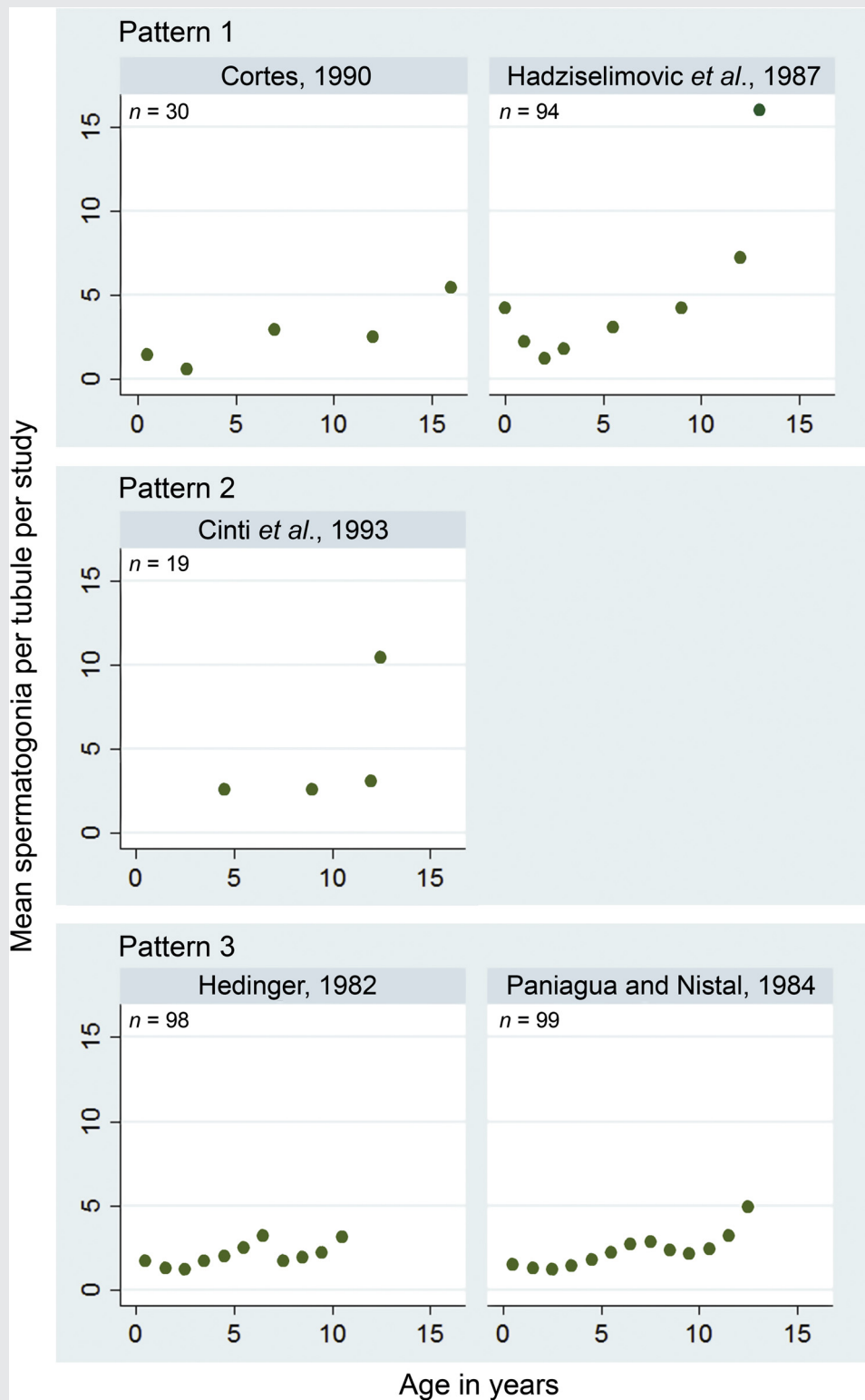
Acknowledgments: The authors thank Andreas Meissner (andrologist) and Annemieke A. de Melker (clinical embryologist) from the Center of Reproductive Medicine at the Academic Medical Center (Amsterdam, the Netherlands) for the critical reading of the manuscript.

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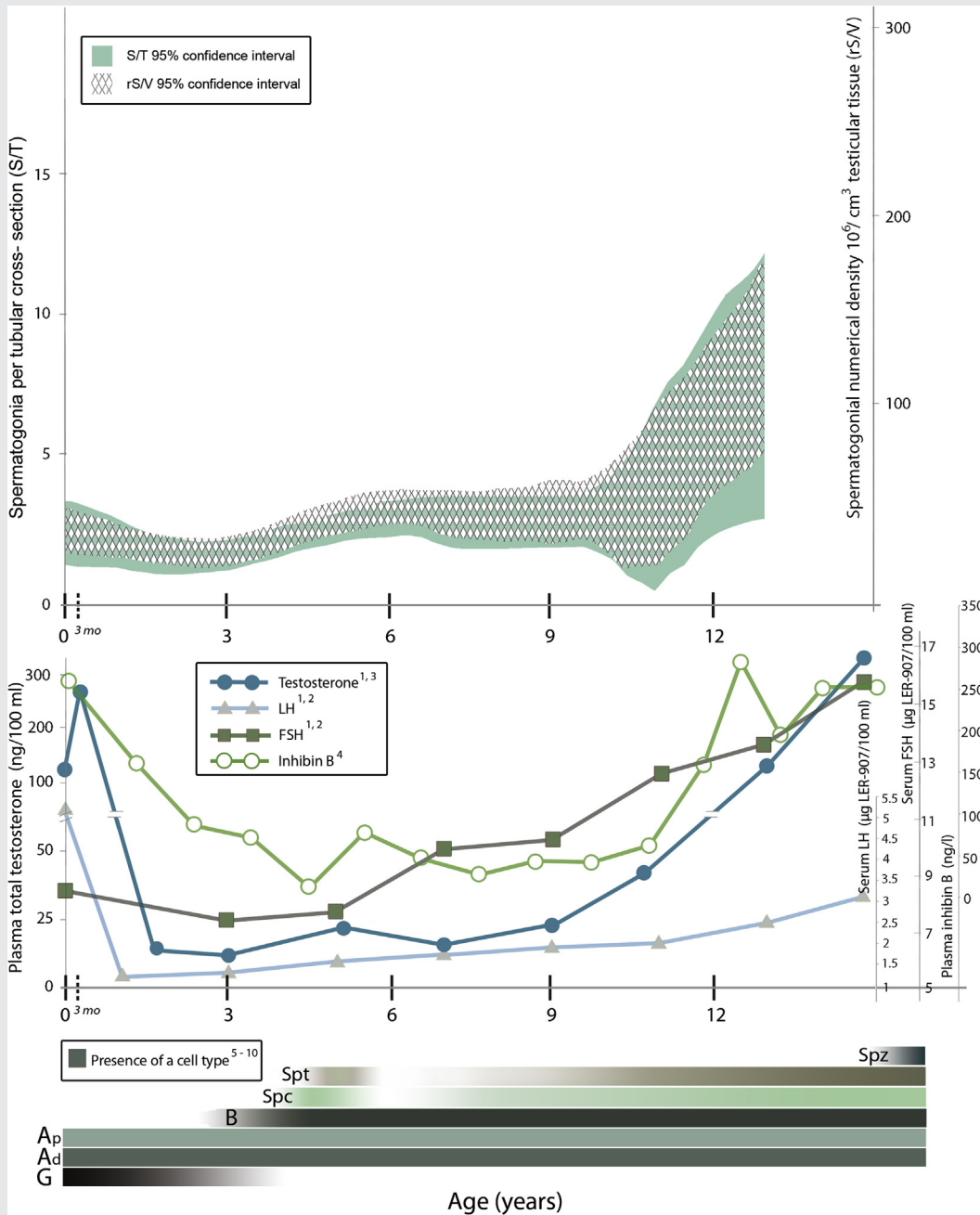
SUPPLEMENTAL FIGURE 1



Spermatogonia per tubular cross section (S/T) distribution from individual reports illustrating three S/T trends as a function of age. Dots represent mean or median values in the original reported studies: Cortes 1990 (17); Hadziselimovic et al. 1987 (19); Cinti et al. 1993 (18); Hedinger 1982 (20); Paniagua and Nistal 1984 (5).

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SUPPLEMENTAL FIGURE 2



Spermatogonia per tubular cross section (S/T) and spermatogonial numerical density per cm^3 (rS/V) as a function of age in relation to inhibin B, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels, as well as differentiation from gonocytes (G) and into more advanced germ cell types from A dark (Ad) and A pale (Ap) into type B spermatogonia (B), spermatocytes (Spc), spermatids (Spt), and spermatozoa (Spz), based on the previous reports: 1 (32), 2 (42), 3 (31), 4 (41), 5 (2), 6 (39), 7 (19), 8 (23), 9 (28), 10 (5).

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