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ORIGINAL ARTICLE ***Reproductive endocrinology***

Safety of standardised treatments for haematologic malignancies as regards to testicular endocrine function in children and teenagers

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STUDY QUESTION: Does standardised treatments used in children and adolescents with haematologic malignancies, including acute lymphoblastic (ALL) or myeloid leukaemia (AML) and non-Hodgkin lymphoma (NHL), affect endocrine function of the developing testes?

SUMMARY ANSWER: Therapy of haematologic malignancies do not provoke an overt damage of Sertoli and Leydig cell populations, as revealed by normal levels of anti-Müllerian hormone (AMH) and testosterone, but a mild primary testicular dysfunction may be observed, compensated by moderate gonadotropin elevation, during pubertal development.

WHAT IS KNOWN ALREADY: Evidence exists on the deleterious effect that chemotherapy and radiotherapy have on germ cells, and some attention has been given to the effects on Leydig and Sertoli cells of the adult gonads, but information is virtually non-existent on the effects of oncologic treatment on testicular somatic cell components during childhood and adolescence.

STUDY DESIGN, SIZE, DURATION: A retrospective, analytical, observational study included 97 boys with haematological malignancies followed at two tertiary paediatric public hospitals in Buenos Aires, Argentina, between 2002 and 2015.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Clinical records of males aged 1–18 years, referred with the diagnoses of ALL, AML or NHL for the assessment of gonadal function, were eligible. We assessed serum levels of AMH and FSH as biomarkers of Sertoli cell endocrine function and testosterone and LH as biomarkers of Leydig cell function.

MAIN RESULTS AND THE ROLE OF CHANCE: All hormone levels were normal in the large majority of patients until early pubertal development. From Tanner stage G3 onwards, while serum AMH and testosterone kept within the normal ranges, gonadotropins reached mildly to moderately elevated values in up to 35.9% of the cases, indicating a compensated Sertoli and/or Leydig cell dysfunction, which generally did not require hormone replacement therapy.

LIMITATIONS, REASONS FOR CAUTION: Serum inhibin B determination and semen analysis were not available for most patients; therefore, we could not conclude on potential fertility impairment or identify whether primary Sertoli cell dysfunction resulted in secondary depleted spermatogenesis or whether primary germ cell damage impacted Sertoli cell function.

WIDER IMPLICATIONS OF THE FINDINGS: The regimens used in the treatment of boys and adolescents with ALL, AML or NHL in the past two decades seem relatively safe for endocrine testicular function; nonetheless, a mild primary testicular endocrine dysfunction

may be observed, usually compensated by slightly elevated gonadotropin secretion by the pituitary in adolescents, and not requiring hormone replacement therapy. No clinically relevant risk factor, such as severity of the disease or treatment protocol, could be identified in association with the compensated endocrine dysfunction.

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Key words: anti-Müllerian hormone / chemotherapy / gonadotropins / hypogonadism / leukaemia / lymphoma / Sertoli cells / testosterone

Introduction

Haematologic malignancies account for almost 40% of paediatric cancers (Armstrong *et al.*, 2016), with an annual incidence of ∼30 cases per million children and adolescents <20 years of age (Hunger and Mullighan, 2015). Acute lymphoblastic leukaemia (ALL), the most prevalent paediatric malignancy, occurs more frequently in children of Latin/Hispanic ancestries compared with other ancestries in the United States (Lim *et al.*, 2014), with a slight predominance in males (Hunger and Mullighan, 2015).

The use of effective chemotherapy and radiotherapy for the management of childhood cancer has resulted in remarkable improvements in overall survival over the past decades. The 5-year survival rate for ALL, acute myeloid leukaemia (AML) and non-Hodgkin lymphoma (NHL) has increased to ∼90% following the development of intensive multi-drug, induction and consolidation regimens including intrathecal chemotherapy (Hunger and Mullighan, 2015). This marked improvement in therapy has led to a growing population of childhood cancer survivors, who are subject to long-term sequelae impacting on health issues, the most frequent being endocrine disorders (Crowne *et al.*, 2015). In particular, cancer treatment can affect reproductive function, and loss of fertility has become an important concern.

The adult testis produces spermatozoa and hormones. Spermatozoa arise from germ cells within the seminiferous tubules, whereas hormones are secreted by specific somatic cell populations: androgens by Leydig cells present in the interstitial tissue and inhibin B by Sertoli cells located within the seminiferous tubules, where they also give functional support to germ cells. In the prepubertal gonad, the germ cell population is represented exclusively by pre-meiotic spermatogonia that self-renew by mitosis, while immature Sertoli cells produce anti-Müllerian hormone (AMH) and inhibin B, and mature Leydig cells are absent owing to the lack of LH stimulation (Rey, 2014, Stukenborg *et al.*, 2018).

A large amount of evidence exists on the deleterious effect that chemotherapy agents and irradiation reaching the testes have on germ cells that have a high mitotic rate (reviewed by Jahnukainen and Stukenborg, 2012, Gebauer *et al.*, 2018 and Kesari *et al.*, 2018). Some attention has been focused on the effects of chemotherapy and radiotherapy on the Leydig (Brämswig *et al.*, 1990, Heikens *et al.*, 1996, Kenney *et al.*, 2012) and Sertoli (Heikens *et al.*, 1996, Bar-Shira Maymon *et al.*, 2004, Bordallo *et al.*, 2004, van Beek *et al.*, 2007, Lahteenmaki *et al.*, 2008, Cuny *et al.*, 2011, Laporte *et al.*, 2011) cell populations of the adult gonads and to late effects observed during adulthood in patients having received oncologic treatments during childhood. Leydig and Sertoli cell failure seems relatively rare after chemotherapy in the adult and involves a significantly higher irradiation dose than that required to cause germ cell damage. Conversely, information is virtually non-existent on the effects of cancer treatment on the somatic cell components of the prepubertal testis (reviewed by Stukenborg *et al.*, 2018 and Allen *et al.*, 2018).

Recent research on the effects of cancer treatment has focused on preventing damage. However, this is challenging for prepubertal males for whom fertility preservation is not possible given that they do not produce spermatozoa that can be cryopreserved. Some centres have started to cryopreserve spermatogonial stem cells, which could be transplanted back into the testes later in life and give rise to sperm (Stukenborg *et al.*, 2018). Nonetheless, normal function of the Sertoli and Leydig cells exposed to cancer treatment would be essential for the success of this therapeutic approach. Furthermore, endocrine testicular function is important for pubertal development, especially for the achievement of a normal growth spurt and bone mass accrual (Sklar *et al.*, 2018).

In this work, we aimed to determine whether the oncologic treatments used in prepubertal and pubertal boys with ALL, AML or NHL, the set of most frequent paediatric cancers, affect the endocrine function of the somatic component of the developing testis. In an observational study on a large cohort of patients treated before or after the age of 10 years, with long-term follow-up, we assessed serum levels of AMH and FSH as direct and indirect biomarkers respectively of Sertoli cell function, and testosterone and LH as direct and indirect biomarkers respectively of Leydig cell function.

Materials and methods

Study design and setting

We performed a retrospective, analytical, observational study at Ricardo Gutiérrez and Pedro de Elizalde Children's Hospitals, two tertiary paediatric public hospitals in Buenos Aires, Argentina. Clinical and laboratory data were extracted from the clinical charts.

The diagnoses of haematologic malignancies were made according to the third and fourth editions of the World Health Organisation Classification of Tumours of Haematopoietic and Lymphoid Tissues (Arber *et al.*, 2016). For patients with ALL, risk stratification and treatment were assigned according to the ALL IC-BFM 2002 intercontinental trial (Stary *et al.*, 2014). Briefly, three risk groups were defined: standard risk (SR), intermediate risk (IR) and high risk (HR), as described in Supplementary Fig. S1.

Patients

Inclusion criteria

All clinical records of males aged 1–18 years, referred with the diagnoses of ALL, AML or NHL from the Units of Haematology to the Divisions of Endocrinology of Ricardo Gutiérrez and Pedro de Elizalde Children's Hospital for the assessment of gonadal function between 2002 and 2015, were eligible.

Exclusion criteria

Clinical records were excluded from the analysis when the malignancy involved the central nervous system or the testes, in cases of trisomy 21 (Grinspon *et al.*, 2011) or in any other condition known to affect the hypothalamic–pituitary–testicular axis (Rey *et al.*, 2013), in order to avoid potential confusion due to known causes of central or primary hypogonadism.

Outcome measures and definitions

Two different analyses were performed. For the cross-sectional analysis, clinical records were included for patients with at least 1 year of follow-up after the end of chemotherapy and one endocrine assessment of testicular function (including serum levels of AMH, testosterone, FSH and LH) between 1 and 8 years after the end of chemotherapy. For the longitudinal study, all clinical records were included. For the analyses of serum hormones, patients were grouped according to pubertal stages as defined by Marshall and Tanner (1970). Owing to the retrospective design of the study, testicular volume, genital development and pubic hair staging were obtained from the history charts as reported by the patient's paediatric endocrinologist. Testicular volume was obtained by comparison with Prader's orchidometer, with the inter-individual variability inherent to the assessment method.

The main outcome measures of the study were the serum concentrations of AMH, testosterone, FSH and LH. Values were expressed in absolute levels and as standard deviation scores (SDS) based on age- and Tanner genital stage-matched reference ranges (see Supplementary Table SI), previously published by our laboratory (Grinspon *et al.*, 2011, Grinspon *et al.*, 2012b). Values of AMH and testosterone below −2 SDS were considered direct markers of affected testicular function. Values of FSH and LH above +2 SDS were considered indirect markers of affected testicular function. The use of SDS allowed the analysis of the whole cohort, whereas the use of absolute levels required a subgroup analysis by age and Tanner genital stage.

For the primary analysis, exposure measures, i.e. chemotherapy and radiotherapy, were analysed together and separately. Due to the retrospective design of our study, we did not have clinical Tanner staging data at the end of treatment, since patients were referred for endocrine assessment after the end of oncologic treatment. We intended to assess separately the effect of cancer treatment on boys who received the whole treatment at a prepubertal period, i.e. when the hypothalamic–pituitary–testicular axis is mainly quiescent (low mitotic index for all cell types), and on boys who were more likely exposed to a reactivated axis (characterised by a higher mitotic index in testicular cells). The classical clinical signs of pubertal onset, i.e. testicular volume and Tanner stages, were not used for categorising patients at the time of the end of treatment because cancer treatment could affect testicular size and pubertal development (Crowne *et al.*, 2015). Like other authors (Romerius *et al.*, 2011), we chose to use an age cut-off of 10 years based on population studies indicating that in boys <10 years of age the likelihood of having started pubertal development is <5% (Tomova *et al.*, 2010) and the classical Tanner study indicating that at 9.5 years <2.5% of boys have reached G2 (Marshall and Tanner, 1970). Therefore, two subgroups were considered, according to the age at which chemotherapy or radiotherapy ended: <10 years old (Group A) and ≥10 years old (Group B).

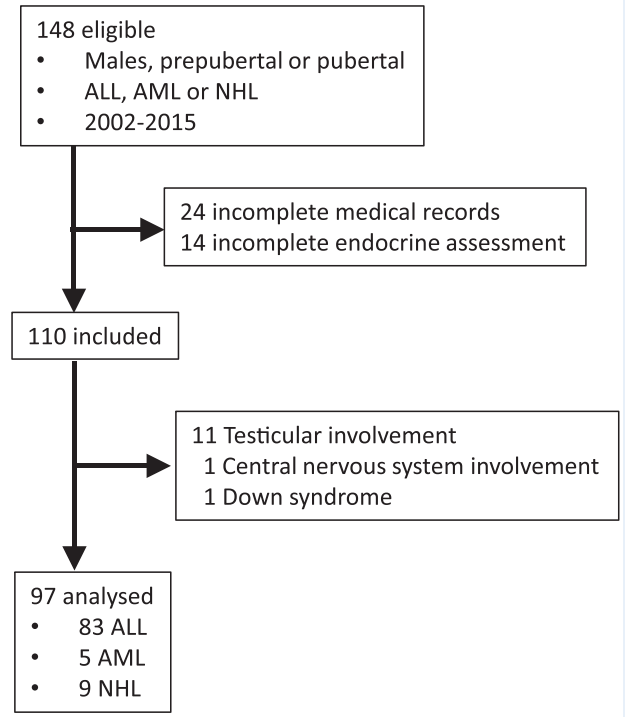

Figure 1 Flow chart of patient inclusion in the study.

Hormone assays

Hormonal values were extracted from the clinical charts and the laboratory Cobas® Infinity system (Roche). The AMH assay for all samples (Ricardo Gutiérrez and Pedro de Elizalde Children's Hospitals) was centralised in the Ricardo Gutiérrez laboratory. Gonadotropin and testosterone measurements were performed in both hospitals, using the same methods.

AMH

Serum AMH was determined using an enzyme-linked immunoassay specific for human AMH (EIA AMH/MIS®, Beckman-Coulter Co., Marseilles, France), as previously validated by our group (Grinspon *et al.*, 2011, Grinspon *et al.*, 2012b). Intra- and inter-assay coefficients of variation were, respectively, 10.5% and 9.4% for a serum AMH concentration of 700 pmol/l, and 11.1% and 12.8% for a serum AMH concentration of 7 pmol/l. When serum AMH levels were undetectable, a value of 1 pmol/l, corresponding to the limit of quantification (functional sensitivity), was attributed.

Table I Characteristics of the whole study sample.

		Whole sample (n = 97)	Acute Lymphoid Leukaemia (n = 83)	Acute Myeloid Leukaemia (n = 5)	Non-Hodgkin Lymphoma (n = 9)
Classification		-	Pre-B: 4 B (common): 48 Pro-B: 6 T: 8 Non-typified: 17	M3: 3 M4: 1 M5: 1	Lymphoblastic: 3 Burkitt: 4 Diffuse large B-cell: 2
Risk	Group A		Standard: 27 Intermediate: 20 High: 4	-	-
	Group B		Standard: 0 Intermediate: 28 High: 4		
N					
	Group A	58	51	4	3
	Group B	39	32	1	6
Age at diagnosis, yr*					
	All	5.5 (1.1–16.6)	5.2 (1.8–16.6)	4.0 (1.1–9.4)	10.6 (4.7–15.4)
	Group A	3.9 (1.1–7.1)	3.7 (1.8–7.1)	3.4 (1.1–5.0)	6.4 (4.7–6.9)
	Group B	12.1 (8.1–16.6)	12.0 (8.1–16.6)	9.4	12.5 (8.2–15.4)
Age at end of chemotherapy, yr*					
	All	7.7 (2.6–18.6)	7.3 (3.8–18.6)	6.9 (2.6–11.6)	11.1 (5.2–17.4)
	Group A	6.1 (2.6–9.3)	6.0 (3.8–9.3)	5.8 (2.6–8.2)	7.2 (5.2–8.7)
	Group B	13.5 (10.3–18.6)	14.0 (10.3–18.6)	11.6	12.8 (10.6–17.4)
Elapsed time, end of chemotherapy-first endocrine assessment, yr*					
	All	1.1 (0.1–12.9)	0.9 (0.1–12.9)	3.1 (1.8–5.3)	1.1 (0.2–10.4)
	Group A	1.9 (0.1–12.9)	1.6 (0.1–12.9)	2.5 (1.8–5.3)	1.1 (1.1–8.6)
	Group B	0.8 (0.1–11.0)	0.8 (0.1–6.9)	3.9	1.5 (0.2–10.4)
Elapsed time, end of chemotherapy-last endocrine assessment, yr*					
	All	3.1 (0.2–12.9)	2.8 (0.1–12.9)	3.3 (1.9–9.8)	4.8 (0.2–12.7)
	Group A	4.3 (0.1–12.9)	4.0 (0.1–12.9)	3.2 (1.9–9.8)	5.5 (4.8–12.7)
	Group B	2.5 (0.1–12.0)	2.4 (0.1–6.9)	3.9	3.5 (0.2–12.0)

Group A: patients in whom chemotherapy ended before the age of 10 years.
Group B: patients in whom chemotherapy ended at age 10 years or more.
*Values represent median (range).

Gonadotropins

LH and FSH were determined using electro-chemiluminescent immunoassays (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany) as described (Grinspon *et al.*, 2012b). The limits of quantification of both LH and FSH assays were 0.10 IU/l, according to the 2nd National Institute for Biological Standards and Control International Standard 80/552 for LH and the 2nd World Health Organisation International Reference Preparation 78/549 for FSH. Intra- and inter-assay coefficients of variation were 1.1% and 1.8% for LH, respectively, for a mean LH concentration of 2.8 IU/l and 1.4% and 1.5% for a mean LH concentration of 16.9 IU/l. Intra- and inter-assay coefficients of variation were 1.0% and 4.2% for FSH, respectively, for a mean FSH concentration of 14.8 IU/l and 1.1% and 4.1% for a mean FSH concentration of 23.4 IU/l. When serum LH or FSH levels were undetectable, the value of the limit of quantification (functional sensitivity) was attributed.

Testosterone

Testosterone was determined in serum using an electro-chemiluminescent immunoassay (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany) as described (Grinspon *et al.*, 2011). Intra- and inter-assay coefficients of variation were 2.4% and 2.6%, respectively, for a mean testosterone concentration of 176 ng/dl (6.10 nmol/l) and 1.2% and 2.3% for a mean testosterone concentration of 455 ng/dl (15.78 nmol/l). When serum testosterone levels were undetectable, a value of 10 ng/dl (0.347 nmol/l), corresponding to the limit of quantification (functional sensitivity), was attributed.

Statistical analyses

Data distribution was assessed for normality using the Shapiro–Wilk test. Results are expressed as median and range. Because non-Gaussian distribution was found in most cases, nonparametric tests were used

Table II Characteristics of the study sample included in the cross-sectional analysis.

		Whole sample (n = 61)	Acute Lymphoid Leukaemia (n = 50)	Acute Myeloid Leukaemia (n = 5)	Non-Hodgkin Lymphoma (n = 6)
Classification		-	Pre-B: 3 B (common): 32 Pro-B: 4 Early B: 1 T: 4 Non-typified: 6	M3: 3 M4: 1 M5: 1	Burkitt: 4 Diffuse large B-cell: 2
Risk	Group A		Standard: 19 Intermediate: 10 High: 2	-	-
	Group B		Standard: 0 Intermediate: 17 High: 2		
N					
	Group A	37	31	4	2
	Group B	24	19	1	4
Age at diagnosis, yr*					
	All	5.8 (1.1–16.6)	4.0 (2.0–16.6)	4.0 (1.1–9.4)	11.4 (4.7–14.7)
	Group A	3.7 (1.1–7.1)	3.7 (2.0–7.1)	3.4 (1.1–5.0)	5.8 (4.7–6.9)
	Group B	10.6 (8.1–16.6)	10.6 (8.1–16.6)	9.4	12.5 (10.6–14.7)
Age at end of chemotherapy, yr*					
	All	8.0 (2.6–18.6)	8.0 (4.0–18.6)	6.9 (2.9–11.6)	11.7 (5.2–15.0)
	Group A	5.9 (2.6–9.3)	5.9 (4.0–9.3)	5.8 (2.6–8.2)	6.2 (5.2–7.2)
	Group B	12.7 (10.3–18.6)	12.7 (10.3–18.6)	11.6	12.8 (11.1–15.0)
Age at endocrine assessment, yr*					
	All	11.7 (5.7–20.1)	11.5 (6.0–20.1)	10.0 (5.7–15.5)	14.1 (8.3–16.0)
	Group A	9.4 (5.7–14.9)	9.4 (6.0–14.9)	8.3 (5.7–12.2)	11.0 (8.3–13.8)
	Group B	15.3 (11.6–20.1)	15.5 (11.6–20.1)	15.5	14.8 (13.1–16.0)
Elapsed time, end of chemotherapy-endocrine assessment, yr*					
	All	2.8 (1.0–8.6)	2.8 (1.0–7.6)	3.1 (1.8–5.3)	1.9 (1.1–8.6)
	Group A	3.1 (1.1–8.6)	3.1 (1.3–7.6)	2.5 (1.8–5.3)	4.8 (1.1–8.6)
	Group B	2.2 (1.0–7.0)	2.5 (1.0–7.0)	3.9	1.9 (1.1–2.8)

Group A: patients in whom chemotherapy ended before the age of 10 years.
Group B: patients in whom chemotherapy ended at age 10 years or more.
*Values represent median (range).

for comparisons. Fisher's exact test was used to compare categorical variables. To assess the existence of association between chemotherapy agent cumulative doses and gonadal dysfunction, a logistic regression model was used, considering cumulative doses as continuous variables and gonadal dysfunction as dichotomised (FSH or LH $>+2$ SDS considered as gonadal dysfunction; $\leq+2$ SDS considered as normal). Odds ratios and 95% confidence intervals for the final model were reported. The level of significance was set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism version 8.02 for Windows (GraphPad Software, San Diego, CA, USA) and STATA 13 (StataCorp LLC, College Station, TX, USA).

Ethical issues

Research was conducted in accordance with principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of the Ricardo Gutiérrez and Pedro de Elizalde Children's Hospitals, Buenos Aires. Because the study was based on a retrospective clinical chart review with descriptive purposes and no anticipated effect on prognosis or therapeutic management of the patients whose charts were included, the need for a written informed consent was waived.

Results

Characteristics of the study sample

Our database contained 148 eligible males with a diagnosis of ALL, AML or NHL, endocrinologically assessed between 2002 and 2015 (Fig. 1). Due to incomplete medical records or endocrine assessment, 38 could not be included and 13 patients were excluded because they were highly likely to have hypothalamic–pituitary–testicular involvement prior to the initiation of treatment for ALL, AML or NHL: 11 with testicular and 1 with central nervous system involvement of

Table III **Treatments of the patients included in the study, according to the age at end of treatment.**

	Whole cohort		ALL - Standard Risk		ALL - Intermediate Risk		ALL - High Risk		ALL - Bone Marrow Transplantation *		NHL		AML	
Age Group (end of treatment)	**A (<10 yr)**	**B (≥10 yr)**	**A (<10 yr)**	**B (≥10 yr)**	**A (<10 yr)**	**B (≥10 yr)**	**A (<10 yr)**	**B (≥10 yr)**	**A (<10 yr)**	**B (≥10 yr)**	**A (<10 yr)**	**B (≥10 yr)**	**A (<10 yr)**	**B (≥10 yr)**
n	58	39	26	0	19	26	4	2	2	4	3	6	4	1
Cranial radiotherapy, Gy	13.8 ± 2.9 (n:7)	13.6 ± 2.7 (n:12)	(n:0)	(n:0)	12.3 ± 0.4 (n:2)	13.2 ± 2.7 (n:5)	14.0 ± 3.5 (n:3)	15.3 ± 3.8 (n:2)	12.0(n:1) (n:1)	14.0 ± 3.5 (n:3)	18.0 (n:1)	(n:2)	(n:0)	(n:0)
Chemotherapy (cumulative)														
Cyclophosphamide (g/m^2)	2.92 ± 0.63	3.25 ± 0.75	2.71 ± 0.47	-	2.96 ± 0.55	3.07 ± 0.46	4.17 ± 1.22	3.99 ± 1.40	3.00 ± 0.00	4.66 ± 0.42	2.98 ± 0.00	2.30 ± 0.42	3.00 ± 0.00	3.00
Ifosfamide (g/m^2)	7.05 ± 2.48	4.87 ± 3.11	-	-	-	-	8.11 ± 0.09	7.87 ± 0.18	5.00 ± 4.24-	5.02 ± 1.99	8.00 ± 0.00	3.60 ± 3.40	-	-
Methotrexate IV (g/m^2)	13.16 ± 16.70	13.99 ± 7.04	8.56 ± 3.16	-	10.43 ± 5.24	11.96 ± 6.30	16.04 ± 6.97	20.70 ± 0.99	81.40 ± 42.43	17.91 ± 5.85	20.00 ± 0.00	19.33 ± 11.02	-	21.40
Methotrexate oral (g/m^2)	1.44 ± 0.72	1.39 ± 0.62	1.80 ± 0.81	-	1.32 ± 0.36	1.44 ± 0.62	1.13 ± 0.25	1.40 (n:1)	-	1.12 ± 0.53	1.31 ± 0.00	1.06 ± 0.12		-
6-Mercaptopurine (g/m^2)	28.67 ± 12.79	2 5.87 ± 8.24	31.95 ± 16.74	-	27.41 ± 7.26	26.18 ± 7.19	20.49 ± 3.78	25.35 ± 4.03	22.50 ± 0.00	17.67 ± 13.16	24.00 ± 0.00	24.17 ± 3.59	22.50 ± 0.00	22.50
6-Thioguanine (g/m^2)	1.13 ± 0.73	1.39 ± 2.22	1.09 ± 0.46	-	1.57 ± 0.85	1.50 ± 0.90	0.78 ± 0.18	0.68 ± 0.00	-	1.94 ± 1.28	0.52 ± 0.00	0.82 (n:1)	-	-
C-Arabinoside (g/m^2)	1.90 ± 0.65	2.22 ± 1.03	1.75 ± 0.44	-	2.07 ± 0.65	2.03 ± 0.76	2.41 ± 0.33	2.18 ± 0.57	2.70 ± 0.00	3.66 ± 1.59	2.46 ± 0.00	2.20 ± 1.25	0.30 ± 0.00	1.20
Doxorubicin (g/m^2)	124.9 ± 21.5	130.9 ± 37.8	117.8 ± 13.12	-	135.7 ± 30.49	133.1 ± 25.48	130.2 ± 11.40	140.0 ± 14.14	120.0 ± 0.00	137.0 ± 32.20	118.2 ± 0.00	117.6 (n:1)	120.0 ± 0.00	120.0
Daunorubicin (g/m^2)	132.9 ± 103.4	160.3 ± 91.0	93.74 ± 39.28	-	128.4 ± 19.84	128.6 ± 16.74	205.5 ± 36.06	168.0 ± 11.31	460.0 ± 424.3	215.0 ± 77.60	178.5 ± 0.00	344.0 ± 240.7	160.0 ± 0.00	160.0
Vincristine (mg/m^2)	17.99 ± 7.57	16.53 ± 6.85	15.89 ± 5.65	-	16.21 ± 3.21	14.26 ± 3.38	26.93 ± 5.51	24.75 ± 3.18	39.0 ± 14.85	26.01 ± 13.58	32.16 ± 0.00	17.99 ± 8.50	15.00 ± 0.00	15.00
L-Asparaginase (U/m^2)	110 786 ± 61 492	128 380 ± 82 554	87 371 ± 29 066	-	105 695 ± 37 694	95 783 ± 31 447	173 632 ± 102 726	254 652 ± 7564	280 000 ± 70 711	216 663 ± 157 066	265 375 ± 0	187 347 ± 166 386	80 000 ± 0	80 000
Etoposide (mg/m^2)	991 ± 44.7	851.4 ± 233.6	-	-	-	620 (n:1)	1015 ± 48.76	1028 ± 39.60	-	750.0 ± 288.7	1000 ± 0.00	817.5 ± 307.6	950.0 ± 0.00 0.00	-
Dexamethasone (g/m^2)	0.36 ± 0.38	0.35 ± 0.29	0.22 ± 0.03	-	0.37 ± 0.53	0.22 ± 0.08	0.81 ± 0.01	0.53 ± 0.46	0.91 ± 0.42	0.88 ± 0.32	0.81 ± 0	0.67 ± 0.50	0.21 ± 0	0.21
Meprednisone (g/m^2)	2.78 ± 1.23	2.66 ± 1.18	2.86 ± 1.39	-	2.80 ± 1.11	2.60 ± 1.05	2.41 ± 1.18	1.79 ± 0.01	1.80 ± 0	2.66 ± 2.09	3.51 ± 0	3.54 ± 0.51	1.80 ± 0	1.80
Pegaspargase (IU/m^2)	2006 ± 1549	1505 ± 833.3	1679 ± 588.4	-	1000 ± (n:2)	1600 ± 894.4	5000 (n = 1)	-	-	8598 ± 10 746	-	1029 (n:1)	-	-
Erwinase (IU/m^2)	60 000 (n:1)	60 000 (n:1)	-	-	-	-	60 000 (n = 1)	-	-	60 000 (n = 1)	-	-	-	-
Idaurubicin (mg/m^2)	50.0 ± 0 (n:2) (n:2)	51.0 (n:1)	-	-	-	-	-	-	-	-	-	-	50.0 ± 0 (n:2) (n:2)	51.0 (n:1) (n:1)
Mitoxantrone (mg/m^2)	40.0 ± (n:2)	20.0 (n:1)	-	-	-	-	-	-	-	-	-	-	40.0 ± 0 (n:2)	20.0 (n:1)

ALL: Acute Lymphoblastic Leukaemia; AML: Acute Myeloid Leukaemia; NHL: Non-Hodgkin Lymphoma.
*These patients also received total body irradiation (9–12 Gy) for bone marrow transplantation conditioning.
Values represent mean ± SD.

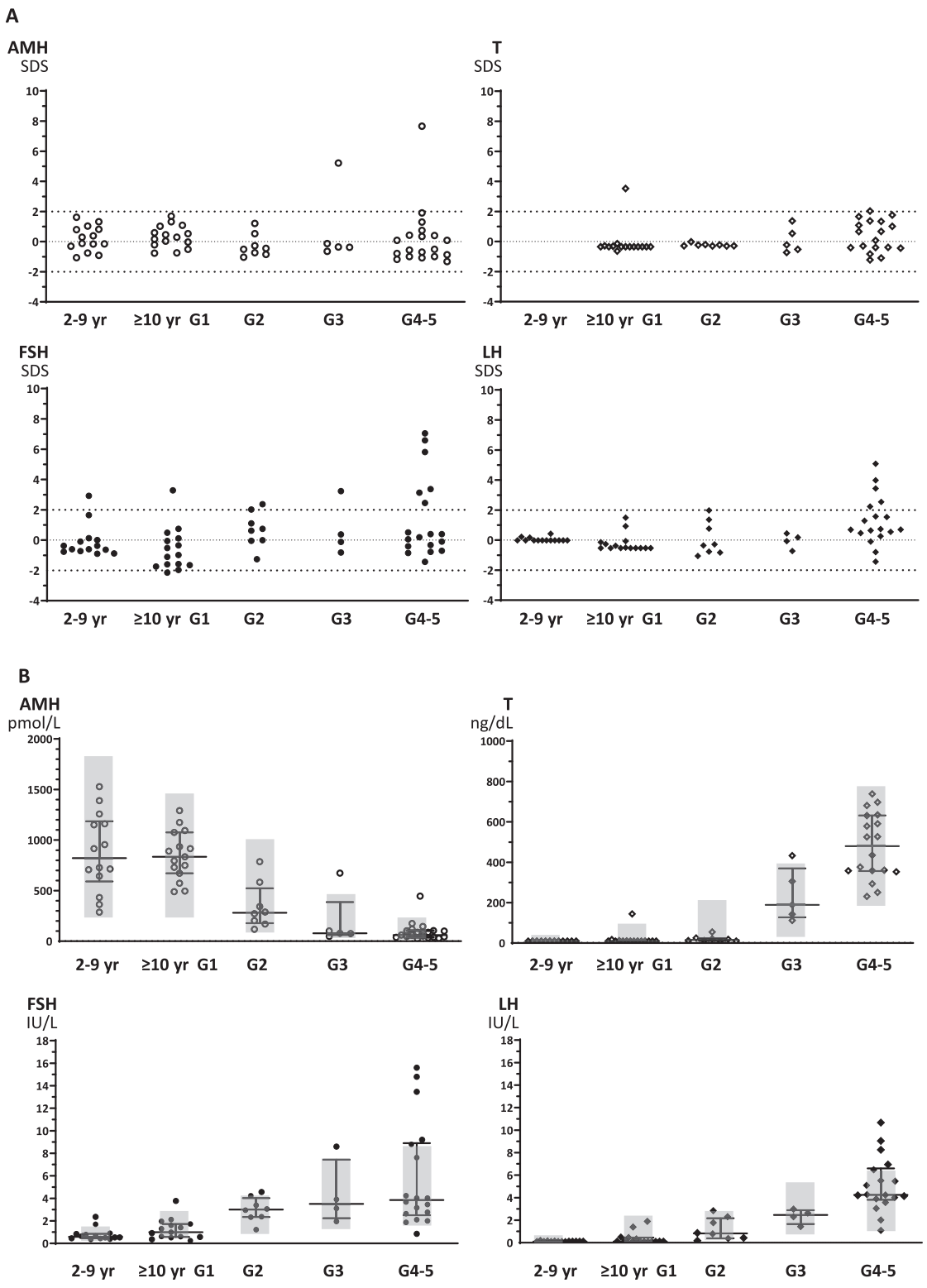

Figure 2 Serum levels of AMH, testosterone and gonadotropins in the 61 boys with ALL, AML or NHL included in the cross-sectional study. Patients are grouped according to age or pubertal Tanner stage at the moment of hormone determination. One value per patient obtained no earlier than 1 year after the end of chemotherapy. (**A**) Values expressed as SDS for age and Tanner genital stage. (**B**) Absolute values; the grey areas represent normal reference levels for age and Tanner genital stage. Both A and B are according to Grinspon *et al.*, 2011. ALL: acute lymphoblastic leukaemia; AML: acute myeloid leukaemia; NHL: non-Hodgkin lymphoma.

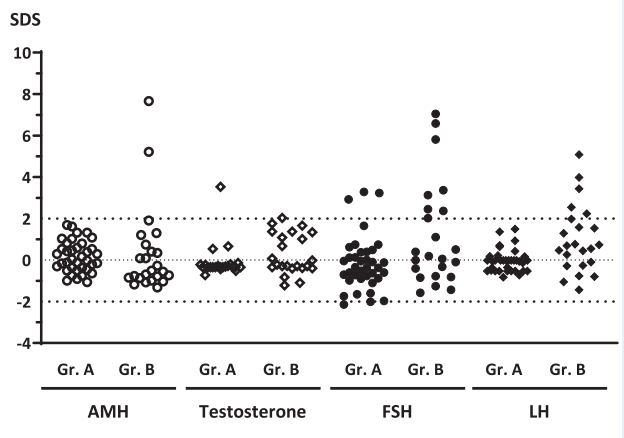

Figure 3 Serum levels of AMH, testosterone and gonadotropins, expressed as SDS for age in the 61 boys with ALL, AML or NHL included in the cross-sectional study. Values are expressed as SDS for age (see Supplementary Table SII). One value per patient obtained no earlier than 1 year after the end of chemotherapy. Group A: patients in whom chemotherapy ended before the age of 10 years. Group B: patients in whom chemotherapy ended at age 10 years or more. Dotted lines represent 0, +2 and −2 SDS for age and Tanner genital stage. ALL: acute lymphoblastic leukaemia; AML: acute myeloid leukaemia; NHL: non-Hodgkin lymphoma.

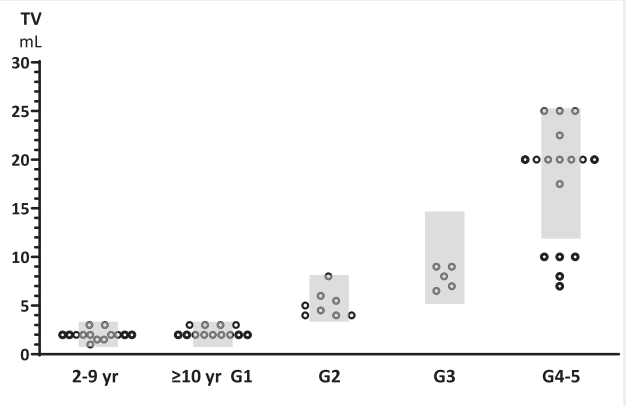

Figure 4 Mean bilateral testicular volume in the 61 boys with ALL, AML or NHL included in the cross-sectional study. Patients are grouped according to age or pubertal Tanner stage at the moment of clinical assessment of testicular volume. One value per patient obtained no earlier than 1 year after the end of chemotherapy. The grey areas represent normal reference values, according to (Grinspon *et al.*, 2011). ALL: acute lymphoblastic leukaemia; AML: acute myeloid leukaemia; NHL: non-Hodgkin lymphoma.

the malignant disease and 1 patient with trisomy 21 (Grinspon *et al.*, 2011). Therefore, 97 paediatric male patients, i.e. 65.5% of those in our database, were analysed: 83 with ALL, 5 with AML and 9 with NHL. As expected, common B-cell ALL was the most prevalent form. Patients classified with SR and IR, and thus receiving a similar treatment protocol, were the vast majority: they represented 90% of patients with ALL and 77% of all patients in the study (Table I). Regarding hospital origin, 76 patients were recruited at Ricardo Gutiérrez Children's Hospital (65 ALL, 2 AML and 9 NHL) and 21 at Pedro de Elizalde Children's Hospital (18 ALL, 3 AML).

As expected, patients with ALL and AML were slightly younger at diagnosis and at end of chemotherapy than those with NHL. Most patients with ALL (61.4%) or with AML (80%), whereas only 33.3% of patients with NHL, were younger than 10 years at the end of treatment (Group A). The age at endocrine assessment for the cross-sectional part of the study was also lower in patients with ALL and AML: 38.0% and 40.0% respectively were still younger than 10 years when hormonal studies were performed after at least 1 year of follow-up after the end of the last chemotherapy phase (Table II). Bone marrow transplantation (BMT) preconditioning, including total body irradiation (12–18 Gy), was performed in six cases of ALL, and radiotherapy (12–24 Gy) was performed in 11 cases of ALL and 3 of AML (Table III and Supplementary Table II). The cumulative doses of chemotherapy agents are described in Table III: patients with HR ALL and with AML or NHL received ~2-fold higher cumulative doses of IV methotrexate and 10-fold higher cumulative doses of C-arabinoside, and were the only ones who received ifosfamide and etoposide. Pegasparagase and erwinase were rarely used, and idarubicin and mitoxantrone were used exclusively in patients with AML.

Three patients received levothyroxine supplementation due to hypothyroidism, and one patient received treatment with growth hormone.

Cross-sectional study

There were 61 patients, with at least 1 year of follow-up after the end of chemotherapy and one endocrine assessment of testicular function between 1 and 8 years after the end of chemotherapy, included in the cross-sectional analysis (Table II). Laboratory results from the first assessment available in that period were used. Because serum values of hormones of the gonadal axis vary with age in normal boys during infancy and childhood, values were analysed using SDS for age and pubertal stage groups in the overall evaluation. In the age and pubertal stage subgroup analyses, absolute serum levels and SDS were used.

When the analysis was performed according to the age and Tanner pubertal stage at endocrine assessment, FSH and/or LH elevation was observed in 13 patients (Fig. 2). Gonadotropin elevation was mild to moderate and was noticed mainly in the older groups (Tanner stages 4–5). Eleven of them had suffered from ALL (three SR and eight IR) and two had suffered from NHL (Burkitt). Only two of these patients with gonadotropin elevation had required total body irradiation and BMT.

Serum levels of AMH, a direct Sertoli cell biomarker, were within the normal levels in all 37 patients treated for ALL, AML or NHL before the age of 10 years (Group A) (Figs 2 and 3). FSH was also normal in the vast majority, and only slightly above the reference range for age in three cases (Fig. 3). Two of them were already >10 years old when hormone levels (FSH 3.8 and 8.6 IU/l) were first assessed at least 1 year after the end of chemotherapy and had testosterone levels (12 and 143 ng/dl, respectively) compatible with pubertal onset (Fig. 2). All three had a diagnosis of SR ALL, and none of them had received radiotherapy or BMT preconditioning.

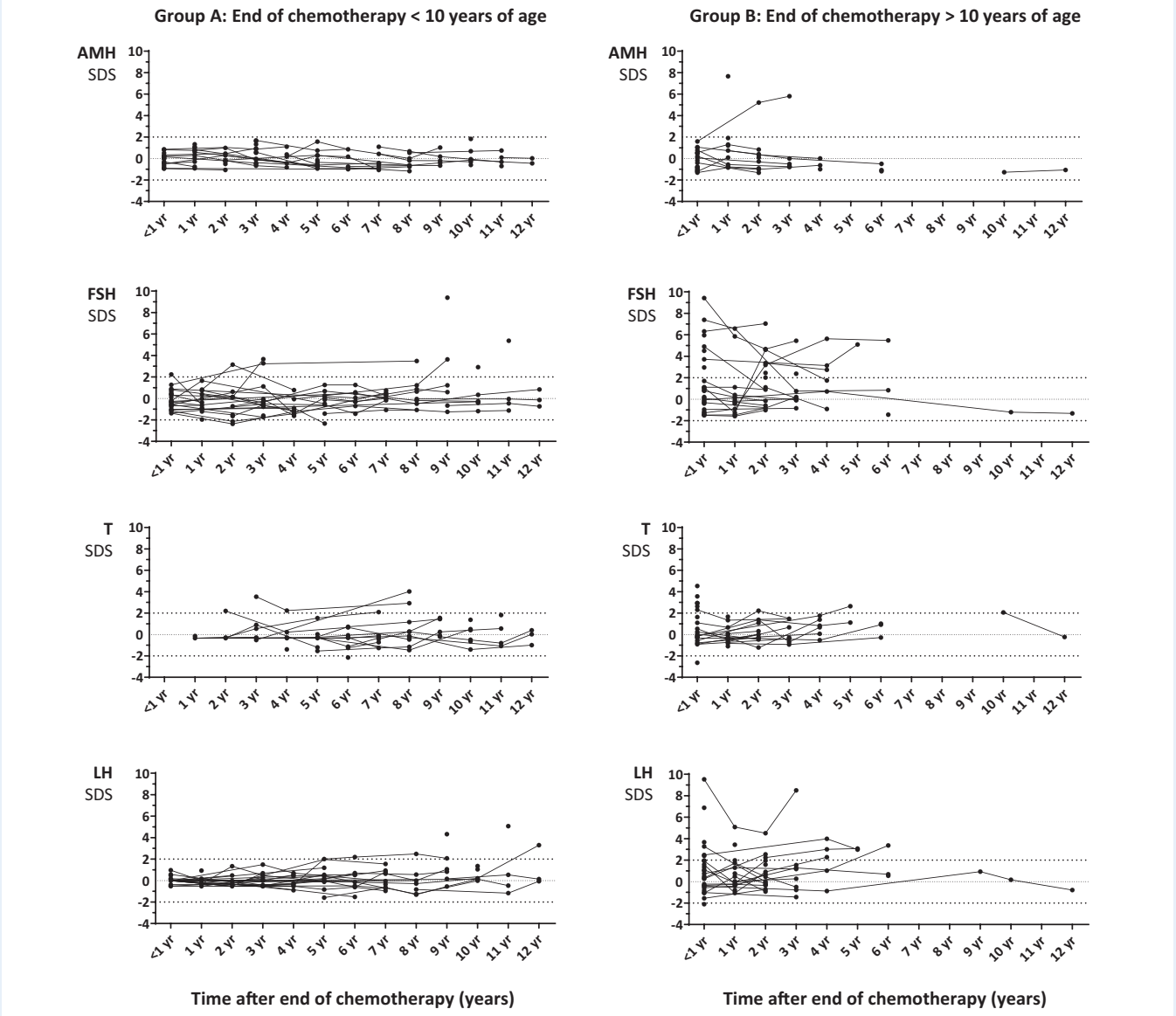

Figure 5 Progression with time after the end of chemotherapy of serum levels of AMH, testosterone and gonadotropins, expressed as SDS for age and Tanner genital stage in the 97 boys with ALL, AML or NHL included in the longitudinal follow-up study. Values are expressed as SDS for age and Tanner genital score, according to Grinspon *et al.*, 2011. Group A: patients in whom chemotherapy ended before the age of 10 years. Group B: patients in whom chemotherapy ended at age 10 years or more. ALL: acute lymphoblastic leukaemia; AML: acute myeloid leukaemia; NHL: non-Hodgkin lymphoma.

Serum levels of testosterone and LH, reflecting Leydig cell function, were within the normal levels in all patients treated for ALL, AML or NHL before the age of 10 years (Group A). However, one patient was first assessed at the age of 11.7 years: since testicular volume was 2 ml bilaterally, he was clinically classified as G1, yet hormonal values were already typical of G2 (LH 1.9 IU/l, FSH 0.9 IU/l, testosterone 144 ng/dl). Testicular volume was slightly below normal (10 ml) in only one patient of this group at Tanner stage G4 (Fig. 4), yet he had normal hormone levels.

Given that primary gonadal damage may not elicit gonadotropin elevation during childhood, we performed a subanalysis of those patients of Group A for whom the endocrine function was assessed during puberty, at Tanner stage ≥G3: one of six patients had elevated FSH and all had normal LH. Altogether, these results suggest that treatment for childhood cancer before the age of 10 years did not significantly affect testicular endocrine function assessed >1 year after the end of treatment in the majority of the cases.

In the 24 patients in whom treatment ended at an age above 10 years (Group B), i.e. more likely to have a reactivated hypothalamic–pituitary–gonadal axis, AMH was never found below the reference range but FSH was elevated in eight cases, suggesting the occurrence of a compensated primary Sertoli cell insufficiency. In two patients, one at Tanner stage G3 and one at G4, serum AMH was slightly above

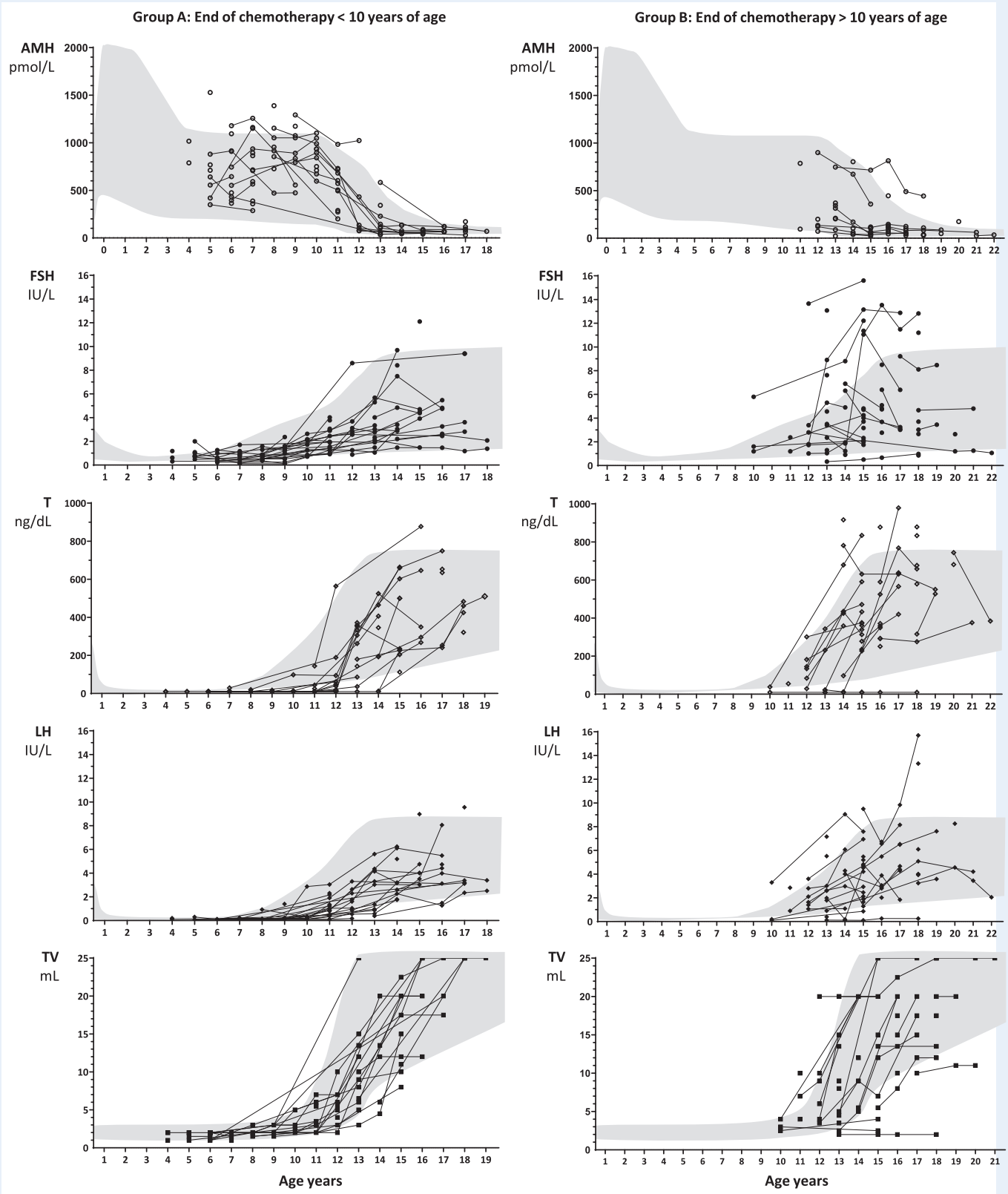

Figure 6 Progression with age of absolute serum levels of AMH, testosterone and gonadotropins in the 97 boys with ALL, AML or NHL included in the longitudinal follow-up study. Group A: patients in whom chemotherapy ended before the age of 10 years. Group B: patients in whom chemotherapy ended at age 10 years or more. The grey areas represent normal reference values, according to Grinspon *et al.*, 2011. ALL: acute lymphoblastic leukaemia; AML: acute myeloid leukaemia; NHL: non-Hodgkin lymphoma.

Table IV Logistic regressions for chemotherapy agent cumulative doses as independent variables and FSH or LH as dichotomized variables (> +2 SDS considered as affected; ≤ +2 SDS considered as normal) in 97 patients.

	FSH (SDS)			LH (SDS)		
	Odds Ratio	95% CI	*P*	Odds Ratio	95% CI	*P*
Cyclophosphamide (g/m^2)	1.42	0.58–3.51	0.45	2.68	0.55–13.07	0.22
Ifosfamide (g/m^2)	0.73	0.35–1.54	0.41	**0.04**	**0.004–0.57**	**0.017**
Methotrexate IV (g/m^2)	1.09	0.98–1.21	0.12	1.49	0.99–2.25	0.054
Methotrexate oral (g/m^2)	3.25	0.78–13.36	0.10	9.63	0.55–169.17	0.12
6-Mercaptopurine (g/m^2)	0.92	0.83–1.02	0.13	0.84	0.68–1.03	0.09
6-Thioguanine (g/m^2)	0.92	0.22–3.86	0.91	**0.001**	**0.0001–0.45**	**0.026**
C-Arabinoside (g/m^2)	1.37	0.23–8.11	0.73	**730.22**	**2.80–190 279**	**0.020**
Doxorubicin (g/m^2)	1.00	0.97–1.02	0.93	**1.14**	**1.00–1.30**	**0.044**
Daunorubicin (g/m^2)	1.00	0.99–1.02	0.37	0.97	0.91–1.02	0.19
Vincristine (mg/m^2)	1.10	0.93–1.30	0.26	1.20	0.83–1.75	0.33
L-Asparaginase (g/m^2)	1.00	0.99–1.00	0.41	1.00	0.99–1.00	0.68
Etoposide (mg/m^2)	1.00	0.99–1.00	1.00	**1.01**	**1.00–1.03**	**0.037**
Erwinase (IU/m^2)	1.00	0.99–1.00	0.56	0.99	0.99–1.06	0.99
Pegaspargase (U/m^2)	0.99	0.99–1.00	0.18	0.48	0.99–1.00	0.48

the upper normal range (Fig. 3B); the observation was not considered clinically significant, although a mild degree of Sertoli cell immaturity cannot be ruled out.

Similarly, testosterone levels were within the reference range in all 24 cases, but LH were slightly elevated in five patients receiving treatment after the age of 10 years (Group B), probably indicating a compensated Leydig cell dysfunction (Fig. 3). All these patients were at pubertal stages Tanner G4–5 (Fig. 2). Testicular volume was below normal in five patients at pubertal stages Tanner G4–5 (Fig. 4). Two of them had an elevation of both gonadotropins, one of whom had a relapse leading to BMT.

Longitudinal study

All 97 patients were included in the longitudinal analysis. Median elapsed time between the end of treatment and the first hormonal assessment was 3.1 years in the whole study sample, ranging from 0.1 to 11.9 years (Table I). Longitudinal data were available for 58 patients who completed treatment before the age of 10 years (Group A) and for 39 who received treatment beyond the age of 10 years (Group B). Serum AMH was never <2 SDS for up to 12 years of follow-up (Fig. 5) or below the reference range for age (Fig. 6) in patients of Groups A or B, during the whole follow-up. In two cases (Fig. 5), serum AMH was slightly above the upper normal limit for age. Whether this represents a slightly delayed pubertal development could not be ascertained with the available data. Testosterone remained below the reference range in only one pubertal patient.

In Group A, eleven of 58 patients showed at least one serum FSH and/or LH value above the normal range. The elevation was transient in two cases and persistent in nine (15.5%): FSH was elevated in eight patients (13.8%), suggesting a compensated dysfunction of the tubular component, and LH was elevated in four (6.9%), which was indicative of compensated Leydig cell dysfunction (Supplementary Table II). Nine patients had ALL (six SR and three IR), two of whom had been exposed to BMT preconditioning (one SR and one IR); these two patients had both gonadotropin levels above the normal range for their respective pubertal stage. Testicular volume remained below the expected value in one patient at age 15 years. In another case, testicular volume was initially low but recovered as pubertal development progressed (Fig. 6).

In Group B, 17 of 39 patients showed at least one elevated gonadotropin value. In 14 cases (35.9%), the elevation was present in the last available assessment: FSH was elevated in 11 (28.2%) and LH in 10 (25.6%) (Supplementary Table II). Thirteen patients had ALL (11 IR and 2 HR), two of whom had been exposed to BMT preconditioning (two IR), and one had NHL. No patient with AML was affected. Testicular volume remained below the expected value in five cases (Fig. 6).

A subanalysis was performed to compare the proportion of gonadotropin elevation between patients of Group A with follow-up until Tanner stage ≥G3 ($n = 23$) and Group B ($n = 39$). We found no statistically significant difference regarding FSH elevation (Group A, 21.7%; Group B, 28.2%; Fisher's exact test, $P = 0.40$) or LH elevation (Group A, 17.4%; Group B, 25.6%; Fisher's exact test, $P = 0.42$).

We assessed the association between cumulative doses of chemotherapy agents and the occurrence of gonadal dysfunction. Multiple regression analysis showed that no statistically significant associations were found when patients were categorised into affected Sertoli cell dysfunction (FSH >+2 SDS) or not (FSH ≤+2 SDS) (Table IV). For Leydig cell dysfunction (i.e. LH >+2 SDS), exposure to C-arabinoside appeared as a significant risk factor, whereas exposures to doxorubicin and etoposide marginally increased the odds ratio. Conversely, thioguanine and ifosfamide were associated with a decreased risk of Leydig cell dysfunction (Table IV). The incidence

of gonadotropin elevation was similar in the three risk groups of ALL: SR, 22.2%; IR, 33.3%; and HR, 37.5%.

Discussion

In this large study, with prolonged follow-up, we show that oncologic therapy for haematologic malignancies in boys and adolescents did not provoke an overt primary hypogonadism, as revealed by the persistence of normal levels of direct biomarkers of Sertoli cells (AMH) and Leydig cells (testosterone). However, a compensated Sertoli and Leydig cell dysfunction was evidenced by a mild to moderate gonadotropin elevation during pubertal development in up to 35.9% of the cases. No clinically relevant risk factor, such as severity of the disease or treatment protocol, could be identified in association with the compensated endocrine dysfunction.

Most studies assessing gonadal toxicity of cancer therapy in male adolescents and adults have focused on germ cell toxicity, and identified alkylating agents (busulphan, cyclophosphamide and ifosfamide) in high doses and cis-platinum as high or moderate risk for spermatogenic damage. Our cohort included only patients exposed to the ALL IC-BFM 2002 intercontinental trial protocol adopted by the Argentine Group for the Treatment of Acute Leukaemia (GATLA, Grupo Argentino de Tratamiento de la Leucemia Aguda; Sociedad Argentina de Hematología, 2013). This therapy regimen uses mostly drugs classified as of low risk for spermatogenic toxicity and doses of alkylating agents, like cyclophosphamide and ifosfamide, which are below the expected toxicity risk in the large majority of patients.

The diagnosis of hypogonadism in the male requires the assessment of the different cellular populations of the testis: Leydig, Sertoli and germ cells. The classical definition used in the adult male, equating hypogonadism to low androgen production, is not adequate for male patients of prepubertal age, a period of life when testosterone levels are normally very low or undetectable (Rey *et al.*, 2013, Salonia *et al.*, 2019, Young *et al.*, 2019, Grinspon *et al.*, 2019). Conversely, biomarkers of Sertoli cells, the most active component of the prepubertal testis, are more suitable for the assessment of gonadal function (Josso *et al.*, 2013). In the present study, we used serum AMH to examine the potential adverse effect of oncologic therapy in prepubertal boys with ALL, AML or NHL. AMH is a widely accepted biomarker of the prepubertal Sertoli cell (Lee *et al.*, 1997, Ankarberg-Lindgren *et al.*, 2011, Grinspon *et al.*, 2012a, van Brakel *et al.*, 2017, Grinspon *et al.*, 2018a) and of granulosa cells of small ovarian follicles, proving to be a useful marker of gonadotoxicity in girls with cancer (Brougham *et al.*, 2012).

No alteration in serum AMH levels was observed in prepubertal patients evaluated at least 1 year after the end of chemotherapy, radiotherapy or even BMT. In adults, Sertoli cells are expected to be resistant to most chemotherapy agents because they have a mature, stable, non-proliferating phenotype (Dere *et al.*, 2013, Stukenborg *et al.*, 2018). Conversely, from birth to the initial stages of puberty, Sertoli cells proliferate in response to FSH, with two peaks in infancy and peripuberty (Sharpe *et al.*, 2003), and then mature in response to androgens (O'Shaughnessy *et al.*, 2009, Edelsztein *et al.*, 2016, Edelsztein and Rey, 2019), which could make them more susceptible to chemotherapy or irradiation (Kelnar *et al.*, 2002, Stukenborg *et al.*, 2018). Our results indicate that neither of these processes are significantly affected in prepubertal boys treated for haematologic malignancies.

In patients of pubertal age, AMH and testosterone levels were also within the normal range in almost every case. However, when evaluated beyond Tanner stage G3, i.e. in the context of a reactivated hypothalamic–pituitary–testicular axis, we found an increased prevalence of compensated testicular dysfunction. This was especially revealed by an elevation of serum FSH, an indirect sign of Sertoli cell damage. Concordantly, decreased testicular volume was more frequently observed in patients during the latest pubertal stages (Tanner G4–5). Whether FSH elevation and reduced testicular volume were due to a primary Sertoli cell damage or to germ cell depletion cannot be answered with our data. Sertoli cells proliferate in response to FSH at the very beginning of puberty, then they mature in response to the increase in intratesticular androgen concentration (Grinspon *et al.*, 2018b). Sertoli cell maturation is characterised by a cessation of mitotic proliferation, a decrease in AMH production and the development of subcellular structures involved in cell–cell interaction between Sertoli cells, driving the development of the blood-testis barrier, and between Sertoli and germ cells, ultimately responsible for the initiation and stabilisation of adult spermatogenesis. Both pubertal Sertoli and germ cell populations contribute to an increase in inhibin B levels (Andersson *et al.*, 1998, Lahteenmaki *et al.*, 1999, Rohayem *et al.*, 2017), which exert a negative feedback on pituitary FSH secretion. One limitation of our study, due to its retrospective design, is the lack of systematic determination of serum inhibin B in all patients of the cohort. Nevertheless, as mentioned, both Sertoli and germ cells contribute to the pubertal increase in circulating inhibin B and testicular volume. It is therefore difficult to identify the primary damage: whether primary Sertoli cell dysfunction results in secondary depleted spermatogenesis or whether primary germ cell damage impacts on Sertoli cell function and inhibin B secretion. Another limitation of our study is that semen analysis was not performed in those patients with long-term follow-up, which precludes us form concluding on potential fertility impairment. Based on observations made in prepubertal boys exposed to chemotherapy, reduced spermatogonial quantity may be a major contributor to explain the elevation of serum FSH (Masliukaite *et al.*, 2016, Poganitsch-Korhonen *et al.*, 2017). Finally, although some of our patients received cranial radiotherapy, radiation doses were always below 30 Gy, which do not increase the risk of hypothalamic–pituitary–testicular dysfunction (Crowne *et al.*, 2015, Gebauer *et al.*, 2018).

In this study, our aim was to determine whether the oncologic treatments used in boys and adolescents with ALL, AML or NHL, the set of most frequent paediatric cancers, produce a persistent impairment of the endocrine function of Sertoli and Leydig cells in a period of life characterised by significant developmental changes in the testes. To this end, we included in the cross-sectional analysis patients who had their testicular function assessed at least 1 year after the end of cancer treatment. We estimated that 1 year was sufficient for somatic cells to recover after the discontinuation of chemotherapy. Patients susceptible to receive testicular irradiation were not included. Owing to the retrospective design of our study, testicular function was first assessed after variable periods (from 1 to 8 years) after the end of treatment. We chose to perform the analysis using the first testicular endocrine assessment available after 1 year had elapsed from the end of chemotherapy, and not assessments after longer-term follow-up,

to avoid, whenever possible, the confounding effect of developmental changes occurring in puberty. Since Sertoli cell endocrine function was our main focus, we used AMH levels as a direct biomarker. Serum AMH is normally high in prepubertal boys and declines from Tanner stage G2 onwards due to the physiological inhibition of its expression induced by intratesticular testosterone (Rey, 1998, Edelsztein *et al.*, 2018). By selecting the earliest follow-up sample, we reduced the potentially confounding effect of the physiological decline of AMH due to pubertal onset. Also, to minimise this effect, we compared hormone levels in our patients with normative data previously validated for age and Tanner stage (Grinspon *et al.*, 2011, Grinspon *et al.*, 2012b).

Our series included mainly boys with a diagnosis of ALL, while the AML and NHL groups were small. The reason for including the latter, together with patients with ALL, was that drug regimens used are similar, and our aim was to assess the effect of treatment rather than the risk of gonadal dysfunction in different childhood cancers. Although big series of patients with only one diagnosis and only one treatment would be the ideal, we believe that ours is one of the most homogeneous series on the subject. Indeed, most series on the effect of oncologic treatment of childhood on testicular function include adult patients with many different types of solid and haematologic cancers, where the treatments are completely different. Intentionally, we did not include in this study patients with other types of cancer.

Leydig cells, while sensitive to radiation therapy when high doses (>24 Gy) need to be used (Jahnukainen *et al.*, 2011), seem to be more resistant to chemotherapy. Indeed, the patients of our cohort showed no clinically relevant damage of Leydig cell function, as demonstrated by normal testosterone and LH levels during pubertal development in the vast majority of cases, with endocrine assessments up to 12 years after the end of oncologic treatment. A compensated Leydig cell dysfunction was observed in a few cases, as reflected by LH elevation whereas a severe hypoandrogenism, requiring testosterone replacement therapy, was seen in only one case with IR ALL, who received standard chemotherapy. Multiple regression identified high doses of c-arabinoside as a potential risk factor, although its effect size needs to be unequivocally proved. In adult males exposed to chemotherapy during childhood, an increased proportion of hypoandrogenism has been observed, especially in association with an increased fat mass and insulin level (Greenfield *et al.*, 2007). The difference between the prevalence of hypoandrogenism observed in adult survivors of childhood cancer and the adolescents in our study might reflect the influence of time or of progressive overweight during adult life. Other studies have identified up to 31% of Leydig cell failure in adult survivors of childhood cancer (Hudson *et al.*, 2013). The higher proportion of Leydig cell dysfunction may be due to the inclusion of all childhood cancer types, not only haematologic malignancies, some of which require very aggressive chemotherapy or radiotherapy affecting testicular function primarily or secondarily to hypothalamic-pituitary impairment as a consequence of high-dose cranial radiation protocols (Crowne *et al.*, 2015).

FSH and LH levels were within the normal range in a large majority of boys whose treatment ended before the age of 10 years. Given that gonadotropins may not increase during prepuberty in response to gonadal damage, we performed a subanalysis in those patients in whom endocrine assessments were available during puberty. We found no statistically significant differences in the prevalence of elevated gonadotropins, which could reveal a compensated primary hypogonadism, between patients treated before and after 10 years of age. However, our study may be underpowered in this analysis, since only part of the cohort treated before the age of 10 years (Group A) reached advanced puberty during follow-up. In a few cases, the elevation of gonadotropins was temporary. Long-term follow-up might identify a subset of patients with transient gonadotropin elevation with subsequent normalisation by the end of pubertal development.

In conclusion, the regimens used in the treatment of patients with ALL, AML or NHL in the past two decades seem not to provoke an overt damage of testicular somatic components, as revealed by normal levels of AMH and testosterone. However, a mild primary testicular dysfunction may be observed, usually compensated by slightly elevated gonadotropin secretion by the pituitary, which generally does not require hormone replacement therapy.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

R.P.G., G.B., L.A.A. and R.A.R. conceived the study design; R.P.G., M.A., S.P., G.B., M.S., M.M.B., M.G., P.B., A.K., G.O.E., S.G., M.G.R., I.B., L.A.A. and R.A.R. collected clinical and laboratory data; R.P.G., M.A., S.P., G.B., M.S., A.J.B., L.A.A. and R.A.R. analysed the data; R.P.G., L.A.A. and R.A.R. drafted the manuscript; all authors approved the final version.

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Conflict of interest

R.A.R., R.P.G. and P.B. have received honoraria from CONICET (Argentina) for technology services using the AMH ELISA. L.A.A. is part-time employee of CSL Behring Argentina. The other authors have no conflicts of interest to disclose.

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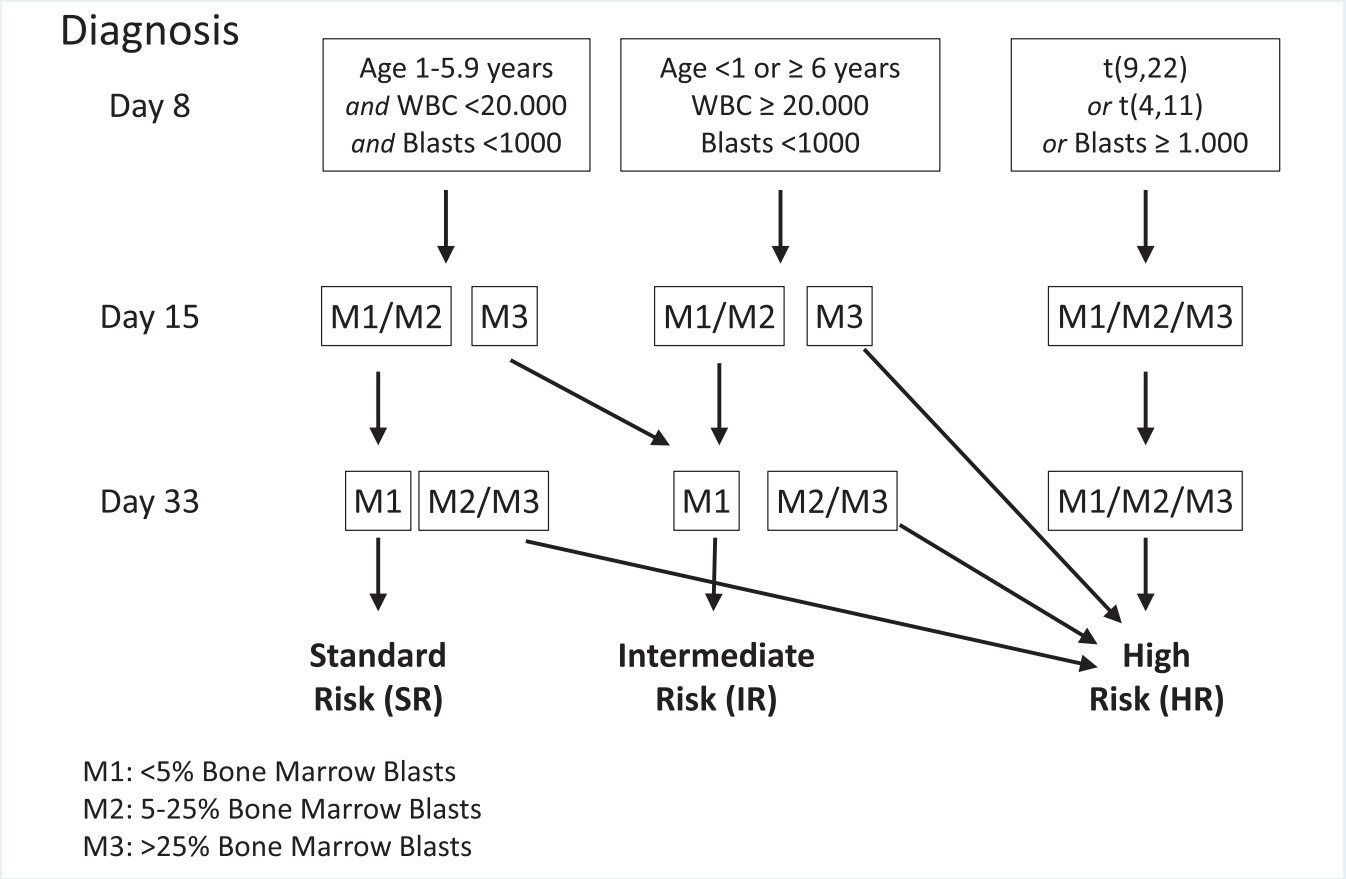
SUPPLEMENTARY DATA



Supplementary Figure SI Risk stratification and treatment assigned for patients with acute lymphoblastic leukaemia (ALL) according to the ALL IC-BFM 2002 intercontinental trial. Standard risk (SR) definition required the fulfilment of all the following criteria: prednisone good response ($<1 \times 10^9$/l blasts in peripheral blood on day 8 after 7 days of prednisone and one dose of intrathecal methotrexate on day 1), age 1–6 years, initial WBC $<20 \times 10^9$/l, and M1 (<5% blasts) or M2 (5–25% blasts) marrow on day 15, and M1 marrow on day 33. Intermediate risk (IR) was defined as prednisone-good response, age <1 year or ≥6 years, and/or WBC $\geq 20 \times 10^9$/l and M1 or M2 marrow on day 15 and M1 marrow on day 33, or SR criteria but M3 (≥25% blasts) marrow on day 15 and M1 marrow on day 33. High risk (HR) definition required at least one of the following criteria: prednisone poor response ($\geq 1 \times 10^9$/l blasts in peripheral blood on day 8 after 7 days of prednisone and one dose of intrathecal methotrexate on day 1), IR and M3 marrow on day 15, M2 or M3 marrow on day 33, t(9;22) (BCR-ABL), or t(4;11) (MLL-AF4). WBC, white blood cells.

Human Reproduction, pp. 1–1, 2019
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SUPPLEMENTARY DATA

Supplementary Table SI Serum gonadotropins, testosterone and AMH levels in 306 healthy males.

Age group		n	AMH (pmol/l) Mean (SD)	FSH (IU/l) Mean (SD)	T (ng/dl) Mean (SD)	LH (IU/l) Mean (SD)
2.0–8.9 yr		95	782 (461)	0.84 (0.52)	10 (0)	0.10 (0.02)
9–18 yr	**G1**	34	741 (327)	1.64 (0.65)	21.8 (34.6)	0.57 (0.89)
	G2	34	419 (301)	2.38 (0.92)	57.5 (164.5)	1.10 (0.88)
	G3	42	121 (114)	3.29 (1.64)	223.1 (154.2)	2.37 (1.26)
	G4	41	75 (40)	3.80 (1.68)	416.3 (154.2)	3.11 (1.40)
	G5	60	92 (50)	3.05 (1.73)	426.0 (158.9)	3.25 (1.52)

Data from: Grinspon RP, Bedecarrás P, Ballerini MG, Iñíguez G, Rocha A, Mantovani Rodrigues Resende EA, Brito VN, Milani C, Figueroa Gacitua V, Chiesa A *et al.* Early onset of primary hypogonadism revealed by serum anti-Müllerian hormone determination during infancy and childhood in trisomy 21. *Int J Androl* 2011;**34**: e487–e498.
To obtain serum testosterone in nmol/l, multiply by 0.03467. To obtain serum AMH in ng/ml divide by 7.14.

Supplementary Table SII **Characteristics of the patients with at least one elevated serum gonadotropin level.**

Patient #	Group	Diagnosis	Risk	BMT	Age at end of treatment (yr)	Age at evaluation (yr)	Genital stage at evaluation	Testicular volume (Right/Left)	AMH (pmol/l)	AMH SDS	FSH (IU/l)	FSH SDS	T (ng/dl)	T (SDS)	LH (IU/l)	LH SDS	COMMENT
70	A	ALL	SR	NO	4.9	5.3	1	1/1	880	0.21	**2.0**	**2.23**	10	0	0.3	0.96	FSH elevation was transient. Last available assessment at 9.8 yr, G1: FSH 0.6 IU/l, LH 0.1 IU/l, T 10 ng/dl, AMH 474 pmol/l
68	A	ALL	SR	NO	5.6	8.4	1	2/2	287	−1.07	**2.3**	**2.92**	10	0	0.2	0.22	Two previous assessments showed normal gonadotropin levels
75	A	ALL	SR	NO	7.9	11.3	1	2/2	573	−0.51	**3.7**	**3.28**	12	−0.28	0.5	−0.07	Only one hormonal assessment available
8	A	ALL	SR	NO	8.0	11.9	1	3/3	604	0.42	**4.0**	**3.66**	10	−0.34	1.1	0.68	Two previous assessments showed normal gonadotro pin levels
53	A	NHL	HR	NO	8.7	12.0	1	2/2	N.A.	N.A.	**3.6**	**3.14**	10	−0.34	1.7	1.34	FSH elevation was transient. At Tanner stage G5: FSH 4.7 IU/l, LH 3.0 IU/l, T 235 ng/dl, AMH 52 pmol/l
55	A	ALL	IR	YES	4.1	15.2	3	12/10	40	−0.71	**12.1**	**5.37**	500	1.81	**8.9**	**5.07**	Only one hormonal assessment available
5	A	ALL	IR	NO	3.8	14.7	4	12/12	57	−0.60	**8.4**	**2.91**	346	−0.49	5.1	1.35	Only one hormonal assessment available
28	A	ALL	IR	NO	5.2	14.8	5	15/12	133	1.02	**9.7**	**3.64**	406	−0.10	**6.2**	**2.10**	Persistent elevation, FSH and LH
20	A	ALL	IR	NO	4.6	16.6	5	20/20	64	−0.45	4.8	0.84	268	−0.99	**8.0**	**3.30**	Persistent elevation, only LH
95	A	ALL	SR	NO	8.9	17.0	5	25/25	30	−1.17	**9.4**	**3.48**	1049	4.02	3.2	0	Persistent elevation, only FSH
64	A	ALL	SR	YES	7.2	17.1	5	N.A.	66	−0.4	**19.6**	**9.39**	653	1.48	**9.5**	**4.32**	Persistent elevation, FSH and LH
41	B	ALL	IR	NO	10.4	13.7	2	8/8	203	−0.74	**4.5**	**2.37**	10	−0.29	0.4	−0.77	Only one hormonal assessment available
87	B	ALL	IR	NO	14.1	14.6	2	3/8	104	−1.07	**6.9**	**4.91**	97	0.24	**4.0**	**3.28**	FSH elevation was transient. At Tanner stage G4: FSH 4.8 IU/l, LH 2.9 IU/l, T 349 ng/dl
69	B	ALL	IR	YES	13.5	15.5	2	4/4	118	−1.03	**4.2**	**2.02**	10	−0.29	0.8	−0.28	Two previous assessments showed normal gonadotropin levels
52	B	ALL	HR	YES	12.1	13.9	3	8/10	23	−0.87	**13.0**	**5.96**	916	4.53	**7.1**	**3.67**	Only one hormonal assessment available

Continued

Supplementary Table SII *Continued*

Patient #	Group	Diagnosis	Risk	BMT	Age at end of treatment (yr)	Age at evaluation (yr)	Genital stage at evaluation	Testicular volume (Right/Left)	AMH (pmol/l)	AMH SDS	FSH (IU/l)	FSH SDS	T (ng/dl)	T (SDS)	LH (IU/l)	LH SDS	COMMENT
72	B	ALL	IR	NO	15.4	16.4	4	20/25	133	1.02	**8.5**	**2.96**	371	−0.33	4.2	0.74	Persistent elevation, only FSH
38	B	ALL	IR	NO	16.1	16.4	4	15/12	45	−0.85	4.7	0.80	878	2.92	**6.7**	**2.40**	Only one hormonal assessment available
40	B	ALL	HR	NO	17.6	18.1	4	20/20	38	−1,00	**11.2**	**4.51**	833	2.63	**13.3**	**6.68**	Only one hormonal assessment available
51	B	ALL	IR	NO	11.2	13.9	5	20/20	89	0.09	**7.6**	**2.45**	231	−1.22	5.5	1.58	Only one hormonal assessment available
14	B	NHL	E2	NO	12.3	15.1	5	20/20	23	−1.32	**15.6**	**7.04**	591	1.08	4.2	0.69	Persistent elevation, only FSH
27	B	ALL	IR	NO	12.7	15.1	5	20/20	104	−0.40	2.0	−0.78	376	−0.29	**6.9**	**2.54**	Persistent elevation, only LH
12	B	ALL	IR	NO	10.3	15.4	5	20/20	N.A.	N.A.	**12.2**	**5.09**	834	2.64	**7.5**	**2.98**	Persistent elevation, FSH and LH
85	B	ALL	IR	NO	12.6	15.6	5	12/12	38	−1.01	**11.3**	**4.6**	227	−1.25	4.5	0.84	FSH elevation was transient. At last assessment, Tanner stage G5: FSH 6.4 IU/l, LH 6.5 IU/l, T 638 ng/dl, AMH 55 pmol/l
1	B	NHL	E2	NO	15.0	16.0	5	20/25	120	0.74	**14.8**	**6.58**	376	−0.83	3.0	−0.10	FSH elevation was transient. At last assessment, Tanner stage G5: FSH 4.8 IU/l, LH 4.2 IU/l, T 376 ng/dl, AMH 62 pmol/l
74	B	ALL	IR	NO	10.8	17.7	5	25/25	29	−1.18	**12.8**	**5.48**	566	0.92	**8.1**	**3.37**	Persistent elevation, FSH and LH
36	B	ALL	IR	YES	14.6	18.7	5	12/12	N.A.	N.A.	**12.8**	**5.44**	658	1.51	**15.7**	**8.50**	Persistent elevation, FSH and LH
61	B	ALL	IR	NO	12.5	19.9	5	10/12	68	0.01	**8.4**	**2.94**	595	1.11	**7.7**	**3.07**	Persistent elevation, only FSH
65	B	ALL	IR	NO	18.6	20.0	5	25/25	175	1.91	2.6	−0.41	681	1.66	**8.2**	**3.44**	Persistent elevation, only LH

ALL: acute lymphoblastic leukaemia, NHL: non-Hodgkin lymphoma, SR: Standard Risk, IR: Intermediate Risk, HR: High Risk, BMT: Bone Marrow transplantation. N.A.: not available.