Anti-Müllerian Hormone Is a Marker of Gonadotoxicity in Pre- and Postpubertal Girls Treated for Cancer: A Prospective Study

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Context: Cytotoxic treatment may accelerate depletion of the primordial follicle pool, leading to impaired fertility and premature menopause. Assessment of ovarian damage in prepubertal girls is not currently possible, but Anti-Müllerian Hormone (AMH) is a useful marker of ovarian reserve in adults.

Objective: The objective of the study was to prospectively evaluate AMH measurement in children as a marker of ovarian toxicity during cancer treatment.

Design and Setting: This was a prospective, longitudinal study at a University Hospital.

Patients: Twenty-two females (17 prepubertal), median age 4.4 yr (range 0.3–15 yr), were recruited before treatment for cancer.

Main Outcome Measures: AMH, inhibin B, and FSH at diagnosis, after each chemotherapy course and during follow-up, were measured. Risk of gonadotoxicity was classified as low/medium (n = 13) or high (n = 9) based on chemotherapy agent, cumulative dose, and radiotherapy involving the ovaries.

Results: Pretreatment AMH was detectable across the age range studied. AMH decreased progressively during chemotherapy (P < 0.0001) in both prepubertal and pubertal girls, becoming undetectable in 50% of patients, with recovery in the low/medium risk groups after completion of treatment. In the high-risk group, AMH became undetectable in all patients and showed no recovery. Inhibin B was undetectable in most patients before treatment and, with FSH, showed no clear relationship to treatment.

Conclusion: AMH is detectable in girls of all ages and falls rapidly during cancer treatment in both prepubertal and pubertal girls. Both the fall during treatment and recovery thereafter varied with risk of gonadotoxicity. AMH is therefore a clinically useful marker of damage to the ovarian reserve in girls receiving treatment for cancer. (J Clin Endocrinol Metab 97: 2059–2067, 2012)

Survival from childhood cancer has improved markedly over the past few decades, with more than 80% of children achieving long-term cure (1). However, this improved survival may be associated with gonadal dysfunc-

tion and infertility in adulthood (2). In females, both chemotherapy and radiotherapy may hasten oocyte depletion with truncated fecundity and premature menopause (2, 3). An accurate assessment of the risk to individual patients of
subfertility or infertility before and after cancer treatment remains a significant challenge.

The effects of radiotherapy are dependent on the dose received, the fractionation schedule, and the field of irradiation (4, 5). The extent of chemotheraphy-induced damage varies according to the agent administered and is also dependent on the cumulative dose received (2, 5). Assessment of ovarian function in adulthood entails clinical evaluation of pubertal status; menstrual history; and biochemical measurements of gonadotrophins (FSH, LH), estradiol, and progesterone. Although this is helpful in postpubertal children both before and after treatment, it is not informative prepubertally because the hypothalamic-pituitary-gonadal axis is quiescent. Counseling young patients and their families on their fertility prospects is therefore very difficult and is based on assessment of treatment planned or received. The availability of a reliable biochemical marker of ovarian reserve, which was readily detectable in prepubertal girls, would be an invaluable clinical tool (6).

Anti-Müllerian Hormone (AMH) is produced in the granulosa cells of growing ovarian follicles up to the early antral stage (7). The number of these follicles is related to the size of the primordial follicle pool, and therefore, AMH represents a marker of ovarian reserve (8). Indeed AMH demonstrates a rise through childhood and adolescence to a peak in the early 20s, with relative stability during the third decade of life (6, 9). AMH then declines during the later reproductive years (10), becoming undetectable before the menopause (11, 12). AMH is stable across the menstrual cycle (8, 13), adding to its utility in postpubertal females.

Adults receiving chemotherapy demonstrate a rapid decline in AMH (14, 15), and recent data suggest prechemotherapy AMH can predict long-term ovarian function (16). Previous studies (17–19) investigating ovarian reserve in adults after treatment for childhood cancer have demonstrated low AMH concentrations, but the role of AMH as an early and sensitive marker of gonadotoxicity in children during and after treatment for cancer has not previously been investigated.

Inhibin B is produced by larger growing follicles and, with FSH, is also a marker of ovarian reserve. Inhibin B concentrations are lower in adult women who had been treated for Hodgkin’s lymphoma during childhood (18), and a pilot study suggested it may have a role as a marker of gonadotoxicity in prepubertal girls (20). However, their value as markers of ovarian activity is limited by very low/undetectable concentrations before puberty and the need for measurement in the early follicular phase thereafter.

In this prospective cohort study on girls treated for cancer, we have measured AMH, inhibin B, and FSH before, during, and after completion of treatment. The aim of our study was to evaluate these biochemical measures as potential markers of early gonadotoxicity in young girls treated for cancer.

**Patients and Methods**

Female patients diagnosed with malignancy and requiring treatment with pulsed chemotheraphy or radiotherapy were enrolled. All were diagnosed and treated at the Royal Hospital for Sick Children (Edinburgh, UK) between April 2003 and June 2007. Patients with Acute Lymphoblastic Leukemia were excluded because they do not receive pulsed treatment, and those with a brain tumor were excluded if they required cranial irradiation, which could affect the hypothalamic-pituitary-gonadal axis. Patients with a gonadal tumor or primary gonadal dysgenesis were also excluded. Consecutive eligible patients were approached. Only one eligible patient declined study entry due to parental concerns about introducing discussions regarding fertility at the time of diagnosis.

Pubertal status was assessed clinically at diagnosis and was classified as prepubertal (Tanner stage 1), midpuberty (Tanner stage 2/3), or late puberty (Tanner stage 4/5) (21). Patients were also classified according to their predicted risk of future gonadotoxicity (low, medium, or high), based on chemotheraphy agent, cumulative dose and radiotherapy involving the ovaries, without knowledge of hormone results. This classification was based on an assessment of the risk of subfertility according to the cancer diagnosis, stage of the disease, and treatment delivered (22). Low risk was estimated as less than 20% probability of future subfertility, medium risk between 20 and 80%, and high risk at greater than 80% (22).

All participants or their guardians provided written informed consent. Ethical approval was obtained from Lothian Research Ethics Committee.

Blood sampling was performed at diagnosis, as a baseline, and then before and after each cycle of chemotheraphy for the duration of treatment. Treatment usually comprised four or more chemotheraphy cycles. Follow-up samples were obtained a minimum of 6 months after completion of treatment.

Blood was collected into lithium heparin tubes and plasma subsequently stored at −20 C to −80 C until analysis. AMH (active Müllerian inhibiting substance/AMH) and inhibin B were measured using sensitive double-antibody ELISA (both Beckman Coulter, Chaska, MN) as previously described (14, 16). FSH was measured by time-resolved immunofluorimetric assay (Delfia, Wallac, Milton Keynes, UK). Assay detection limits were 0.05 ng/ml for AMH, 10 pg/ml for inhibin B, and 0.07 U/liter for FSH. Intra- and interassay coefficients of variation for AMH, inhibin B, and FSH were less than 10%. All samples from each individual patient were analyzed in the same analytical run to minimize variation.

**Data analysis**

Nonparametric statistics were used. Hormone results below the detection limit were expressed as the detection limit for statistical analysis and graphical display. Results are presented as median (range). Spearman rank correlation was used to test the relationship of hormones with age. Longitudinal changes in hormones with time during treatment were assessed by the Kruskal-
Wallis test. Changes during follow-up were assessed by Wilcoxon signed-rank tests. Mann-Whitney U tests were used to compare hormonal results by risk of gonadotoxicity. As AMH rises with age, SD z-scores were calculated to correct for this from our published nomogram (9). AMH concentrations pretreatment and during follow-up were also assessed for age-adjusted normality (i.e., that they lay within age specific 95% confidence intervals). Additional analyses following classification by risk were performed after log transformation of the data, using Student’s t test or ANOVA and post hoc Dunnett’s test for multiple groups, with pretreatment data as the comparator.

Results

Twenty-two female patients were recruited at the time of diagnosis (Table 1). Median age was 4.4 (range 0.3–15) yr, with 17 being prepubertal. In 15 patients treatment included alkylating agents and seven patients received cisplatin. Six patients received radiotherapy to a field that included both ovaries. Radiotherapy was delivered after the completion of chemotherapy in all six patients except for one patient (patient 6), who received emergency radiotherapy to the spine because of cord compression developing shortly after commencing treatment. The predicted risk of future gonadotoxicity, based on a chemotherapy agent, cumulative dose, and radiotherapy involving the ovaries, was low in five patients, medium in eight patients, and high in nine patients (Table 1) (22). Posttreatment follow-up samples were collected in 16 patients; of the remaining six patients, three had died, two were undergoing further treatment.

<table>
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<th>Subject</th>
<th>Diagnosis</th>
<th>Gonadotoxic chemotherapy</th>
<th>Cumulative dose</th>
<th>Radiotherapy to ovaries</th>
<th>Gonadotoxic risk</th>
<th>Outcome</th>
<th>Pubertal stage at start</th>
<th>Age at start (yr)</th>
<th>Age at completion of treatment (yr)</th>
<th>Duration of follow-up (months)</th>
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<td>Pre</td>
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<td>Pre</td>
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<td>High</td>
<td>Alive</td>
<td>Pre</td>
<td>1.2</td>
<td>1.8</td>
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<td>—</td>
<td>Yes, spine</td>
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<td>Relapsed Alive</td>
<td>Pre</td>
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<td>Relapsed Alive</td>
<td>Pre</td>
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<td>Pre</td>
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<td>Pre</td>
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<td>Alive</td>
<td>Pre</td>
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<td>Pre</td>
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<td>Pre</td>
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<td>5.3</td>
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<td>Pre</td>
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<td>Pre</td>
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<td>Alive</td>
<td>Pre</td>
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<td>6.5 g/m²</td>
<td>Yes, direct</td>
<td>High</td>
<td>Pre</td>
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<td>10.4</td>
<td>12</td>
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<tr>
<td>18</td>
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<td>27 g/m²</td>
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<td>Medium</td>
<td>Mid</td>
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<td>3.4 g/m²</td>
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<td>Medium</td>
<td>Late</td>
<td>13.6</td>
<td>14.1</td>
<td>43</td>
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<td>Ifosfamide, Vincristine</td>
<td>8 g/m²</td>
<td>210 mg/m²</td>
<td>No</td>
<td>Medium</td>
<td>Late</td>
<td>14.3</td>
<td>14.7</td>
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<td>4.2 g/m²</td>
<td>210 mg/m²</td>
<td>No</td>
<td>Medium</td>
<td>Late</td>
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<td>15.1</td>
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<td>22</td>
<td>Metastatic rhabdomyosarcoma</td>
<td>Cyclophosphor, Ifosfamide</td>
<td>29 g/m²</td>
<td>9 g/m²</td>
<td>No</td>
<td>High</td>
<td>Late</td>
<td>15.0</td>
<td>16.1</td>
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—, No gonadotoxic agent administered; Pre, prepubertal.

a Dose recorded as per kilogram body weight rather than surface area in view of young age of patient, according to standard chemotherapy prescribing guidelines.
It is possible that the data from the two youngest girls (aged 0.3 and 0.9 yr before treatment) might have been influenced by the neonatal rise in reproductive hormones; however, both showed similar changes to the rest of the cohort (i.e., declining AMH with treatment, no consistent change in FSH), and excluding them did not alter the results.

After completion of treatment, there was biochemical evidence of recovery of ovarian function detected by changes in AMH but not inhibin B or FSH (Fig. 2). Follow-up samples were grouped as taken between 2 and 12 months after completing treatment and beyond 12 months. Median AMH rose progressively from 0.09 (<0.05 to 2.25) ng/ml at the end of treatment to 0.44 (<0.05 to 3.66) ng/ml at 2–12 months (not significant) and to 1.40 (<0.05 to 2.72) ng/ml at longer than 12 months, similar to pretreatment concentrations and significantly higher than at the end of treatment (P = 0.01, Fig. 2A). However, this recovery was not observed in all girls. In five girls in whom AMH was undetectable at the end of treatment, it remained so during follow-up (patients 3, 6, 13, 17, and 22). All of these had been classified as being at high risk of gonadotoxicity, including three who had received radiotherapy involving the ovaries. Two other girls (one with a germ cell tumor, patient 5, and one with Wilms’ tumor, patient 10, both classified as at low risk of gonadotoxicity) had undetectable AMH at the end of treatment but demonstrated recovery to detectable concentrations thereafter. Examples of changes in AMH in individuals are shown in Fig. 3. These all show a fall in AMH during treatment, with recovery in two prepubertal girls (Fig. 3, A and B; classified as low and medium risk, respectively) to concentrations higher than before treatment (P = 0.01, Fig. 2A). How-

FIG. 1. AMH (A), AMH z-score (B), inhibin B (C) and FSH (D) concentrations before treatment (Pre) and after each of the first six courses of chemotherapy. Note that inhibin B data are from only those girls in whom it was detectable before treatment. Median ± interquartile range, except AMH z-score, are mean ± SEM. AMH and FSH, n = 22; inhibin B, n = 5 (data included only where inhibin B was detectable in pretreatment sample). AMH: **, P < 0.01, ***, P < 0.001 vs. pretreatment concentration. Inhibin B: bar and asterisk denote significant difference by ANOVA (P < 0.05). Individual cycle data were not significantly different from pretreatment.

due to relapse, and one patient declined further blood sampling.

Pretreatment AMH was detectable in all patients, median 1.27 (range 0.11–3.75) ng/ml. There was a significant positive relationship with age (r = 0.87, P = 0.001), but no such relationship with age was seen for inhibin B or FSH (r = 0.3 and 0.18, respectively, both not significant). At diagnosis, inhibin B was detectable (55–122 pg/ml) in only five girls, aged 0.3–14.3 yr. Baseline FSH was 1.27 (0.13–5.04) U/liter.

Median AMH fell progressively during treatment, from 1.27 (0.11–3.75 ng/ml) to 0.05 ng/ml (0.05–0.90 ng/ml; P < 0.001, Fig. 1, A and B) over the first six cycles. AMH fell in both prepubertal (1.06 to 0.05 ng/ml at end of treatment, P = 0.002) and pubertal (1.40–0.36 ng/ml, P = 0.001) girls. Age-adjusted AMH analysis (Fig. 1B) showed that AMH was slightly but not significantly below average (i.e., a negative z score) before treatment and fell to an average of −1.5 SD during treatment.

In the five girls in whom inhibin B was detectable before treatment, it decreased from a median of 105 (55–122) ng/ml to 30 (<10 to 50) ng/ml after four cycles of treatment (P = 0.03; data available on only two girls after six cycles, Fig. 1C). It remained undetectable in those girls in whom it was undetectable before treatment. FSH showed no significant change over this period (median pretreatment FSH 1.27 U/liter, 1.57 U/liter after course 6, Fig. 1D).
tuating inhibin B during treatment, followed by a decrease to an undetectable level after treatment.

FSH showed no overall changes during recovery (Fig. 2C). Two of the girls had clearly elevated concentrations of FSH greater than 10 U/liter, indicating gonadal failure; both had undetectable AMH at end of treatment and were classified as at high risk of gonadotoxicity (patients 17 and 22). There was no significant relationship between AMH and FSH at any time point, although there was a trend toward a negative correlation at the end of follow-up ($r = -0.51$, $P = 0.07$).

Data were also analyzed by estimated degree of gonadotoxicity of the treatment administered (see Materials and Methods and Table 1). Nine girls were classified as at high risk, eight at medium risk, and five at low risk. Due to the similarity of the hormone data in the medium- and low-risk groups, these were combined for analysis. AMH fell to undetectable concentrations in all girls in the high-risk group and subsequently showed no recovery (ANOVA $P = 0.002$; Fig. 4A). AMH also fell significantly during treatment in the medium/low-risk group, but this was followed by a significant rise during recovery (end of treatment vs. recovery: $P = 0.005$). Although AMH concentrations were similar in the high- and medium/low-risk groups at diagnosis, at the end of treatment there were clear differences in AMH concentration between these groups ($P < 0.001$). This difference persisted into the follow-up period with lower AMH concentrations in the high-risk group ($P = 0.003$). There was, however, no evidence for a more rapid fall in AMH in the high-risk group, with the fall in AMH becoming significant after treatment cycle 2 in both groups. AMH concentrations during follow-up were also assessed for age-related normality and were low ($z$ score less than $-2$) in three girls in the medium-risk group [patients 19 and 21, both with Hodgkin’s lymphoma (data shown in Fig. 3, E and F), and patient 11, with a rhabdomyosarcoma] and none in the low-risk group.

FSH concentrations at diagnosis or end of treatment did not differ by risk (Fig. 4B) but were higher in the high-risk group at follow-up ($P = 0.04$). Because of the small number of girls with detectable inhibin B concentrations at diagnosis, analysis by gonadotoxic risk stratification was not possible.

The cohort will be followed up long term, but thus far two patients who were prepubertal at recruitment have confirmed premature ovarian failure with significant elevation of FSH on several occasions (patients 13 and 17). Both are in the high gonadotoxicity risk group and had undetectable AMH during chemotherapy and immediately after treatment (data from patient 17 are represented in Fig. 3D) but with no associated elevation of FSH at that time.

**Discussion**

This is the first study we are aware of to measure AMH prospectively from diagnosis to follow up in a pediatric cancer cohort. We have shown that AMH falls during cancer treatment in both prepubertal and pubertal girls. Subsequent recovery was apparent in the majority of girls after the end of treatment but in those deemed to be at highest risk of ovarian failure recovery was absent up to 3 yr after treatment. Hormonal changes in the youngest might have been complicated by the neonatal rises in FSH and AMH (6, 23); however, the changes in the two girls...
aged younger than 1 yr were comparable with those in older girls. Our findings suggest that AMH may be a useful marker of ovarian reserve in young girls treated for cancer. This will have implications for counseling patients before and after treatment and may be helpful in defining which young patients may benefit from pretreatment ovarian cryopreservation.

Significant advances have been made over recent decades in the treatment of childhood cancer (1). This improvement has, in part, resulted from intensification of cytotoxic treatment modalities, particularly with regard to multiagent and high-dose chemotherapy. Although overall survival has increased, so too have adverse effects of treatment, both in the short and long term. The impact of treatment on future fertility is of significant concern, both to parents and patients (24), but it is difficult to predict accurately future fertility, and guidelines based on agent and dose are relatively crude (22). Radiotherapy to a field that includes the ovaries is gonadotoxic in a dose-dependent fashion (4). However, the impact of chemotherapy can be much more difficult to determine. It is known that certain chemotherapeutic agents, such as alkylating agents, are gonadotoxic, but the degree of ovarian damage can be very variable and the mechanisms are poorly understood (25). Although patients receiving chemotherapy may retain fertility, their menopause may be premature, reflecting an accelerated decline in oocyte numbers (2, 3). Thus, their window of fertility may be shortened (26).

An accurate marker of future reproductive function, which can be measured longitudinally before, during, and after treatment, would be of significant benefit to these patients and their families. AMH was detectable in all pretreatment samples, in contrast to inhibin B. This reflects the different growth stages of ovarian follicles producing these hormones. AMH is produced during early stages of follicle growth (7), whereas inhibin-producing stages are later and more gonadotrophin sensitive, and this hormone is consistently detectable only after puberty (27). Our data confirm a progressive rise in AMH during childhood and adolescence (6, 9) and demonstrate a fall during cancer treatment, which was readily detectable in both prepubertal and postpubertal girls. AMH concentrations at the end of treatment reflected gonadotoxicity, being significantly lower in the high-risk group, confirming the value of AMH as a quantitative index of the damage to the ovarian reserve. Furthermore, differential recovery after treatment was also identified, with AMH remaining undetectable in the high-risk group, whereas girls in the low- and medium-risk groups showed recovery to concentrations similar to those before treatment. Whether this translates to a normal duration of reproductive life span will require very long-term follow-up. The recovery in AMH after chemotherapy indicates restoration of the pool of small growing follicles, which in turn reflects the size on the nongrowing, primordial pool (28). The decline in AMH during chemotherapy reflects increased follicular atresia (29), and during follow-up the normal pattern of early follicle growth, present in girls of all ages (30), will be restored. Thus, the absence of a recovery in the high-risk groups indicates a profound loss of the primordial follicle pool unable to generate sufficient small growing follicles to secrete a detectable amount of
AMH. AMH concentrations rise through childhood and adolescence, with an intriguing inflection at the start of puberty, despite the declining size of the primordial pool (6, 9, 31). The relationship between them therefore changes across the reproductive life span (32). Some caution is thus required in comparison of their relationships between for example the neonatal period, childhood, and adulthood. However, the dramatic differences in end of treatment and recovery phase AMH concentrations between groups demonstrated here supports a clear conclusion that there are substantial differences between chemotherapy regimens in their effects on the ovarian reserve.

A recent study from The Netherlands showed that in a cohort of childhood cancer survivors, AMH was reduced in those predicted to be at high risk of gonadal damage (19). Our study confirms these findings and demonstrates that AMH reflects ovarian damage after chemotherapy, and this can be detected before puberty or traditional evidence of premature ovarian failure, with elevated FSH concentrations and low estradiol. AMH may thus represent a reliable and accurate marker of gonadotoxicity secondary to cancer treatment.

In contrast, our data suggest that inhibin B and FSH are not helpful within the prepubertal age group. A previous pilot study suggested that inhibin B might be a potential marker for gonadotoxicity in prepubertal girls (20), but this has not been confirmed here. We found that inhibin B was undetectable in the majority of prepubertal girls at diagnosis, reflecting the very low gonadal activity after the neonatal “minipuberty” (33) until puberty itself. In those patients in whom inhibin B was detectable, it did, like AMH, show a progressive decrease during successive treatment courses in most cases, with recovery during follow-up in a proportion of patients. However, the lack of reliable sensitivity of this marker in prepubertal girls and its marked variation (unlike AMH) during the menstrual cycle in postpubertal girls limit its use as a marker of gonadotoxicity in children and adolescents.

Whereas in postpubertal patients raised FSH concentrations represent a very useful indicator of ovarian failure, the quiescence of the hypothalamic-pituitary-gonadal axis in the prepubertal age group predicts that FSH is of limited value in prepubertal girls. Our data confirm this. Although FSH did rise during treatment in many (but not all) patients in our study and indicated gonadal failure in one patient, with a trend towards recovery on follow-up in most cases, this hormone did not discriminate sufficiently to function as a marker of gonadotoxicity in children.

A limitation of this study is the relatively small number of patients. However, the marked decline in AMH concentrations in response to cytotoxic treatment, with recovery in those patients stratified to be at lower risk of longer-term gonadotoxicity, indicates its value in monitoring ovarian function before, during, and after cancer treatment in the prepubertal age group. The cohort of patients who were assessed to be at high risk of ovarian toxicity in whom there was no recovery of AMH after treatment was completed required further long-term follow-up to confirm that they have failed to recover their ovarian function. However, it is of note that of the four girls in the high-risk group who are still alive, two have confirmed premature ovarian failure. The remaining two are still at prepubertal ages at the present time.

A marker of ovarian function, particularly in the prepubertal age group, would improve future identification of those to whom fertility preservation strategies should be addressed. Ovarian cryopreservation as a method of restoring fertility after gonadotoxic treatment is becoming increasingly used and is the only method appropriate to prepubertal girls (34–36), and criteria have been proposed as a guide to indicate to whom ovarian cryopreservation should be offered (36–38). However, the experience within one center would suggest that these selection criteria do not necessarily target the desired patient group (36). Improved individualization of risk, for which some data are now available supporting the value of AMH in
adult cancer patient care (16), is particularly important in children in whom an invasive laparoscopic procedure is required to collect ovarian tissue for cryopreservation.

In conclusion, this is the first prospective study of AMH in girls receiving treatment for childhood cancer and provides evidence that serum AMH concentrations could be a clinically useful marker of the size of the remaining follicle pool (the ovarian reserve) after cancer in pre- and postpubertal girls.

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