Serum Levels of Anti-Müllerian Hormone as a Marker of Ovarian Function in 926 Healthy Females from Birth to Adulthood and in 172 Turner Syndrome Patients

Casper P. Hagen, Lise Aksglaede, Kaspar Sørensen, Katharina M. Main, Malene Boas, Line Cleemann, Kirsten Holm, Claus H. Gravholt, Anna-Maria Andersson, Anette T. Pedersen, Jørgen Holm Petersen, Allan Linneberg, Susanne Kjaergaard, and Anders Juul

Departments of Growth and Reproduction (C.P.H., L.A., K.S., K.M.M., M.B., A.-M.A., J.H.P., A.J.), Obstetrics and Gynecology (A.T.P.), and Clinical Genetics (S.K.), Copenhagen University Hospital, Rigshospitalet, DK-2100 Copenhagen, Denmark; Department of Pediatrics (L.C., K.H.), Hillerød Hospital, DK-3400 Hillerød, Denmark; Department of Endocrinology and Internal Medicine MEA (C.H.G.), Aarhus Sygehus, Aarhus University Hospital, DK-8000 C Aarhus, Denmark; Research Centre for Prevention and Health (A.L.), Glostrup University Hospital, DK-2600 Glostrup, Denmark

Context: In adult women, anti-Müllerian hormone (AMH) is related to the ovarian follicle pool. Little is known about AMH in girls.

Objective: The objective of the study was to provide a reference range for AMH in girls and adolescents and to evaluate AMH as a marker of ovarian function.

Setting: The study was conducted at a tertiary referral center for pediatric endocrinology.

Main Outcome Measures: We measured AMH in 926 healthy females (longitudinal values during infancy) as well as in 172 Turner syndrome (TS) patients according to age, karyotype (A: 45,X; B: miscellaneous karyotypes; C: 45,X/46,XX), and ovarian function (1: absent puberty; 2: cessation of ovarian function; 3: ongoing ovarian function).

Results: AMH was undetectable in 54% (38 of 71) of cord blood samples (<2; <2–15 pmol/liter) (median; 2.5th to 97.5th percentile) and increased in all (37 of 37) infants from birth to 3 months (15; 4.5–29.5 pmol/liter). From 8 to 25 yr, AMH levels were stable (19.9; 4.7–60.1 pmol/liter), with the lower level of the reference range clearly above the detection limit. AMH levels were associated with TS-karyotype groups (median A *vs.* B: <2 *vs.* 3 pmol/liter, $P = 0.044$; B *vs.* C: 3 *vs.* 16 pmol/liter, $P < 0.001$) as well as with ovarian function (absent puberty *vs.* cessation of ovarian function: <2 *vs.* 6 pmol/liter, $P = 0.004$; cessation of ovarian function *vs.* ongoing ovarian function: 6 *vs.* 14 pmol/liter, $P = 0.001$). As a screening test of premature ovarian failure in TS, the sensitivity and specificity of AMH less than 8 pmol/liter was 96 and 86%, respectively.

Conclusion: AMH seems to be a promising marker of ovarian function in healthy girls and TS patients. (***J Clin Endocrinol Metab*** **95: 5003–5010, 2010)**

Anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance is produced by primary and preantral ovarian follicles (1). In adult women, serum AMH level is thought to be a predictor of the follicle reserve with high AMH levels associated with high number of remaining ovarian follicles (2). A few studies have reported detectable AMH levels in serum from healthy girls at various ages (3, 4), but data are sparse. Little is known

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Abbreviations: AMH, Anti-Müllerian hormone; CV, coefficient of variation; POF, premature ovarian failure; TS, Turner syndrome.

about the physiological role of AMH, especially in girls during infancy, childhood, and adolescence. Inhibin B reflects ovarian function and serum levels are related to the menstrual cycle. Thus, high serum levels of inhibin B are found after ovulation, whereas low or undetectable levels are normal findings during the early follicular phase and in healthy prepubertal girls and young adolescents (5).

Girls with Turner syndrome (TS) have characteristic physical features and ovarian dysfunction as a result of a complete or partial absence of the second X chromosome, with or without mosaicism. The TS phenotype varies, depending on the specific karyotype. The classical X-monosomic TS (45,X) is usually associated with prenatal degeneration of the ovarian follicles and streak gonads (6). The density of ovarian follicles in subjects with mosaic TS (45,X/46,XX) is higher than in X-monosomic TS (7), and approximately 50% of TS girls with mosaicism (45,X/46,XX) have sufficient ovarian function to enter puberty spontaneously (8). The ovarian function of TS patients with miscellaneous karyotypes depends on the type of the X-chromosome abnormality (9).

Despite differences in follicular density, premature ovarian failure (POF) affects most TS patients, and only 5–10% obtain regular menstrual bleedings (8, 10). To our knowledge, only one previous study has reported AMH levels in TS patients, demonstrating that AMH levels correlated with the density of ovarian follicles (histologically examined) in TS girls and adolescents (7).

In this study, we present extensive normative data for circulating AMH levels, determined by an ultrasensitive assay, in females (0–69 yr), including longitudinal values of AMH in infancy. In addition, we report on AMH levels in 172 TS patients according to their age, karyotype, and ovarian function.

Materials and Methods

Healthy females

A total of 926 healthy females participated as controls. Seven hundred seventy-eight girls and adolescents (0–20 yr) were recruited as part of our population-based studies of healthy subjects: 1) Infant girls were included from an ongoing longitudinal cohort of healthy pregnant women and their offspring with blood sampling at 0, 3, and 12 months of age (n = 108), and at 4–6 yr of age (n = 53) (11, 12); 2) 6- to 20-yr-old girls and adolescents (n = 617) were included from the cross-sectional part of the Copenhagen Puberty Study (13, 14); and 3) 148 adult females (20.1–69 yr) were randomly selected from the Danish National Personal Register, as part of the Health 2006 study (15).

One serum sample was obtained from each girl, adolescent, and adult woman (4–69 yr). AMH levels from umbilical cords and infant girls (0–1.5 yr) were recorded in 108 controls. Longitudinal progress of circulating AMH during female infancy was established from multiple measurements (2–5) obtained from 46 controls (97 samples). Samples from both cord blood and 3 months of age were available in 37 individuals.

TS patients

Serum samples were available from a total of 172 patients from three different Danish TS cohorts: cohort I, 38 pediatric and adolescent TS patients seen at the Department of Growth and Reproduction and the Department of Obstetrics and Gynecology, Rigshospitalet; cohort II, 35 pediatric TS patients who were evaluated as part of an ongoing research project at the Department of Pediatrics, Hillerød Hospital; cohort III, 99 adult TS patients participating in an ongoing research project at the Department of Endocrinology and Internal Medicine, Aarhus Sygehus, Aarhus University Hospital. Multiple samples were recorded in 19 patients.

Karyotypes

Diagnosis of TS was confirmed by routine G-band karyotyping that included counting of at least 10 metaphases, three of which were fully analyzed. All karyotypes were validated by one of the investigators (S.K.). Depending on their karyotype, the patients were divided into three groups: group A, 45,X; group B, miscellaneous karyotypes; and group C, 45,X/46,XX.

Clinical examination

A thorough clinical examination was performed in all healthy girls and adolescents as well as in all TS patients, aged 0–25 yr. All TS patients were examined by pediatric endocrinologists. We have categorized TS patients (12–25 yr) into three groups at the time of AMH measurement.

TS patients were defined as having the following conditions.

Group 1 had absent spontaneous puberty in case of induction of puberty by hormone replacement therapy or prepubertal [breast stage 1 (B1), according to Tanner classification (16)] at older than 12 yr of age.

Group 2 had spontaneous puberty with cessation of ovarian function in case of spontaneous puberty [presence of B2 or higher (palpation of glandular breast tissue) or menarche without previous hormone replacement therapy], subsequently treated with estrogen due to lack of pubertal progression (pubertal arrest) or secondary amenorrhea.

TS patients with absent spontaneous puberty or cessation of ovarian function (groups 1 and 2) were defined as having POF.

Group 3 had spontaneous puberty with ongoing ovarian function in case of spontaneous puberty and ongoing pubertal progression or regular spontaneous menstrual bleeding.

AMH assay

Nonfasting blood samples were drawn between 0800 and 1700 h from an antecubital vein, clotted, centrifuged, and serum was stored at −20 C until hormone analyses were performed. All samples from controls and patients were analyzed after maximum 4 yr of storage in the freezer at −20 C.

All serum AMH measurements were performed in the same laboratory using the same assay. AMH levels were determined using the Immunotech Coulter enzyme immunometric assay (Immunotech, Beckman Coulter Ltd., Marseilles, France). In our hands, the sensitivity of the assay, defined as the mean concentration of the zero standard + 3 SD, was 2.0 pmol/liter. AMH levels were stable after 10 d storage of serum and unprocessed whole blood at 4 and 22 C, respectively. AMH in serum did not

change after 10 freeze-thaw cycles, and dilution of samples resulted in a linear decline in AMH levels. The intraassay coefficients of variation (CVs) were less than 7.8, 5.4, and 6.4% at 13, 123, and 231 pmol/liter, respectively. The interassay CVs were less than 11.6, 10.9, and 9.1% at 19, 99, and 209 pmol/liter, respectively, based on results from the first 152 assays (corresponding to three batches). In the two following batches, several of the low and medium controls were above +2 SD. All samples analyzed within these batches adjusted using batch-specific correction factors. A total of nine samples from TS patients and 38 samples from healthy infants were adjusted.

Inhibin B

We report inhibin B values from TS patients (12–25 yr) in whom AMH and inhibin B levels were available in the same serum sample. Inhibin B levels were measured by double-antibody immunometric assays (Serotec, Oxford, UK). The detection limit was 20 pg/ml and the intra- and interassay CVs were less than 16%. In TS cohorts I and II, part of the data has been described previously (19).

Statistics analyses

Reference curves for the skewed distribution of AMH as a function of age were calculated. The curves were obtained using generalized additive models for location, scale, and shape (18). This technique takes into account both the dependence on age and the skewed distributions of the hormone. The results were verified using locally weighted regression quintiles, a completely nonparametric technique. The curves represent the median, the 2.5th percentile, and the 97.5th percentile corresponding to the mean, the mean − 1.96 SD, and the mean + 1.96 SD, respectively.

In case of more than one AMH value in a single TS patient (8–25 yr), the mean AMH level was used in statistical evaluations.

AMH levels in cord blood, at 3 months of age and at 12 months of age, were compared using a Mann-Whitney *U* test. For other age intervals, the age dependency of AMH was assessed using Spearman correlations. To compare the prevalence of spontaneous puberty between TS karyotype groups, Fisher's exact test was used. AMH levels between groups of TS patients, 8–25 yr (karyotype groups and ovarian function groups), were compared using a Mann-Whitney *U* test. Receiver-operating characteristic curve was used to assess which cutoff level of AMH that resulted in the highest sensitivity and specificity of AMH as a screening test for premature ovarian failure in TS patients.

Ethical considerations

The studies on healthy girls were approved by the local ethical committee (no. KF 01 282214 and V200.1996/90) and conducted in accordance with the Second Helsinki Declaration. All children and parents received written information and were invited to an information meeting. The study was presented as a study on growth and puberty timing. All healthy girls and their parents gave informed consent. All participants in the Health 2006 study signed a written informed consent before the beginning of the study, and the study was registered (www.clinical-.trials.com; unique ID KA20060011). Data on patients with TS were collected as part of routine clinical follow-up (cohort I) as part of an ongoing research project (H-Ø-2004-2-24G, registered at www.clinical.trials.com; unique ID NCT00134745) (cohort II), or as part of an ongoing research project (no. 20010248; registered at the www.clinical.trials.com; unique ID NCT00624949) (cohort III).

Results

AMH levels in healthy females

Cord blood

AMH levels in cord blood were undetectable (54%, 38 of 71) or very low (<2 pmol/liter; <2–15.5 pmol/liter) (median; 2.5th to 97.5th percentile) (Fig. 1).

Infancy

AMH levels at 3 months of age were significantly higher (15 pmol/liter; 4.5–29.5 pmol/liter) than levels in cord blood (<2 pmol/liter; <2–15.5 pmol/liter) ($P < 0.001$) and at 12 months of age (8 pmol/liter; 3.0–18.9 pmol/liter) ($P < 0.001$). In the longitudinal follow-up, all infant girls (37 of 37) demonstrated a marked postnatal rise of AMH levels (Fig. 1).

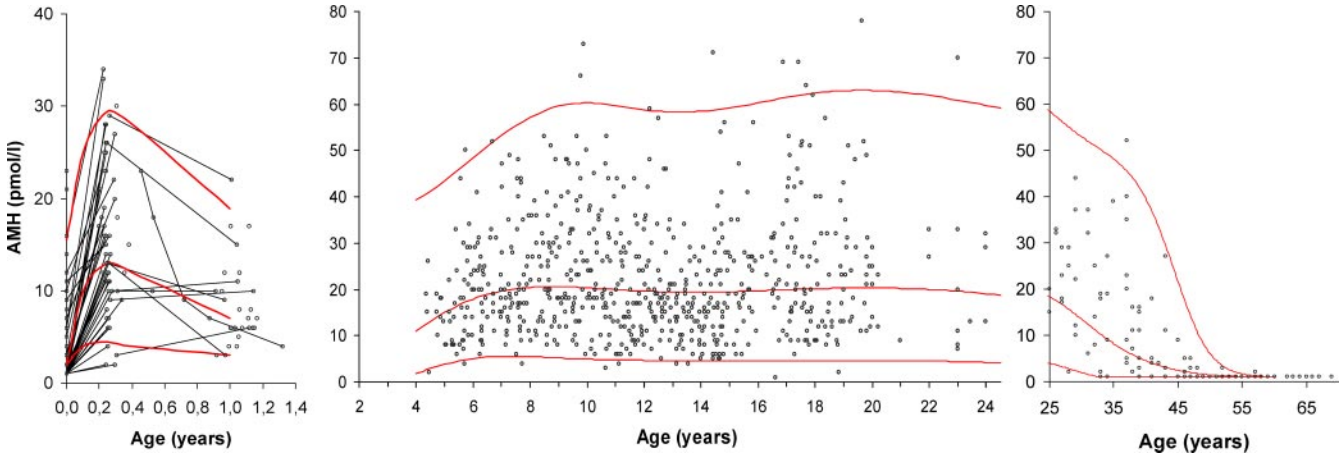

FIG. 1. Serum AMH (picomoles per liter) in 926 healthy infants, girls, adolescents, and adult women. Longitudinal values during infancy are connected with *black lines*. The *red curves* represent the median, the 2.5th percentile, and the 97.5th percentile.

Four to 8 yr of age

A linear increase of AMH levels was noted from 4 yr (10.9; 1.9–39.2 pmol/liter) to 8 yr of age (20.4; 5.5–57.1 pmol/liter) (r = 0.27; $P = 0.001$) (Fig. 1).

Eight to 25 yr of age

From mid childhood to early adulthood, AMH levels were relatively constant (19.9; 4.7–60.1 pmol/liter) (mean of median; mean of 2.5th to 97.5th percentile) (r = −0.046; $P = 0.285$) (Fig. 1). There were no significant fluctuations in mean AMH (and SD) levels between the pubertal stages (data not shown).

From 25 to 69 yr of age

After 25 yr of age, median AMH levels declined to undetectable levels (<2 pmol/liter) at 46.7 yr of age (Fig. 1).

AMH were detectable in serum from 746 of 747 infants (99.9%), girls and young adults (0–32 yr). Three adults older than 48 yr (5%) had detectable AMH levels, and the oldest woman with detectable AMH was 57 yr old.

AMH levels in TS patients

Descriptive characteristics of TS patients according to age and karyotype are shown in Table 1. Spontaneous puberty was demonstrated in 9, 56, and 100% in TS groups A, B (excluding gonadectomized patients), and C, respectively (A *vs.* B: $P < 0.001$; B *vs.* C: $P = 0.021$). In group B, spontaneous puberty and ongoing ovarian function was not restricted to patients with specific karyotypes. Forty-six percent of the TS patients (12–25 yr) who entered puberty spontaneously subsequently experienced cessation of ovarian function.

From 0 to 25 yr

Eighty-five percent of X-monosomic TS patients (34 of 40) had AMH levels less than −2 SD or undetectable AMH (Fig. 2A). Fifteen percent (six of 40) had AMH levels in the reference range. In patients with miscellaneous karyotypes, 43% (12 of 28) (excluding gonadectomized patients) had AMH levels in the reference range (Fig. 2B, *blue*). All patients (10 of 10) subjected to gonadectomy (11–47 yr) had undetectable AMH levels (Fig. 2B, *orange*). All TS patients (10 of 10) with 45,X/46,XX demonstrated AMH levels in the reference range (Fig. 2C). AMH levels were significantly different between TS karyotype groups (median; range of AMH in TS, group A *vs.* B: <2; <2–11 *vs.* 3; <2–33 pmol/liter, $P = 0.044$; in TS group B *vs.* C: 3; < 2–33 *vs.* 16; 8–58 pmol/liter, $P < 0.001$).

From 25 to 69 yr

Six percent (five of 88) of Turner women had AMH levels greater than 2 pmol/liter. Although values in group C were sparse, AMH levels seemed dependent on karyotype (Fig. 2, A–C).

TABLE 1. Descriptive characteristics of TS patients according to age and karyotype

TS karyotype	n < 12 yr	n 12–25 yr	n > 25 yr
Group A: 45,X	8	32	51
Group B: miscellaneous karyotype			
Without Y- chromosome material			
45,X/46,X,i (X)		1	
45,X/46,X,i (Xq)		2	9
45,X/46,X,i (X) (q11)		3	1
45,X/46,X,r (X)	1	8	4
45,X/46,X,der (X)		1	
45,X/46,X,del (X) (p11)		1	1
45,X/46,X,del (X) (q11)			2
45,X/46,X,idic (X)			1
45,X/46,X,idic (X) (p11)		1	
45,X/46,X,idic (Xq)			1
45,X/46,X,idic (X) (q21)		1	
45,X/46,X, +mar		1	1
45,X/46,XXp+			1
46,XX,del (X) (p11.1)		1	1
46,XX,del (X) (p22.1)		1	
46,XX,del (X) (q22.2)		1	
46,XX,del (X) (q23)		1	
46,X,I (Xq)			4
46,X,I (X) (q11)			1
46,X,idic (Xq)		2	
45,X/47,XXX		1	
45,X/46,XX/47,XXX			1
45,X/46,XX/46,X,idic (Xq)			1
45,X/46,X,i (Xq)/47,X,i (Xq), I (Xq)			1
45,X/46,X,i (Xq)/46,X,r (X)			1
45,X/46,X,r (X)/46,XX		1	
Containing Y-chromosome material			
46,X,r (Y)		1	
45,X/46,XY		3	2
45,X/46,X,r (Y)			1
45,X/46,X,dic (Y) (q12)		1	
45,X/46,X,i (Yq?)			1
45,X/45,X,t (Y; 22)	1		
Group C: 45,X/46,XX	2	8	2
Total	12	72	88

AMH correlated with ovarian function

AMH levels correlated significantly with remaining ovarian function in TS patients from 12 to 25 yr of age (Fig. 3) (median; range of AMH in patients with absent spontaneous puberty *vs.* patients with cessation of ovarian function was <2; <2–7 *vs.* 6 pmol/liter; <2–11 pmol/liter, $P = 0.004$; and in patients with cessation of ovarian function *vs.* patients with ongoing ovarian function, AMH was 6; <2–11 *vs.* 14; 2–36 pmol/liter, $P = 0.001$). In patients with cessation of ovarian function, there was no correlation between AMH levels and time from pubertal arrest or cessation of menses (Spearman coefficient, r = 0.475; $P = 0.165$). Twenty-five percent of patients (13 of 53) with

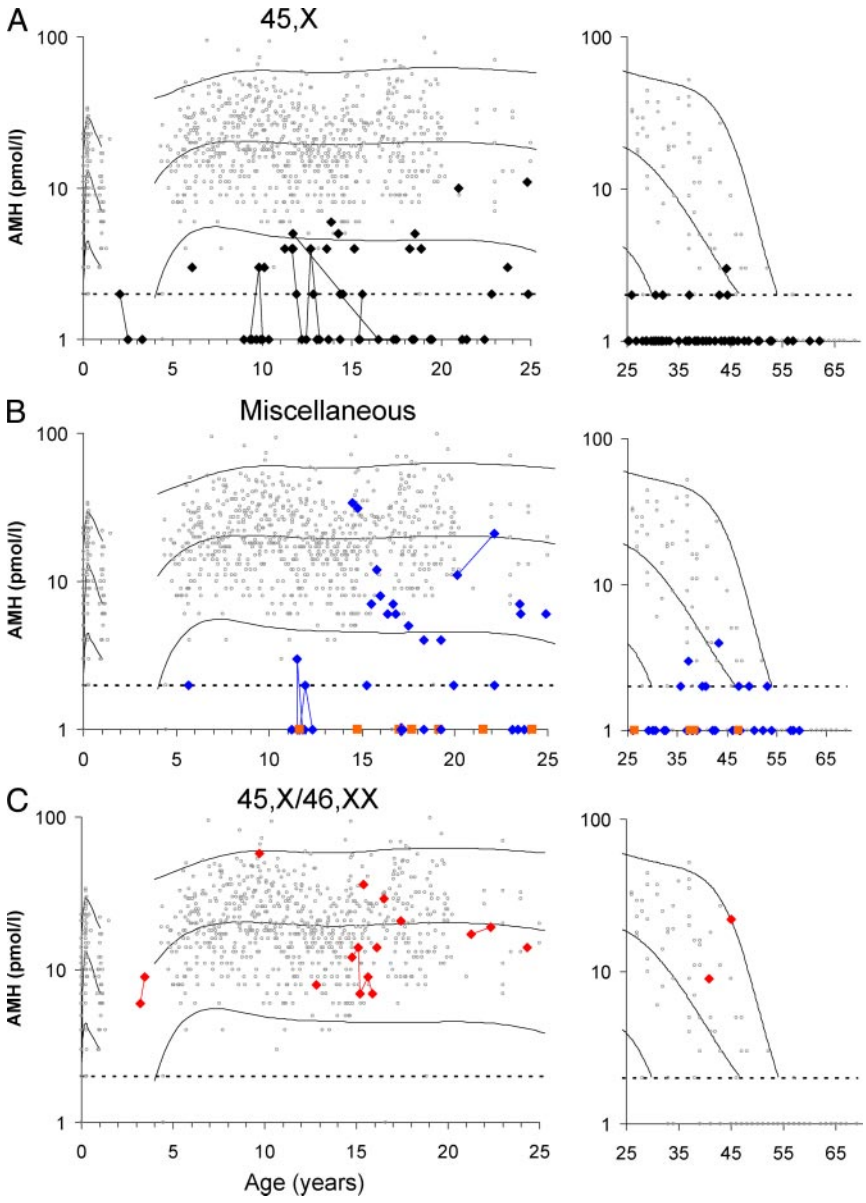

FIG. 2. AMH levels in patients with TS compared with a reference range based on 926 healthy Danish females. *Lines* represent median, the 2.5th percentile, and the 97.5th percentile. *Dotted lines* represent the detection limit of the assay. *Black*, Patients with 45,X monosomy (A); *blue*, miscellaneous TS karyotypes (B); *orange*, gonadectomized patients (B); *red*, 45,X/46,XX karyotype (C).

POF (absent spontaneous puberty or cessation of ovarian function) at time of AMH measurement had levels low in the reference range (6; 5–11 pmol/liter). The sensitivity and specificity of serum AMH as a screening test of POF in TS depend on the cutoff value of AMH. Based on the receiver-operating characteristic curve, a cutoff value of AMH at 8 pmol/liter was established. The sensitivity (probability of AMH $<$ 8 pmol/liter if the patient had POF) was 96% (51 of 53) and the specificity (probability of AMH $\geq$ 8 pmol/liter if the patients had ongoing ovarian function) was 86% (12 of 14). The positive predictive value (probability of POF if the patient had AMH $<$ 8 pmol/liter) was 96% (51 of 53).

Inhibin B levels in TS patients

Forty-nine patients (12–25 yr) had inhibin B and AMH measured in the same serum sample. Thirty-nine of 39 patients with POF and AMH less than 8 pmol/liter had undetectable inhibin B. Thirty-three percent (three of nine) of patients with ongoing ovarian function and AMH 8 pmol/liter or greater had undetectable inhibin B levels. One of one patient with ongoing ovarian function and AMH less than 8 pmol/liter had a detectable inhibin B level of 55 pg/ml.

Discussion

We present extensive normative data on serum AMH levels in 926 healthy infants, girls, and adolescents. To evaluate AMH as a marker of ovarian function, we report AMH levels in 172 TS patients. In healthy subjects, AMH levels rise during infancy, whereas levels are stable from childhood to early adulthood. In TS patients, AMH levels correlate significantly with specific karyotype and with remaining ovarian function. AMH seems to be an excellent marker of ovarian function in girls and adolescents.

Previous reports of AMH in healthy females have been limited by small numbers of subjects, inclusion of subjects with somatic illness, limited age ranges, or low-sensitivity assays (3, 4). In contrast to previous studies, our calculated lower limit of the normal range is clearly separated from the detection limit of the assay during childhood.

To our knowledge, our present longitudinal data in female infants are the first to demonstrate a marked increase in AMH levels from birth to 3 months of age, which is in line with previous cross-sectional findings (4). Activation of the hypothalamic-pituitary-ovarian axis during the so-called minipuberty at the first months of life in girls has previously been demonstrated (12), but AMH is not considered to be up-regulated by gonadotropins (1). AMH expression is limited to primary and preantral follicles (19, 20), and AMH inhibits both initial follicle recruitment (primordial to primary follicles) and FSH-de-

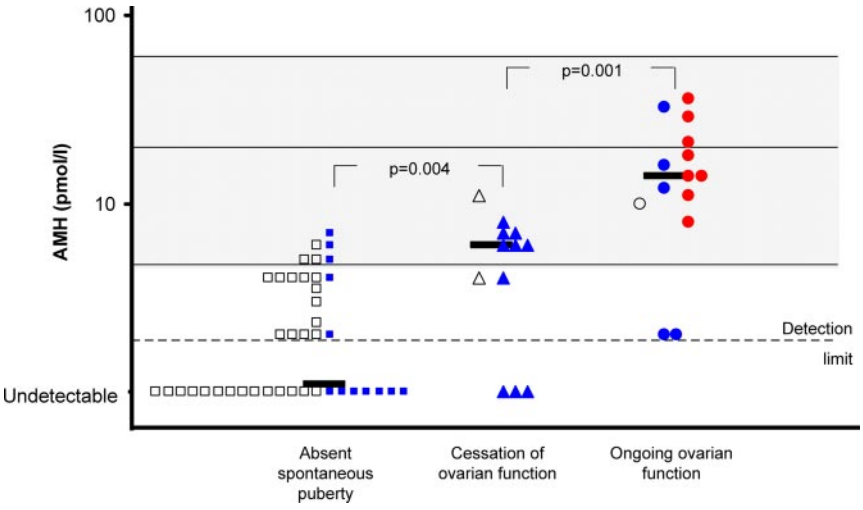

FIG. 3. AMH levels and ovarian function at time of AMH measurement in patients with TS, aged 12–25 yr. The reference range is marked by the *hatched area*, and *lines* represent the median, the 2.5th percentile, and the 97.5th percentile. *Dotted line* represents the detection limit of the assay. *Squares*, Patients with absent puberty; *triangles*, patients with cessation of ovarian function; *circles*, patients with ongoing ovarian function. *Thick black bars*, Median of AMH. *Black*, Patients with 45,X; *blue*, miscellaneous karyotypes; *red*, 45,X/46,XX.

pendent follicle growth (preantral and antral follicles) (21, 22). We speculate that rising levels of AMH during minipuberty may be an ovarian response to prevent FSH-induced follicle growth at a time of life when further differentiation of follicles would be inappropriate. In mice, AMH expression seems to be dependent on the specific developmental stage of the oocyte (23), but the mechanisms responsible for the rising AMH during human female infancy and from 4 to 8 yr remains to be elucidated.

The reference range of serum AMH outlined by the curves of 2.5th and 97.5th percentiles is wide throughout the entire stable period from 8 to 25 yr, in which healthy girls demonstrate AMH levels from 4.5 to 62 pmol/liter. There may be several explanations of this large variation in AMH levels observed in healthy girls and adolescents. Compared with other known biochemical markers of residual ovarian follicles in adults, such as FSH and inhibin B, AMH seems to better reflect the continuous decline of the follicle pool with age (24). Thus, variations in AMH levels during childhood may theoretically predict the duration of any given girl's reproductive life span, assuming that the speed of the continuous follicle loss is comparable between individuals. Some TS patients with POF had detectable AMH in the lower part of the reference range, and some healthy girls with similarly low AMH could therefore represent patients with concealed hypergonadotropic hypogonadism, at risk of premature ovarian failure. However, this is purely speculative, and longitudinal studies are required to confirm this hypothesis. Another explanation for the AMH variability in healthy females could be related to the presence of polycystic ovary syndrome (25, 26) and differences in body mass index and insulin levels (4, 27).

Differences in residual ovarian function among TS patients in relation to karyotype have previously been suggested (7, 8, 28) and are well in line with our present observations. In patients with 45,X who present with AMH in the reference range and apparently normal ovarian function, one cannot exclude the possibility that mosaicism with a normal cell line, not detected in peripheral blood, predominates in the ovaries (29). Some TS females have detectable levels of inhibin B without apparent menstrual cycling (30), and occasionally women with 45,X TS become pregnant without ever experiencing menarche or menstrual cycling (31). This highlights the obvious need for a useful clinical tool for the assessment of ovarian reserve. Our findings of a strong correlation between AMH levels and remaining ovarian function in TS patients, as well as the very low AMH levels in adult TS patients, support the hypothesis that AMH is a surrogate marker of the individual follicle pool (24, 32). A reevaluation of the raw data presented in the publication of Borgström *et al.* (7) reveals that AMH levels correlated significantly with the density of ovarian follicles (confirmed by histology) (Spearman coefficient, $r = 0.438$; $P = 0.003$). The TS cohort of girls and adolescents in that study was clinically comparable with our patients (prevalence of spontaneous puberty 34%).

The high predictive value of low serum AMH in relation to ovarian failure in TS patients suggests that AMH may be a clinically useful marker of ovarian failure in girls. Compared with other markers of ovarian failure during childhood and adolescence, AMH seems to have several advantages. Changes in AMH levels are observed in early phases of ovarian failure in young women (33), and there is no significant fluctuation of AMH during the menstrual cycle (34). At the time of expected puberty, primary ovarian failure usually gives rise to undetectable inhibin B and FSH hypersecretion, but during midchildhood gonadotropin secretion in girls with ovarian failure are not always elevated compared with controls (35). Furthermore, FSH levels are influenced by hormone replacement therapy. In contrast to AMH, undetectable inhibin B is a normal finding in 18% of healthy females aged 12–25 yr (5), which evidently affects the positive predictive value of undetectable inhibin B in relation to ovarian failure. Multiple measurements of exclusively undetectable inhibin B during childhood are predictive of premature ovarian failure in a

subpopulation of TS patients also included in this study. Whether a combination of AMH, inhibin B, and FSH could add to the specificity of the AMH screening test of ovarian failure during midchildhood remains to be elucidated.

In conclusion, we suggest that rising AMH levels during infancy in healthy girls may prevent inappropriate development of follicles by high FSH levels at the time of the postnatal gonadotropin surge. We found that AMH levels did not change in childhood and adolescence but showed large interindividual variability. AMH correlated significantly with adolescent ovarian function in TS and showed excellent sensitivity and specificity in the prediction of remaining ovarian function in such patients. Thus, AMH seems to be a promising marker of ovarian function in healthy girls and patients with TS and may be helpful in counseling of TS patients with regard to their fertility potential.

Acknowledgments

Address all correspondence and requests for reprints to: Casper Hagen, M.D., Department of Growth and Reproduction, GR, Rigshospitalet Section 5064, Blegdamsvej 9, DK-2100 Copenhagen O, Denmark. E-mail: casper.hagen@rh.regionh.dk.

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