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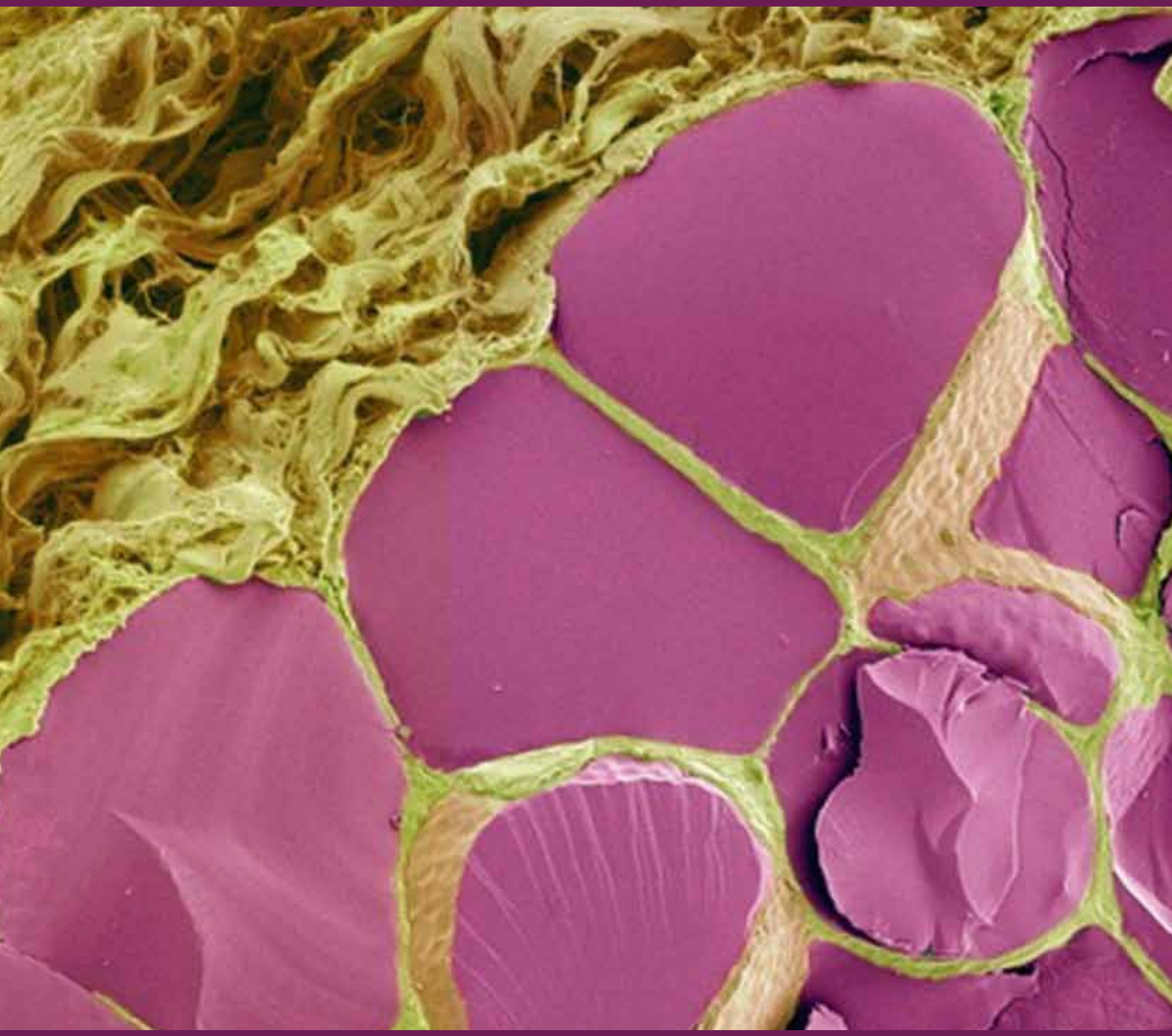
Anti-Mullerian Hormone: Usefulness in Clinical Practice

Guest Editors: Kai J. Buhling, Petra Stute, and Volker Ziller



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Editorial Board

Contents

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Editorial

Anti-Mullerian Hormone: Usefulness in Clinical Practice

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Anti-Mullerian hormone (AMH) is a protein that inhibits the development of the mullerian ducts (paramesonephric ducts) in the male embryo. AMH prevents the development of the mullerian ducts into the uterus and other mullerian structures in the first 8 weeks of gestational age. AMH is produced by granulosa cells of the ovary during the reproductive years and controls the formation of primary follicles by inhibiting excessive follicular recruitment by FSH.

Whereas AMH is nearby undetectable in females at birth, the level appears to be fairly stable in healthy young women from puberty to the age of 30 years.

Some endocrine disorders are correlated with elevated (PCOS) or lower AMH levels (premature ovarian insufficiency). Additionally some studies have shown that the dose of stimulation in assisted reproduction therapy (ART) is negatively correlated to serum AMH levels. Due to this association some authors stated that serum AMH reflects well the ovarian follicular reserve.

The aim of the special issue is to summarize the actual view and the clinical aspects of this interesting hormone in different reproductive life stages.

The colleagues N. Josso et al. and M. L. Johansen et al. give an actual statement about the use of AMH in the pediatrics endocrinology.

A. La Marca et al. have focused on the inter- and intraindividual changes and especially the impact of ethnicity and body mass index as well as smoking behaviours on the AMH levels. They also highlight the impact of hormonal suppression of the ovarian function and its influence on the AMH levels.

R. Tal and D. B Seifer give a review about the racial and ethnical differences.

One manuscript deals with the usefulness of AMH in the diagnosis of ovarian epithelial cancer. Another manuscript describes the AMH levels as a possible marker of reproductive function after different cancers in young women after chemotherapy.

Since AMH is also present in male, two manuscripts have been selected concerning this topic: the paper by E. Matuszczak et al. gives an overview about the physiology and pathology of AMH levels in male and the second one, written by R. P. Grinspon et al., deals with the AMH levels in precocious puberty treated with GnRH.

We believe that AMH and it is clinical usefulness are a topic that is of importance in scientific and clinical practice. This special issue provides important facts and adds to the actual discussion about the clinical usefulness of AMH. We also appreciate gaining some overview about the meaning of AMH in males.

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Research Article

Anti-Müllerian Hormone as a Sensitive Marker of Ovarian Function in Young Cancer Survivors

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We evaluated ovarian function by measuring the levels of anti-Müllerian hormone (AMH), estradiol, and gonadotropins in 83 young women treated for cancer during childhood and adolescence, and classified according to post-treatment gonadal toxicity versus 38 healthy females. *Results*. The mean AMH values were lower in the entire cohort independently of the risk group as compared to the control, whereas FSH was elevated only in the high risk group. The lowest AMH values were noted in patients after bone marrow transplantation (BMT) and those treated for Hodgkin lymphoma (HL). Nineteen patients (22.9%) had elevated FSH. They all had low AMH values. Lowered AMH values (but with normal FSH and LH) were observed in 43 patients (51.8%). There was no effect of age at the time of treatment (before puberty, during or after puberty) on AMH levels. *Conclusion*. Our results show the utility of AMH measurement as a sensitive marker of a reduced ovarian reserve in young cancer survivors. Patients after BMT and patients treated for HL, independently of age at treatment (prepuberty or puberty), are at the highest risk of gonadal damage and early menopause.

1. Introduction

The use of combined chemo- and radiotherapy for childhood cancer treatment has led to an increased survival rate and posed new challenges concerning health problems, organ damage, and quality of life after anticancer therapy. The function of different tissues and organs may be differently impaired by aggressive therapy. Gonads are particularly exposed to the deteriorating effects of certain chemotherapeutics and radiotherapy; on the other hand, when survivors reach adulthood, they wish to have their own biological children [1–3]. Fertility after anticancer therapy is a very important problem known by oncologists and endocrinologists as well as by cancer survivors themselves.

In women, gonadotoxic therapy damages the primordial follicles in the ovaries, which can lead to premature menopause. Very aggressive therapy such as myeloablative therapy prior to bone marrow transplantation (BMT) or surgery/ovariectomy can lead to total sterility, whereas indirect irradiation of the ovaries and chemotherapy can result in a lowered ovarian reserve [4–6].

In the last years, the measurement of anti-Müllerian hormone (AMH) has been used as an informative marker of the ovarian reserve. AMH is a product of granulosa cells of preantral and early antral follicles, capable of growing. In healthy women, AMH measurement is useful for the determination of the reproductive life span and the time of future menopause [7, 8].

The aim of our study was to determine the ovarian reserve in young women after anticancer treatment during childhood and adolescence, using the protocols with different degrees of gonadotoxicity.

2. Patients and Methods

We recruited 83 young women, cancer survivors, from the Department of Pediatric Oncology and Hematology (outpatient clinic), Medical University of Bialystok. At diagnosis,

they were from 0.9 to 17.8 years old ($x = 10.5 \pm 5.21$), and, at examination, they were 18.78 ± 4.98 years old. They had been treated for Hodgkin lymphoma, HL ($n = 22$), Wilms tumor ($n = 11$), soft tissue sarcoma, STS ($n = 7$), neuroblastoma ($n = 2$) germinal tumors ($n = 7$), acute myeloblastic leukemia ($n = 4$), acute lymphoblastic leukemia ($n = 22$) chronic myeloblastic leukemia ($n = 2$), and non-Hodgkin lymphoma ($n = 6$). They were all treated according to international protocols; 20 received irradiation for the infradiaphragmatic area (10 with HL), irradiation for the central nervous system (CNS)—9 patients with leukemia, 6 received bone marrow transplantation (1—total body irradiation, TBI—12 Gy).

All patients were classified according to a possible degree of gonadotoxicity proposed by Wallace et al.; the risk of disturbed fertility or infertility depends on diagnosis, stage, and type of treatment (alkylating agents, radiotherapy to the pelvis/ovaries). The probability of infertility in the low risk group is less than 20% and in the medium risk group between 20 and 80%, whereas, in the high risk group, is greater than 80%.

At diagnosis, forty-five patients were in Tanner stage T1-2, $n = 5$ in T3, and $n = 33$ in T4-5. On examination, one female presented with primary amenorrhea, 65 had normal, regular menses, and 17 had irregular menses or oligomenorrhea (11 were classified as high risk group, 2 as low risk, and 4 as middle risk group). Six patients have their biological children (five treated for HL, one treated for non-Hodgkin lymphoma, NHL).

Details concerning diagnosis, age at the time of therapy, type of therapy, the interval between the end of therapy, and measurements of hormone levels are presented in Table 1, taking into consideration the risk groups proposed by Brougham and Wallace [9].

Control group was composed of 38 healthy females aged 20.68 ± 4.34.

3. Hormone Level Measurements

Serum concentrations of FSH, LH, and E2 were measured in the same laboratory using the commercially available immunoenzymatic kits; serum AMH levels were determined with the EIA AMH/MIS kit (Immunotech, Beckman Coulter Company/Marseille, France). All hormonal measurements were performed in the early follicular phase (2–4 days of menstrual cycle) and stored at −80°C.

The study was approved by the local Medical Ethics Committee. Hormone measurements were made after an informed consent was obtained from the patients.

4. Results

We found higher FSH and lower AMH levels in the entire group as compared to the control group ($P = 0.001$; $P = 0.001$, resp.), whereas the mean levels of estradiol and LH were normal. When the study group was subdivided according to the risk of gonadotoxicity, the levels of FSH were elevated only in the high risk group (18.11 ± 28.7 mIU/mL versus 5.36 ± 1.89 mIU/mL, $P = 0.005$), whereas, in the middle and low risk groups, they were comparable with the control group. AMH values were lower than those in the control group in all the three risk groups (HR group 14.14 ± 13.26 pmol/L ($P = 0.001$); MR group 14.82 ± 16.2 pmol/L ($P = 0.019$); LR group 19.44 ± 13.96 pmol/L ($P = 0.053$)). Mean serum LH and estradiol values did not differ between the risk groups and control (see Table 2).

The HR group was analyzed separately: patients diagnosed with HL irradiated and nonirradiated for the infradiaphragmatic region, patients treated for solid tumors with radiation to the infradiaphragmatic area, and patients after bone marrow transplantation. In these subgroups, AMH values were lower than those in the control group, being the lowest in patients after bone marrow transplantation (3.37 ± 2.32 pmol/mL). FSH levels were the highest in females after BMT (42.55 ± 26.55 mIU/mL) and elevated in females treated for HL with inverted Y irradiation. The values of LH and estradiol did not differ between the HR group and control (except the patients after BMT) (see Table 3).

There were 19 females (22.9%) in the study group with elevated FSH levels (>10 mIU/mL), AMH lower than 12.5 pmol/L, and normal LH levels; 12/19 derived from the HR group. They all presented low AMH values. Lowered AMH levels (yet with normal FSH and LH) were observed in 43 patients (51.8%).

We found no influence of age at the time of treatment (before puberty, during or after puberty), although AMH was lower in patients treated after puberty (13.04 ± 12.06 pmol/L) than during puberty (15.43 ± 13.65 pmol/L) and before puberty (18.52 ± 14.93 pmol/L).

5. Discussion

Combined anticancer treatment has improved the prognosis for young patients and at the same time has enabled us to recognize different late effects of the treatment. Diminished fertility or infertility and early menopause are the major side effects lowering life quality among cancer survivors. According to Childhood Cancer Survivor Study (CCSS), premature menopause occurs in 8% survivors and depends on age, dose of irradiation to the ovaries, and cumulative dose of alkylating agents [10]. Brougham and Wallace classified the most common cancers treated during childhood and adolescence according to the risk of subfertility resulting from gonadotoxicity. The high risk group includes patients after TBI, megachemotherapy, tumors located in pelvis and irradiated, metastatic soft tissue sarcomas, and Hodgkin lymphomas treated with alkylating agents. The risk of impaired fertility in this group is more than 80%, as compared to less than 20% in the low risk group [9]. Our knowledge concerning the toxicity of anticancer treatment enlarges, and treatment protocols change, not only for better outcome and improved survival but also for a reduction in side effects. We categorized our patients according to the type of malignancy and treatment, mainly the area of radiotherapy and total dose of alkylating agents.

To evaluate ovarian function we analyzed the levels of FSH, LH, estradiol, and AMH. In males, it is easier to evaluate

Table 1: Characteristic of patients classified according to different risk groups and related treatment.

Diagnosis	*n*	Age at diagnosis (y)	Age at exam (y)	Time off treatment (y)	Chemotherapy (doses of gonadotoxic chemotherapeutics)	Radiotherapy
HR	38					
HL	22	15.2 ± 2.6	21.35 ± 4.4	6.03 ± 3.8		
IIA, IIB	12				3 × MVPP + 3 × B-DOPA Dacarbazine 900 mg/m^2 Procarbazine 3000 mg/m^2 Nitrogen mustard 36 mg/m^2	Supradiaphragm 20 Gy
IIIA	7				3 × MVPP + 3 × B-DOPA	Supradiaphragm 20 Gy Infradiaphragm 15 Gy
IIIB	3				4 × MVPP + 4 × B-DOPA Dacarbazine 1200 mg/m^2 Procarbazine 4000 mg/m^2 Nitrogen mustard 48 mg/m^2	Supradiaphragm 20 Gy Infradiaphragm 15 Gy
BMT	6				Busulphane 3 × 16 mg/kg/day Cyclophosphamide 4 × 0.2 g/kg/day	
CML	2					
AML	2					TBI 12 Gy (1 pt)
ALL	2					
STS II/III	3				CWS 90 Ifosfamide 37.5 g/m^2 (Actinomycin D, vincristine) Dacarbazine 2.25 g/m^2 Cyclophosphamide 5 g/m^2	Supradiaphagm 20 Gy (1 pt) Infradiaphragm (49.6 Gy—1 pt, 25 Gy—1 pt)
NHL III	1				LMB Cyclophosphamide 5.8 g/m^2	Infradiaphragm 15 Gy
Wilms tumor	6				SIOP (Actinomycin D, vincristine, and epirubicine)	Infradiaphagm 15 Gy—2 pts 20 Gy—2 pts
III-5 V-1					Ifosfamide 36, actinomycin D, and vincristine	25 Gy—1 pt 40 Gy—1 pt
MR	12	8.04 ± 5.5	18.36 ± 5.7	8.7 ± 4.4		
AML	2				AML—BFM 90 Cytarabine 44.7 g/m^2	CNS—18 Gy
STS II	3				CWS 96 Ifosfamide 37.5 g/m^2—1 pt 42 g/m^2—1 pt 48 g/m^2—1 pt	Supradiaphragm 20 Gy
NBL II	2				PACE Cisplatin—2 g/m^2	—
NHL II	5				BFM 95 Cyclophosphamide 3 g/m^2 Ifosfamide 8 g/m^2	—
LR	33	7.8 ± 5.3	17.25 ± 5.0	9.0 ± 4.8		
ALL	22				BFM 90 Cyclophosphamide—3.0 g/m^2 Without cht—4	CNS 12 Gy—5 pts CNS 18 Gy—2 pts
Germinal tumors	7				TGM 95 (Cisplatin, ifosfamide, and etoposide)	Unilateral ovariectomy (7)
Wilms tumor II	5				Actinomycin D, vincristine	

HR: high risk group, MR: medium risk group, LR: low risk group, HL: Hodgkin lymphoma, BMT: bone marrow transplantation, CML: chronic myeloid leukemia, AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, STS: soft tissue sarcoma, NHL: non-Hodgkin lymphoma, NBL: neuroblastoma, CNS: central nervous system, and TBI: total body irradiation.

Table 2: Serum levels of FSH, LH, E2, and AMH in female cancer survivors according to risk groups and comparison to control group.

Study group	n	Hormones	X	M	SD	P
Whole group	83	FSH (mIU/mL)	12.24	7.01	19.41	0.001
HR	38		18.11	7.64	28.71	0.005
MR	12		7.43	7.16	2.77	0.964
LR	33		8.95	6.44	9.63	0.679
Control	34		5.36	5.15	1.89	
Whole group	83	LH (mIU/mL)	7.73	4.49	11.32	0.449
HR	38		10.23	4.10	17.01	0.106
MR	12		5.34	4.42	4.33	1
LR	33		6.44	4.77	5.15	0.924
Control	34		5.52	4.68	4.1	
Whole group	83	E2 (pg/mL)	45.42	32.5	54.72	0.057
HR	38		52.21	33.29	69.20	1
MR	12		51.39	17.60	75.46	0.999
LR	33		38.38	33.20	28.99	0.491
Control	34		53.32	47.39	41.14	
Whole group	83	AMH (pmol/L)	16.7	11.58	14.13	0.001
HR	38		14.14	9.89	13.26	0.001
MR	12		14.82	8.54	16.19	0.019
LR	33		19.44	19.00	13.96	0.053
Control	34		27.03	25.26	12.31	

HR: high risk, MR: medium risk, LR: low risk, FSH: follicle-stimulating hormone, LH: luteinizing hormone, E2: estradiol, AMH: anti-Müllerian hormone, X: average value, and M: median.

Table 3: Hormone levels in female cancer survivors classified to the high risk group.

Analyzed HR group	n	Hormones	X	M	SD	P
HL rtx −	12	FSH (mIU/mL)	7.07	7.0	2.78	0.647
HL rtx +	10		8.53	6.9	3.25	0.045
BMT	6		42.55	42.55	26.55	0.034
Solid tumors	9		10.55	7.9	14.68	0.068
Control	34		5.35	5.15	1.89	
HL rtx −		LH (mIU/mL)	6.13	3.85	6.67	0.283
HL rtx +			3.63	3.00	2.05	0.483
BMT			26.31	15.20	28.23	0.001
Solid tumors			6.45	7.0	5.93	0.494
Control			5.52	4.68	4.1	
HL rtx −		E2 (pg/mL)	69.72	42.79	101.82	0.628
HL rtx +			47.34	33.09	32.24	0.578
BMT			26.25	22.74	17.26	0.021
Solid tumors			40.91	17.6	47.29	0.670
Control			53.32	47.39	41.14	
HL rtx −		AMH (pmol/L)	23.17	25.10	15.25	0.042
HL rtx +			14.15	11.90	8.10	0.037
BMT			3.37	2.62	2.32	0.001
Solid tumors			19.21	8.14	16.30	0.05
Control			27.03	25.26	12.31	

HR: high risk, HL: Hodgkin lymphoma, rtx: radiotherapy, BMT: bone marrow transplantation, FSH: follicle-stimulating hormone, LH: luteinizing hormone, E2: estradiol, AMH: anti-Müllerian hormone, X: average value, and M: median.

gonadal function by analyzing spermiograms and hormone measurements; in females, assessment of oocyte depletion and premature ovarian failure is more difficult. AMH seems to be very useful to determine the ovarian reserve, better than the evaluation of the antral follicle count by vaginal ultrasonography or the measurement of FSH and inhibin B. The AMH level is relatively constant from mid-childhood to early adulthood, without fluctuations between pubertal stages [7, 11–13]. Elevated FSH levels were observed only in the HR group, whereas lower AMH (compared to the control group) was found in the total cohort independently of risk group. More than half (51.8%) of the patients had low AMH levels, whereas 22.9% presented with elevated FSH values. Abnormalities were most pronounced in patients after BMT, who had very low AMH values and elevated FSH. Total body irradiation and/or high doses of alkylating agents, such as cyclophosphamide or busulphane, led to ovary dysfunction. Similar results have been reported by Miyoshi et al., who found low AMH levels in 53% and high FSH in 30% of childhood cancer survivors [14]. Fong et al. observed accelerated loss of primordial follicles in females after TBI before stem cell transplantation [15]. The treatment for HL also leads to high risk of premature menopause [16, 17]. De Bruin et al. found a 12-fold higher risk of early (before the age of 40) menopause in HL survivors treated with procarbazine as an element of chemotherapy as compared to those irradiated for the supradiaphragmatic areas or paraaortic nodes [18]. Like in our study, van Beek et al. found that hormone levels were not influenced by age at treatment (before versusduring puberty) [19]. Different results have been presented for women treated at an age older than 30 years since AMH levels fall gradually due to a reduced oocyte pool [20, 21]. In the patients treated for HL, lower AMH values were noted for the irradiated and nonirradiated infradiaphragmatic areas, whereas elevated FSH levels were observed only in irradiated females. Those who are irradiated for the infradiaphragmatic region received 3 or 4 cycles with procarbazine (MOPP), and those who are nonirradiated received only two cycles. In the former, the gonadotoxic effect might result from the combined effect of chemo- and radiotherapy. Patients treated for solid tumors, irradiated for the abdomen, also presented with lower AMH levels; in that group, only one female was irradiated (44 Gy) for the pelvic area—she had primary amenorrhea and received hormonal therapy. Irradiation directly for the ovaries, especially in the total dose >15 Gy, seems to be the most important factor deteriorating gonadal function [22], although according to Wallace et al. the LD50 for human oocyte is <2 Gy [23].

We observed lowered AMH levels not only in the HR group but also in the MR or LR group, which indicates that all types of anticancer treatment affected gonadal function, even when low doses of chemotherapeutics were used. Patients treated for acute lymphoblastic leukemia, classified to the low risk group, show subtle ovarian disorders (lower estradiol levels) [24] and some of them are infertile after anticancer treatment [25]. In a prospective study performed during and after cytotoxic treatment, Brougham et al. observed progressive lowering of AMH during treatment and recovery in the low and middle risk groups between 2 and 12 months following therapy completion, thus indicating possible restoration of the pool of growing oocytes. This recovery was not observed in the high risk group, suggesting a profound loss of the primordial follicle pool [26].

Sixty-five out of 83 survivors had normal regular menstrual cycle, one had primary amenorrhea, and 17 had irregular menses or oligomenorrhea; the latter group included patients after BMT after HL treatment (HR group). The group with normal menstrual cycles contained patients with lower AMH levels and with a diminished ovarian reserve.

We found elevated FSH in the early follicular phase with normal LH and estradiol levels; this situation is characteristic of premature ovarian failure and can appear even 20 years prior to menopause [19, 27, 28]. Taken together, lowered mean AMH values in the entire cohort and a monotropic rise in FSH indicate the possibility of premature menopause [29]. Our results suggest older biological ovarian age as compared to the chronological one. Six patients had their biological children; five were treated for HL and were classified to the HR group. The peak incidence for HL is observed in older adolescents, most often over 15 years of age. Female cancer survivors should be informed that their "fertility window" is shorter than that of the general population [30].

In conclusion, our results show the utility of AMH measurement as an early, sensitive marker of a reduced ovarian reserve in young cancer survivors. Patients after conditioning therapy prior to BMT as well as patients treated for HL, independently of age at treatment (prepuberty or puberty), are at the highest risk of gonadal damage and early menopause.

Conflict of Interests

The authors declare that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

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Review Article

Anti-Müllerian Hormone: A Valuable Addition to the Toolbox of the Pediatric Endocrinologist

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Anti-Müllerian hormone (AMH), secreted by immature Sertoli cells, provokes the regression of male fetal Müllerian ducts. FSH stimulates AMH production; during puberty, AMH is downregulated by intratesticular testosterone and meiotic germ cells. In boys, AMH determination is useful in the clinical setting. Serum AMH, which is low in infants with congenital central hypogonadism, increases with FSH treatment. AMH is also low in patients with primary hypogonadism, for instance in Down syndrome, from early postnatal life and in Klinefelter syndrome from midpuberty. In boys with nonpalpable gonads, AMH determination, without the need for a stimulation test, is useful to distinguish between bilaterally abdominal gonads and anorchism. In patients with disorders of sex development (DSD), serum AMH determination helps as a first line test to orientate the etiologic diagnosis: low AMH is indicative of dysgenetic DSD whereas normal AMH is suggestive of androgen synthesis or action defects. Finally, in patients with persistent Müllerian duct syndrome (PMDS), undetectable serum AMH drives the genetic search to mutations in the AMH gene, whereas normal or high AMH is indicative of an end organ defect due to AMH receptor gene defects.

1. Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS) or factor (MIF), is a member of the transforming growth factor-β (TGF-β) secreted essentially by fetal and prepubertal Sertoli cells and to a lesser amount by granulosa cells of small follicles. AMH plays a biological major role in shaping the male reproductive tract by triggering the regression of male fetal Müllerian ducts while androgens, secreted by the Leydig cells present in the interstitial tissue, are responsible for the stabilization of the Wolffian ducts and their differentiation into male accessory organs as well as for the virilization of the urogenital sinus and the external genitalia. In males lacking AMH, the persistence of Müllerian derivatives coexists with the development of normal male external genitalia. It follows that clinical applications of AMH in pediatric endocrinology are essentially diagnostic and restricted to boys. In recent years, AMH has gained great importance in gynecology and obstetrics, due to its value as a marker of ovarian reserve but this clinical application does not concern pediatricians and will not be considered here. Several AMH ELISA kits are commercially available as discussed elsewhere in this issue.

2. Ontogeny and Regulation of Testicular AMH Production

AMH is a homodimeric glycoprotein member of the TGF-β family. It is initially secreted as a precursor, subsequently cleaved to yield 110 kDa N-terminal and 25-kDa C-terminal homodimers, which remain associated as a biologically active noncovalent complex [1]. Dissociation of the noncovalent complex occurs at the time of binding to type II AMH receptor and is required for biological activity [2]. The major site of AMH production is the immature Sertoli cell. In the late fetal

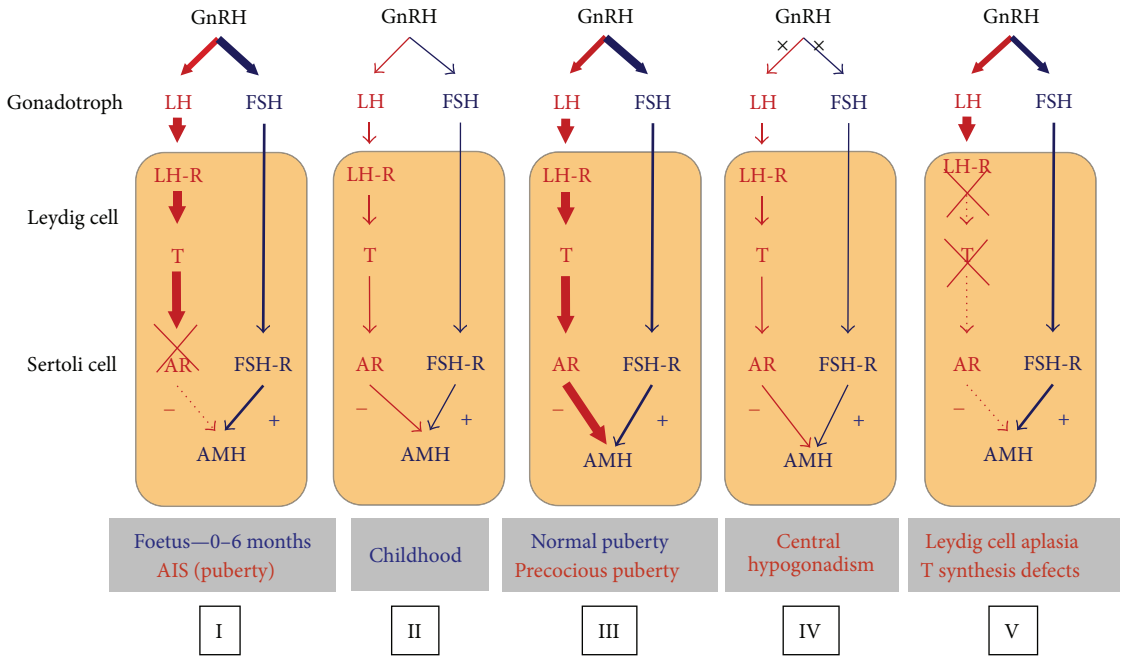

Figure 1: Regulation of testicular AMH secretion by gonadotropins and androgens. In general, the hypothalamus regulates LH and FSH secretion by the gonadotroph through the gonadotropin-releasing hormone (GnRH). LH acts on the LH receptor (LH-R) present in Leydig cells, inducing testosterone (T) secretion. FSH acts on the FSH receptor (FSH-R) present in Sertoli cells. The hypothalamic-pituitary-gonadal axis is active in the foetus and early infancy, is quiescent during childhood, and is reactivated at puberty. FSH is a moderate inducer of AMH secretion, whereas T, acting through the androgen receptor (AR), is a potent inhibitor of AMH production. In the normal foetus and infant, as well as in patients with the androgen insensitivity syndrome (AIS), the lack of AR expression results in high AMH production by Sertoli cells (I). During childhood, there is a physiologic hypogonadotropic state resulting in very low T; AMH levels remain high, but somewhat lower probably due to the lack of FSH stimulus (II). In normal or precocious puberty, T prevails over FSH, resulting in AMH inhibition (III). In congenital central hypogonadism, AMH is lower than in the normal boy because of the longstanding lack of FSH from foetal life; however, at pubertal age, the inhibitory effect of T is also absent, and AMH remains higher than in normal puberty (IV). In Leydig cell-specific primary hypogonadism (Leydig cell aplasia or hypoplasia due to LH-R defects or defects of steroidogenesis), the inhibitory effect of androgens is absent, and AMH levels are high. The orange area represents the testis. Thickness of lines is in correlation with hormone effect on its target. From [5], Copyright Karger AG, 2010, with permission.

and postnatal ovary, it is also produced by granulosa cells of developing follicles, essentially preantral and small antral follicles [3, 4].

In the male, AMH is a specific functional marker of the immature Sertoli cell. AMH expression is initiated at the time of fetal differentiation of the seminiferous cords, by the end of the 7th embryonic week, and remains at high levels until the onset of puberty, except for a transient decline in the perinatal period [6, 7]. AMH expression is triggered by SOX9, which binds to the AMH promoter; subsequently, SF1, GATA4, and WT1 further increase AMH promoter activity (reviewed in [8]). The onset of AMH expression and its basal expression level throughout life are independent of gonadotropins. However, FSH stimulates testicular AMH production by both inducing Sertoli cell proliferation and upregulating AMH transcription [9]. The latter is mainly mediated by the classical pathway involving Gsα and adenylyl cyclase increase of cyclic AMP levels, which stimulates protein kinase A (PKA) activity, subsequently involving transcription factors SOX9, SF1, GATA4, NFκB, and AP2 [10–12]. During puberty, AMH production is inhibited by the increase of intratesticular testosterone concentration and the onset of germ cell meiosis (Figure 1) (reviewed in [13, 14]). The inhibitory effect of androgens on AMH overcomes the positive effect of FSH after pubertal onset. On the contrary, androgens cannot inhibit AMH production in the fetal and neonatal testis, where Sertoli cells do not yet express the androgen receptor [15–17].

3. AMH in Boys with Hypogonadism

Gonadotropin and testosterone, which are high in the 3–6 months after birth, normally decrease to very low levels until the onset of puberty. Therefore, their usefulness as markers of the function of the hypothalamo-pituitary-gonadal axis in the boy is limited. On the contrary, AMH determination is extremely useful, since Sertoli cells remain active during infancy and childhood [18, 19]. Serum AMH reliably reflects the presence and function of testes in prepubertal boys, without the need for any stimulation test [18, 20, 21]. In this section, we address how the different disorders causing hypogonadism affect AMH testicular production.

3.1. Central Hypogonadism. Serum AMH is low in infants with congenital central hypogonadism. Treatment with FSH

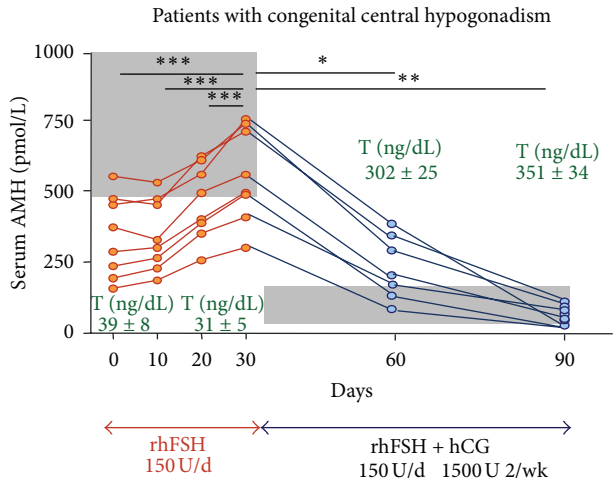

Figure 2: AMH levels in central hypogonadism. Serum AMH was low for Tanner stage I (prepubertal) in patients with previously untreated central hypogonadism. Initial treatment with recombinant human FSH (rhFSH) during 30 days resulted in an elevation of serum AMH in all 8 patients, while testosterone (T) remained at prepubertal levels. Shaded area represents normal AMH for Tanner I stage, according to T levels observed in these patients. Subsequent addition of hCG treatment resulted in an elevation of T which provoked a decline in serum AMH. Shaded area represents AMH values for Tanner IV-V stages, according to T levels observed in the treated patients. From [5], Copyright Karger AG, 2010, with permission.

results in an elevation in serum AMH in correlation with an increase in testis volume [22]. In patients of pubertal age with untreated congenital central hypogonadism, serum AMH is elevated for age—because the insufficient testosterone production is unable to downregulate AMH, but lower than expected for patient's Tanner stage [23, 24]—reflecting the lack of FSH stimulus. FSH treatment results in an increase in serum AMH; subsequent treatment with hCG induces androgen production, which provokes a physiological decline in AMH (Figure 2). Interestingly, inhibition of AMH does not occur when patients are treated with exogenous testosterone, which reflects that intratesticular testosterone levels remain low [24].

3.2. Primary Hypogonadism. In patients with sex-chromosome aneuploidies resulting in Klinefelter syndrome (47,XXY), no overt signs of hypogonadism are evident during infancy and childhood: AMH, inhibin B, and FSH levels are normal. However, from midpuberty Sertoli cell function deteriorates progressively, resulting in extremely low or undetectable AMH and inhibin B levels, very high FSH, and small testis volume [25].

Unlike Klinefelter syndrome, the somatic aneuploidy of Trisomy 21 (Down syndrome) results in early-onset primary hypogonadism in a large proportion of cases. Serum AMH is low from infancy [22].

Patients with Prader-Willi syndrome have hypogonadism leading to small genitalia and arrested pubertal development, classically attributed to hypothalamic dysfunction. However, recent investigations have demonstrated that the disorder may also be due to primary hypogonadism, with low AMH and testosterone levels associated with normal to moderately elevated gonadotropins [26–28] or to a combined form of hypogonadism, with low testicular hormones and inadequately normal gonadotropins [27, 29].

The X-linked form of adrenal hypoplasia congenita associated with hypogonadism resulting from mutations in the DAX1 gene is another example of combined (central + primary) hypogonadism. These patients have low serum AMH and inhibin B and defective androgen response to hCG, indicative of a primary testicular failure. At pubertal age, gonadotropin levels remain inadequately normal in spite of the lack of negative feedback resulting from low inhibin B and testosterone, which indicates that gonadotrope function is also impaired [30].

3.3. Cryptorchidism. Cryptorchidism is a sign that can be present in many disorders of different etiologies, most of which remain elusive [31, 32]. Dissociated testicular dysfunction primarily affecting the tubular compartment seems to be the underlying pathophysiology in cases presenting with low AMH [33] and inhibin B [34] but with normal testosterone and INSL3 [35] during early infancy and childhood. In other cases, no significant changes in hormone levels could be detected [36]. The apparently contradictory results are most probably due to the heterogeneity of the cryptorchid patients with underlying conditions of different etiologies and prognoses. Bilateral cryptorchidism with nonpalpable gonads should be distinguished from anorchia. Vanishing or regression of testicular tissue occurring in late fetal life, once sex differentiation has occurred, is associated with male genitalia, micropenis, and hypoplastic scrotum. Later in postnatal life, anorchia should be distinguished from bilateral cryptorchidism with abdominal testes. Serum AMH is undetectable in anorchid boys but detectable in boys with abdominal gonads (Figure 3) [20, 21, 37].

In Noonan syndrome, cryptorchidism occurs in approximately 2/3 of the cases. During childhood, reproductive hormones are within the expected range. Pubertal onset is delayed; by mid- to late puberty, gonadotropin levels increase over the normal range and AMH and inhibin B decline to subnormal levels in patients with a history of cryptorchidism but remain within normal levels in those with descended testes [38].

4. AMH in Disorders of Sexual Differentiation

The development and differentiation of the sex organs during fetal life involve three successive steps: (1) the early morphogenesis of the gonadal and genital primordia, which is identical in XY and XX embryos; (2) the differentiation of the gonadal ridge into a testis or an ovary; (3) the differentiation of the primordia of the internal and external genitalia, which are virilized by the action of androgens and AMH or feminized in their absence.

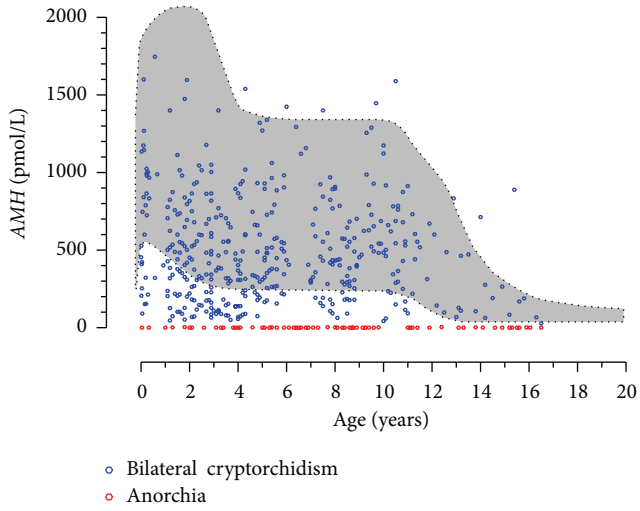

Figure 3: Serum AMH levels are useful to distinguish between bilateral cryptorchidism with abdominal testes and anorchism. Serum AMH is undetectable in anorchid patients; in patients with bilaterally abdominal testes, serum AMH is always detectable ranging from subnormal to normal values according to the functional status of the gonads. Shaded area represents the normal serum AMH range (3th–97th centiles), according to [22].

Based on the recognition of the cause of abnormal sex organ development in patients bearing a Y chromosome (Table 1), disorders of sex development (DSD) may be divided into (a) malformative DSD, where abnormal morphogenesis of the genital primordia occurs in early embryonic life; (b) dysgenetic DSD, due to abnormal gonadal differentiation resulting in insufficient secretion of androgens and AMH; and (c) nondysgenetic DSD, in which the abnormal sex hormone-dependent genital differentiation results from specific defects in the production or action of androgens or AMH.

4.1. AMH in Malformative DSD. Defects in the early morphogenesis of the Müllerian or Wolffian ducts, the urogenital sinus, or the primordia of the external genitalia, for example, cloacal malformations, isolated hypospadias, or aphallia, usually occur in eugonadal patients. Therefore, serum AMH and testosterone levels are within the expected range for sex and age. From a practical standpoint, nonendocrine related DSD should be considered when there is inconsistency in the development of the different elements of the genitalia. For instance, isolated hypospadias, with no other signs of hypovirilization, in patients with normal AMH and androgen levels is most probably due to early morphogenetic defects [39, 40]. In most cases, endocrine-unrelated malformations of the genitalia are associated with other somatic dysmorphic features, like in Robinow syndrome due to *ROR2* mutations, Pallister-Hall syndrome due to *GLI3* mutations, or many other polymalformative associations of unknown etiology. "Idiopathic" persistence of Müllerian derivatives (PMDS) in patients with a normal AMH level, mutation-free AMH, and AMH receptor genes may belong to the same category (see below).

4.2. AMH in Dysgenetic DSD. Gonadal dysgenesis established in the first trimester of fetal life represents the earliest form of primary hypogonadism and prevents the normal hormone-driven differentiation of the sex organs. In the fetus carrying a Y chromosome, gonadal dysgenesis results in female or ambiguous genitalia, reflecting the degree of testicular hormone deficiency. Serum AMH is low or undetectable, depending on the amount of testicular tissue remaining [41] (Table 1 and Figure 4). Serum AMH observed in a newborn with ambiguous genitalia should be compared with reference levels for the adequate age period to avoid overdiagnosis of dysgenetic DSD. AMH levels are transiently lower during the first 2-3 weeks after birth in the normal newborn [6, 7, 22]; when in doubt, a repeat measurement to assess the evolution of serum AMH may be helpful [42].

In 45,X or 45,X/46,XX patients, gonads are reduced to fibrous streaks or develop into dysgenetic ovaries. Serum AMH levels reflect the amount of small follicles present in these gonads and predict the occurrence of spontaneous pubertal onset [43].

Ovotesticular DSD is a particular type of gonadal dysgenesis where both testicular and ovarian tissues are present. The most frequent karyotypes are 46,XX or mosaicism including at least one XY lineage. The degree of virilization is usually commensurate with the amount of testicular tissue. In XX patients, the differential diagnoses are congenital adrenal hyperplasia, aromatase deficiency, and androgen-secreting tumors. An increased level of serum AMH is specific of ovotesticular DSD [41]; in the other conditions serum AMH is in the female range. In contrast, androgen assay is not useful for diagnosis, since androgens are always above normal female levels.

4.3. AMH in DSD due to Defects in Androgen Synthesis or Action. While gonadal dysgenesis affects the production of both androgens and AMH, DSD may also result from a specific defect impairing the endocrine function of Leydig cells. In this case, there is a "dissociated" or "cell-specific" form of fetal-onset primary hypogonadism (reviewed in [5]), as opposed to gonadal dysgenesis leading to whole gonadal failure. Deficiency of androgen synthesis results in the occurrence of female or ambiguous external genitalia and no uterus.

4.3.1. Leydig Cell Aplasia/Hypoplasia and Steroidogenic Protein Defects. Leydig cell aplasia, due to inactivating mutations of the LH/CG receptor, and defects in proteins or enzymes involved in testicular steroidogenesis result in complete lack or insufficiency of androgen production by the testes. Consequently, hypovirilization or feminization of genitalia occurs as in dysgenetic DSD. Both dissociated primary hypogonadism specifically affecting Leydig cells and dysgenetic DSD have low testosterone levels in serum, yet it is possible to distinguish them by measuring AMH. While AMH is low or undetectable in dysgenetic DSD, as described above, it is normal/high in steroidogenic defects because the androgen inhibitory effect on AMH is lacking and the elevation of serum FSH upregulates AMH secretion [41], particularly in

Table 1: Etiopathogenic classification of disorders of sex development (DSD) in patients with a Y chromosome.

Etiopathogenic classification	Serum AMH	Serum T
(A) Malformative DSD		
Defective morphogenesis of the wolffian ducts		
Congenital absence of the vas deferens (Cystic Fibrosis)	Normal	Normal
Defective morphogenesis of the urogenital sinus and of the primordia of the external genitalia		
Cloacal malformations, aphallia, and isolated hypospadias	Normal	Normal
(B) Primary hypogonadism (early fetal-onset)		
(B.1) Dysgenetic DSD: whole testicular dysfunction		
Complete gonadal dysgenesis		
Y chromosome aberrations		
DSS duplications, 9p deletions (DMRT1/2?), 1p duplication (WNT4?)		
Gene mutations: SRY, CBX2, SF1, WT1, SOX9, DHH, MAMLD1, TSPYL1, DHCR7, and so forth	Undetectable	Undetectable
Partial gonadal dysgenesis		
Same as complete gonadal dysgenesis	Low	Low
Asymmetric gonadal differentiation		
45,X/46,XY, an other mosaicism, or Y chromosome aberrations	Low	Low
Ovotesticular gonadal differentiation		
46,XX/46,XY; an other mosaicism	Low	Low
(B.2) Nondysgenetic DSD: cell-specific dysfunction		
Leydig cell dysfunction		
Mutations in LH/CG-R, StAR, P450scc, P450c17, POR, cytochrome b5, 3β-HSD, and 17β-HSD	High in neonates and in pubertal age, normal in childhood	Low/undetectable
Sertoli cell dysfunction		
AMH gene mutations	Low/undetectable	Normal
(C) End-organ failure		
(C.1) Androgen end-organ failure		
Impaired DHT production		
5α-Reductase gene mutations	Normal	Normal
Androgen insensitivity syndrome (AIS)		
Androgen receptor mutations	Partial AIS: high in neonates, normal in childhood, and inadequately high at pubertal age Complete AIS: normal/low in neonates, normal in childhood, and very high at pubertal age	Normal/high
(C.2) AMH end-organ failure		
AMHR-II mutations	Normal	Normal

3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; AGD: asymmetric gonadal differentiation; AMH: Anti-Müllerian hormone; AMHR2: Anti-Müllerian hormone receptor type 2.

the first 3–6 months after birth and at pubertal age in those cases where gonadectomy has not yet been performed (Table 1 and Figure 4). It should be noted that AMH may be within the normal male range in these patients during childhood.

4.3.2. Deficiency of 5α-Reductase. Steroid 5α-reductase is the key enzyme for the conversion of testosterone to dihydrotestosterone (DHT). The androgen receptor has a higher affinity for DHT than for testosterone. In the absence of

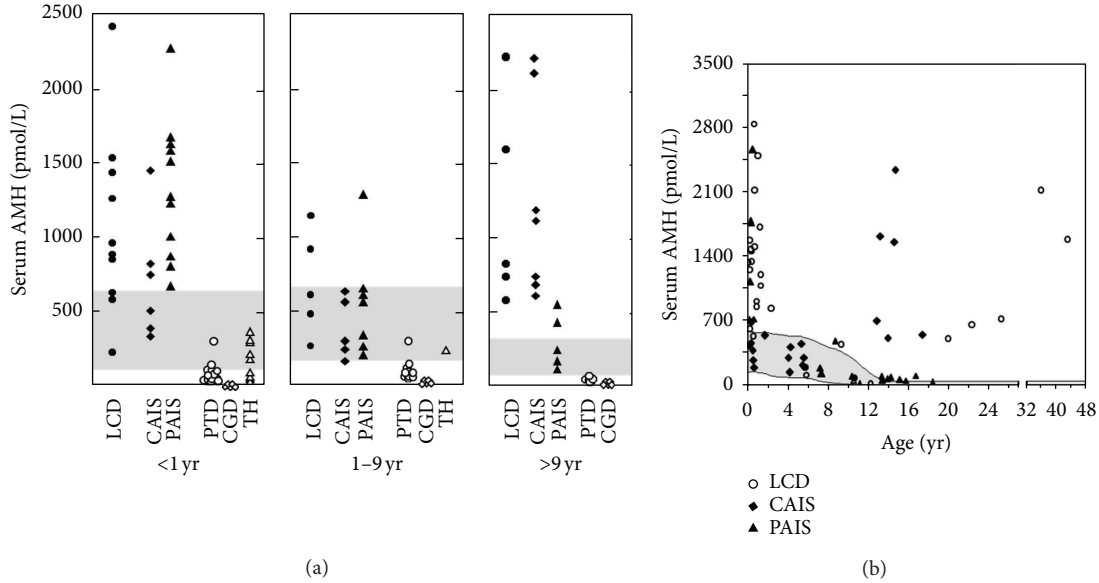

Figure 4: Serum AMH in disorders of sex development (DSD). (a) Serum AMH levels in patients with DSD. LCD: Leydig cell defects, including Leydig cell aplasia or hypoplasia and steroidogenic enzyme mutations; CAIS: complete androgen insensitivity syndrome; PAIS: partial androgen insensitivity syndrome; PTD: partial testicular dysgenesis, including asymmetrical gonadal differentiation; CGD: complete gonadal dysgenesis; TH: true hermaphroditism or ovotesticular DSD. The shaded areas represent the normal levels. Data is obtained from [41]. Copyright, The Endocrine Society, 1999. (b) Serum AMH levels in patients with DSD due to defects in androgen production (Leydig cell defects, LCD) or action (complete or partial androgen insensitivity syndrome, AIS). The shaded area represents the normal levels. Data is obtained from [44]. Copyright, The Endocrine Society, 1994, with permission.

5α-reductase activity, the Wolffian ducts differentiate normally because the adjacent testes supply sufficiently high local testosterone concentrations. Conversely, more distant androgen-dependent organs, like the urogenital sinus and the external genitalia, need testosterone conversion to DHT for adequate virilization. The Müllerian ducts regress normally because Sertoli cell AMH production is not affected. Testosterone levels are normal, and serum AMH is also within the normal male range. Because there are normal testicular androgen concentration and androgen receptor expression and FSH is not elevated, serum AMH is not increased in these patients [45] (Table 1).

4.3.3. Androgen Insensitivity Syndrome (AIS). Androgen insensitivity due to mutations in the androgen receptor is the most frequent cause of lack of virilization in eugonadal XY patients. The testes differentiate normally, and both Sertoli and Leydig cells are functionally normal from an endocrine standpoint. Owing to end-organ insensitivity to androgens, Wolffian ducts regress, and the urogenital sinus and the external genitalia fail to virilize. Müllerian ducts do not develop, reflecting normal AMH activity. Complete AIS results in a female external phenotype, whereas partial AIS presents with ambiguous genitalia.

The pituitary-gonadal axis shows different features during the first three months of life in complete and partial AIS. In the newborn with complete AIS, FSH remains low, which probably explains why serum AMH is not as high as expected [46]. Conversely, in partial AIS, gonadotropins as well as AMH are elevated (Table 1 and Figure 4) [44, 46]. As in DSD due to defects of steroid synthesis, serum AMH remains within the normal male range during childhood [41]. At pubertal age, provided gonadectomy has not been performed, a difference is again observed between partial and complete AIS. In complete AIS, serum AMH increases to abnormally high levels, whereas in partial AIS the elevation of intratesticular testosterone concentration is capable of inducing

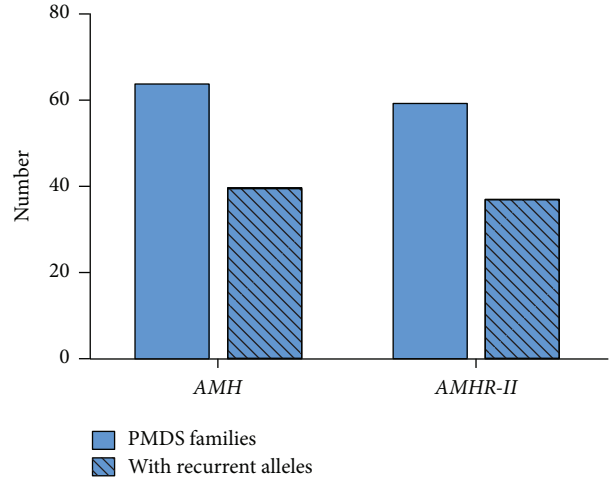

Figure 5: Recurrent alleles in families with persistent Müllerian duct syndrome (PMDS). Number of PMDS families and number of families with recurrent alleles. PMDS: persistent Müllerian duct syndrome; *AMH*: anti-Müllerian hormone gene; *AMHR-II*: anti-Müllerian hormone receptor type II gene.

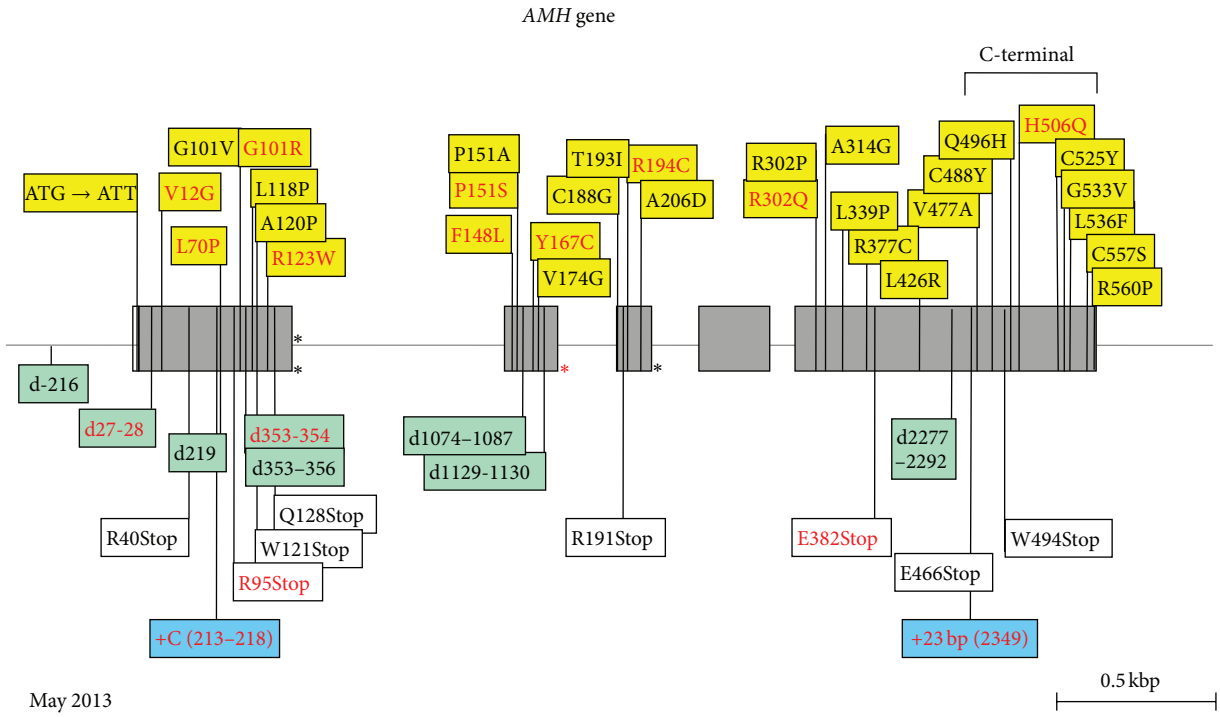

Figure 6: Mutations of the *AMH* gene in PMDS. Exons are shaded. All recurrent mutations are indicated in red. Missense mutations are in yellow boxes; note that the first mutation destroys the translation initiation site. Asterisks represent splice mutations; the red asterisk at the beginning of the second intron indicates a mutation detected in three different families all from Northern Europe. Deletions (marked "d") are in green boxes, insertions (marked "+") are in blue boxes, and nonsense mutations are in white boxes. A deletion mutation in the promoter is shown. C-terminal: coding for bioactive C-terminal domain of the AMH molecule. Base numbering is from major transcription initiation site, −10 bp from A of ATG.

an incomplete inhibition of AMH expression. Nonetheless, AMH levels are inadequately high for the concomitant circulating testosterone [41].

4.4. AMH in the Persistent Müllerian Duct Syndrome (PMDS). PMDS is characterized by the persistence of Müllerian duct derivatives, uterus, Fallopian tubes, and upper vagina, in otherwise normally virilized 46,XY males. Approximately 85% of cases are due to mutations of the AMH or AMHR-II gene; in roughly equal proportions, 15% are idiopathic. All the information provided is current up to May 2013.

4.4.1. AMH Deficiency: PMDS due to AMH Gene Mutations

Clinical and Anatomical Features. Because of their normal external male phenotype, patients are assigned at birth to the male gender without hesitation, in spite of the fact that one or both testes are not palpable in the scrotum. When cryptorchidism is unilateral, the contralateral scrotal sac contains a hernia, in addition to the testis. Preoperative diagnosis of PMDS is best reached by laparoscopy [47, 48]. However, unless an elder brother has been diagnosed with the condition, persistence of Müllerian derivatives is usually discovered unexpectedly during a surgical procedure for cryptorchidism and/or hernia repair.

Testes and the vasa deferentia adhere to the walls of uterus and vagina [49]. Their location depends upon the mobility of the Müllerian structures. Often, the broad ligament which anchors the uterus to the pelvis is abnormally thin, allowing the Müllerian derivatives to follow one testis through the inguinal canal and into the scrotum, resulting in "*hernia uteri inguinalis*." The testis on the opposite side may already be present in the same hemiscrotum, a condition known as "*transverse testicular ectopia*;" this rare condition is associated with PMDS in 30% of cases [50]. Very rarely, transverse testicular ectopia is the only anatomical abnormality observed in patients homozygous for an AMH or AMHR-II mutation; no Müllerian derivatives can be detected [51].

The PMDS testis is only loosely anchored to the bottom of the processus vaginalis; the gubernaculum is long and thin, resembling the round ligament of the uterus and exposing the mobile testis to an increased risk of torsion [52] and subsequent degeneration [53]. Alternatively, the Müllerian derivatives may remain anchored in the pelvis, preventing testicular descent [54] and giving rise to bilateral cryptorchidism. The presence of these midline structures may be missed if cure is attempted through inguinal incisions. The apparent rise in the incidence of PMDS over recent years may be due to the increased use of laparoscopy in patients presenting with bilateral impalpable testes.

Prognosis. Pubertal development is normal; however, incontrovertible evidence of paternity is lacking. Infertility may result from aplasia of the epididymis or germ cell degeneration due to long standing cryptorchidism. However, excising

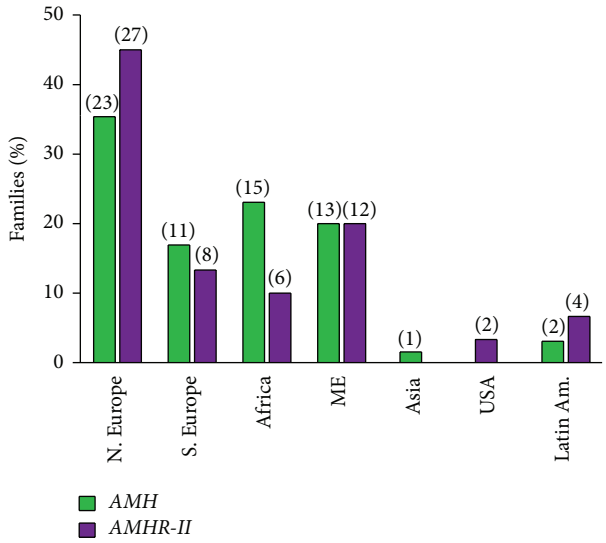

Figure 7: Ethnic origin of PMDS families. Results are expressed as percentages of total number of families with, respectively, AMH or AMHR-II mutations. The number of families is shown between parentheses. Differences between *AMH* and *AMHR-II* mutations are not statistically significant; the predominance of Northern Europe merely reflects a recruitment bias. N. Europe: Northern Europe (including Northern France), S. Europe: Southern Europe (including Southern France), Africa: (mostly Maghreb), ME: Middle East (includes Turkey, Afghanistan, and Pakistan, as per Wikipedia definition), Latin Am.: Latin America (includes Mexico, Central and South America).

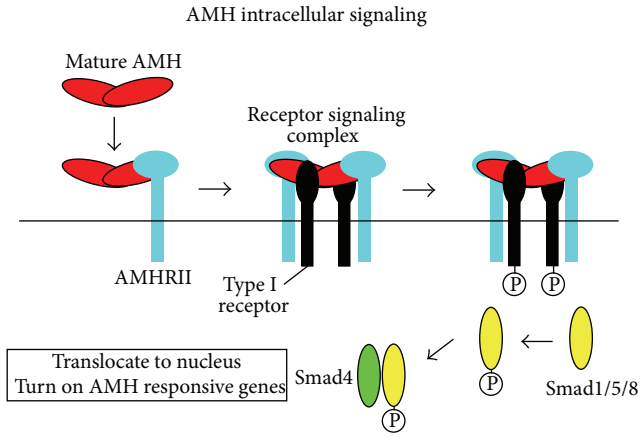

Figure 8: Signaling pathway of the AMH protein. Model showing how processing of AMH may regulate the assembly of the receptor signaling complex. Cleavage of full-length AMH results in a conformational change in the C-terminal domain, indicated by the shape and color change, which allows binding of AMRH-II. Binding of AMHRII induces dissociation of the proregion via a negative allosteric interaction between the receptor- and proregion-binding sites on the C-terminal dimer, indicated by the shape change. Results presented in this paper are consistent with proregion dissociation occurring before type I receptor engagement, but this has not been proven. Type I and II receptor-binding sites on the C-terminal dimer are indicated by either a I or a II; black labels indicate sites on the front of the dimer, and white labels indicate sites on the back of the dimer. From [2], Copyright The Endocrine Society, 2010, with permission.

the uterus to allow abdominal testes to descend into the scrotum carries significant risks to testicular blood supply. Most authors recommend partial hysterectomy, limited to the fundus and proximal Fallopian tubes or the simple division of Müllerian structures in the midline. Later, in the case of ejaculatory duct defects, intracytoplasmic sperm injection may be helpful. Orchiectomy is required if the testis cannot be brought down because of a 15% risk of cancer, an incidence apparently not higher than that for other abdominal undescended testes (reviewed in [55, 56]). AMH mutations are asymptomatic in young girls.

Biological Features. Testosterone and gonadotropin levels are normal for age. Serum AMH levels are generally very low or undetectable in prepubertal patients [57] due to instability of the mutant protein. This is not restricted to mutations coding for the bioactive C-terminus [58]: a 3D model of the C-terminus has been generated, using BMP2 and BMP7 as templates, providing insights into the impact of 3′ mutations upon secretion and action. One single mutation suspected of disturbing the interaction of the molecule with its type I receptor, ALK3, coexisted with a normal serum AMH concentration [58]. Thus, a normal serum AMH, albeit very rare, does not absolutely rule out the possibility of a pathogenic AMH gene mutation; however, this hypothesis cannot be entertained unless the AMHR-II gene has been totally exonerated.

Molecular Genetics. The human AMH gene, first cloned in 1986 [59], contains 5 exons. The 3′ end of the last one is extremely GC rich and shows homology to other members of the TGF-ß family; it codes for the bioactive C-terminal domain of the AMH molecule. The gene is located on the short arm of chromosome 19 [60]. PMDS is usually transmitted as an autosomal recessive trait; AMH mutations are responsible for 52% of the PMDS cases in which genetic defects have been detected. The first reported AMH mutation, a nonsense mutation of the 5th exon, was discovered in 1991 in a Moroccan family [61]. At the time of writing, May 2013, 65 families with AMH mutations (Figure 5), representing a total of 54 different alleles, have been identified (Figure 6). Except for exon 4, all exons coding both the inactive N-terminal proregion and the bioactive C-terminal mature protein are affected. All types of mutations are represented; 63% are homozygous. There is no true hotspot, though 17 abnormal alleles have been detected in more than one family. The ethnic origin of patients with documented AMH mutations is shown in Figure 7. The high proportion of European families is certainly due to a recruitment bias.

4.4.2. Insensitivity to AMH: PMDS due to AMH Receptor Mutations. Like other members of the TGF-ß family, AMH uses two types of membrane-bound serine/threonine kinase receptors for signal transduction. The AMH type II receptor, cloned in 1994 [62, 63], binds specifically to AMH and then

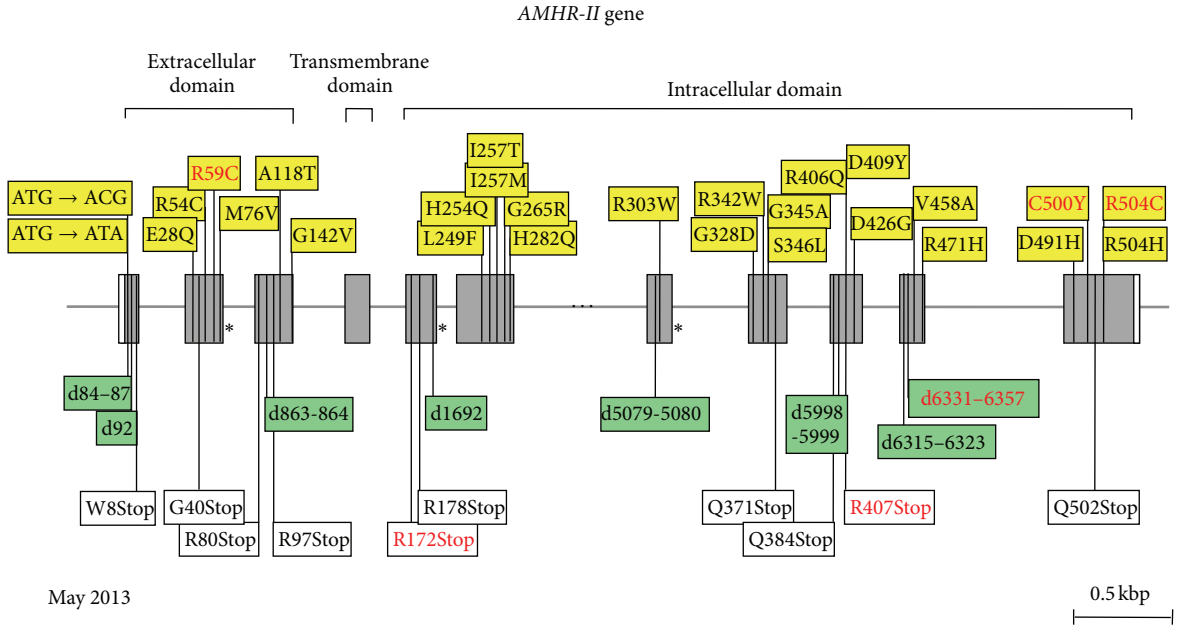

Figure 9: Mutations of *AMHR-II* gene in PMDS. Same representation as in Figure 6. All recurrent mutations are indicated in red. Asterisks represent splice mutations. Missense mutations are represented in yellow boxes, deletions (marked "d") in green boxes, and nonsense mutations in white boxes. The deletion of 27 bases between bases 6331 and 6357 (d6331–6357 in exon 10) is extremely frequent: it is present in 21% of all PMDS families and in 44% of those with receptor mutations. Base numbering is from transcription initiation site, −78 bp from A of ATG.

recruits type I receptor, which phosphorylates intracytoplasmic proteins, the SMADs, allowing them to enter the nucleus to interact with target genes (Figure 8).

ALK2/ACVR1 [64, 65], ALK3/BMPR1A [66], and ALK6/BMPR1B [67], all type I receptors of the BMP family, have been found to interact with the AMH type II receptor. ALK2/ACVR1 [65] and ALK3/BMPR1A [66] have been shown to function redundantly in transducing AMH signal to provoke Müllerian duct regression. Conversely, ALK6/BMPR1B disruption does not affect Müllerian duct regression in male mice [64], and in the immature Sertoli cell line SMAT1 ALK6/BMPR1B inhibits AMH action [68].

Mutations of type II receptor, AMHR-II, are responsible for 48% of PMDS cases with documented genetic abnormalities (Figure 5). Clinical and biological features do not differ from those described above for AMH mutations, apart from the fact that serum AMH level is low/normal. AMH assay cannot discriminate between AMH and AMHR-II mutations in adulthood, because, in both instances, AMH levels are low. Even in childhood, a normal AMH level is not specific for AMHR-II mutations, since approximately 15% of PMDS cases are not associated with either AMH or AMHR-II mutations.

The AMHR-II gene is composed of 11 exons, the first 3 coding for the receptor extracellular domain, exon 4 for most of the transmembrane domain, and the rest for the intracellular domain, where the kinase consensus elements are located. The gene has been mapped to the long arm of chromosome 12 [69]. The first AMHR-II mutation in PMDS, a splice mutation, was reported in 1995 [69]. Since then, 59 families, harboring a total of 49 abnormal AMHR-II alleles, have been studied in our laboratory, and an additional one has been reported in Boston (Figure 9) [70]. All exons except exon 4 may be affected. A 27-base deletion in exon 10 is present in approximately half the families with receptor mutations, nearly all of Northern European origin, suggesting a founder effect. Other recurrent mutations are much less frequent, apart from the nonsense R407Stop in exon 9, detected in 5 cases.

In approximately 15% of PMDS cases, all with a normal level of serum AMH, both the AMH and AMHR-II genes, including their proximal promoters and intronic sequences, are free of mutations. Several were born small and/or presented with various other congenital defects, such as jejunal atresia [71]. Mutations of the AMH type I receptors or cytoplasmic downstream effectors [72] are unlikely since these are shared with the BMPs and required for normal embryonic development. Inactivation [73] or dysregulation [74] of ß-catenin or dysfunction of other factors capable of interfering with AMH action might be involved.

5. Concluding Remarks

Assay of serum AMH now provides the pediatric endocrinologist with a new tool for investigating the function of the prepubertal testis, without the need for hCG stimulation. The assessment of both serum AMH, a marker of Sertoli cell function, and serum testosterone, reflecting Leydig cell function, is a simple and useful tool for the clinician. In DSD patients, when both hormones are below the normal male range, testicular dysgenesis should be suspected. AMH in the male range and low testosterone indicate Leydig cell-specific disorders.

When both hormones are within or above the male range, androgen target organ defects are most likely. Finally, PMDS is a rare etiology of cryptorchidism in boys with virilized external genitalia: in these cases, low or undetectable serum AMH predicts mutations in the AMH gene while normal serum AMH drives attention to the AMHR-II gene. In boys with normally virilized genitalia, serum AMH helps in the assessment of the existence and function of testes. Undetectable AMH is indicative of anorchia, whereas low AMH indicates primary or central hypogonadism.

Abbreviations

AIS:	Androgen insensitivity syndrome
AMH:	Anti-Müllerian hormone
AMHR-II:	AMH receptor type 2
DSD:	Disorders of sex development
hCG:	Human chorionic gonadotropin
PMDS:	Persistent Müllerian duct syndrome.

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Review Article

Anti-Müllerian Hormone and Its Clinical Use in Pediatrics with Special Emphasis on Disorders of Sex Development

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Using measurements of circulating anti-Müllerian hormone (AMH) in diagnosing and managing reproductive disorders in pediatric patients requires thorough knowledge on normative values according to age and gender. We provide age- and sex-specific reference ranges for the Immunotech assay and conversion factors for the DSL and Generation II assays. With this tool in hand, the pediatrician can use serum concentrations of AMH when determining the presence of testicular tissue in patients with bilaterally absent testes or more severe Disorders of Sex Development (DSD). Furthermore, AMH can be used as a marker of premature ovarian insufficiency (POI) in both Turner Syndrome patients and in girls with cancer after treatment with alkylating gonadotoxic agents. Lastly, its usefulness has been proposed in the diagnosis of polycystic ovarian syndrome (PCOS) and ovarian granulosa cell tumors and in the evaluation of patients with hypogonadotropic hypogonadism.

1. Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance, is essential for the involution of the Müllerian ducts (the anlagen of the internal female genitalia) in the male fetus [1–3]. Male sex differentiation is completely dependent on the normal development of testes that produce ample amounts of testosterone and AMH. The two hormones, produced by Leydig cells and Sertoli cells, respectively, represent two distinct pathways in male sex differentiation. Testosterone is responsible for the differentiation of the Wolffian ducts, the urogenital sinus, and the external genitalia. By contrast, AMH does not have any known function in female fetal organogenesis.

Determination of the serum AMH concentration is used in various ways in clinical pediatrics to determine the presence of testicular tissue in patients with cryptorchidism, suspected anorchia, or more severe Disorders of Sex Development (DSD). Also, AMH may be used as a marker of premature ovarian insufficiency (POI). It has been proposed as a marker in polycystic ovarian syndrome (PCOS) [4], as a tumor load marker in ovarian granulosa cell tumors [5] and, lastly, in hypogonadotropic hypogonadism [6].

This paper gives a brief overview of the physiology of AMH and seeks to give clinicians a tool when interpreting results from different assays. The aim is to simplify the use of AMH in clinical pediatrics.

2. Expression and Regulation of AMH

AMH is produced by Sertoli and granulosa cells in the male and female, respectively. AMH is a member of the TGF-β family and is encoded by the *AMH* gene, which contains 5 exons [7] and is located on chromosome 19 p13.3 [8] (see

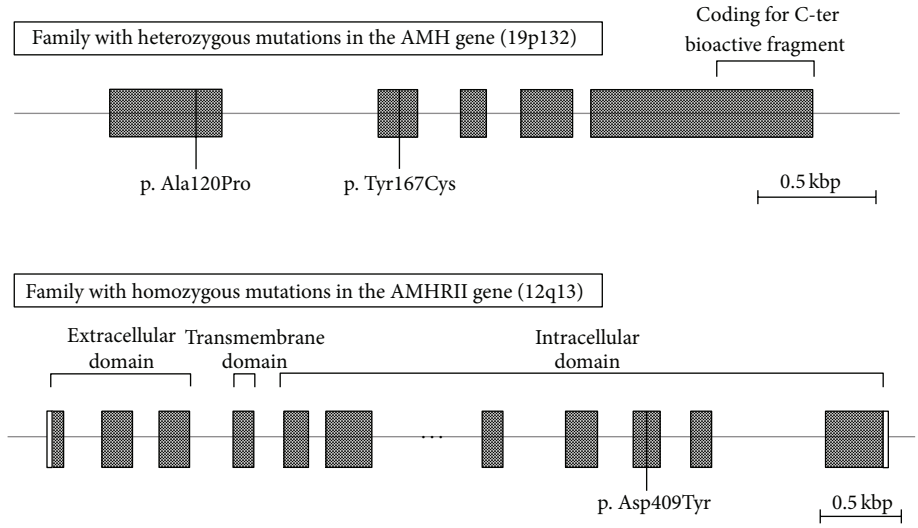

Figure 1: Mutations of the *AMH* gene (Case 1) and the *AMH-RII* gene (Case 2) in two families with boys presenting with PMDS. Two missense mutations in exons 1 and 2 (substitution of alanine by proline and of tyrosine by cysteine, resp.) were found in the *AMH* gene in two brothers presenting with AMH-negative PMDS. A homozygous missense mutation in exon 9 (substitution of aspartic acid by tyrosine) in the *AMH-RII* gene was found in two brothers presenting with AMH-positive PMDS.

Figure 1 for an iconography of the gene). The hormone binds to the specific AMH type II receptor (AMH-RII), which is encoded by a gene found on chromosome 12 p13 (see Figure 1 for an iconography of the gene) [9]. The receptor is a single transmembrane protein with serine-threonine kinase activity, and it is present on the cell membrane of target organs (including the mesenchymal cells of the Müllerian ducts, the granulosa cells of the ovary, and the Sertoli and Leydig cells of the testis) [10].

Several genes play a role in the regulation of AMH production from Sertoli cells. The *SRY* gene, for example, is important for the activation of *SOX9*, which together with steroidogenic factor 1 (SF1) and DAX1 directly stimulates the expression of *AMH* in the fetal testes [14, 15].

3. Male and Female Serum AMH Reference Ranges

We have previously reported serum AMH reference ranges for males and females throughout the entire lifespan measured on the Immunotech Coulter assay [11, 12], (Figure 2). At birth, male cord blood has high levels of AMH (mean 148 pmol/L), whereas AMH is undetectable (54%) or very low (95% CI: <2–16) in cord sera from female infants. At three months of age, AMH levels increase markedly in both sexes, although the concentrations in females (mean 13 pmol/L) remain much lower compared to concentrations in male infants (mean 1047 pmol/L). During childhood, AMH levels are relatively stable in both sexes, boys having approximately 35 times higher levels than girls.

Until pubertal onset, AMH is consequently a sensitive and specific marker of Sertoli cell activity. With the onset of testosterone synthesis in male puberty, serum AMH levels decline rapidly (mean 50 pmol/L), which clearly overlap with the levels seen in healthy females (Figure 2).

4. The Secretion and Function of AMH

4.1. AMH in Males. In males, the Sertoli cells begin to secrete AMH during the 7th week of gestation. Figure 3, modified from a series by Jørgensen et al. [16], clearly shows the high expression of AMH in the fetal testis and furthermore illustrates the reduction of AMH expression in Sertoli cells with increasing age and testosterone production which is also reflected in serum values [12, 17–19]. The adult male testis shows an absence of immunohistochemical staining for AMH. The origin of AMH in adult male serum is unknown, although testicular origin seems most likely despite the expression pattern seen in Figure 3. This is supported by visible AMH expression in slightly undifferentiated Sertoli cells that are occasionally seen in infertile men, often in Sertoli-cell-only tubules [20, 21].

The postnatal surge of AMH, which is seen in Figure 2, is most likely triggered by increasing concentrations of follicle-stimulating hormone (FSH) [22–24]. The pubertal decline is most likely caused by androgens after pubertal activation of the androgen receptor in the differentiated Sertoli cell [6, 17, 21, 25]. Inhibition probably also happens via the synergy with maturing germ cells rather than only through a direct inhibition of AMH transcription [26–28].

4.2. AMH in Females. In female fetuses, AMH is not present in ovarian tissues until the 36th week of gestation [21]. Female AMH is produced by ovarian granulosa cells of the preantral and early antral follicles [29–31]. The circulating levels of AMH in adult women reflect the number of remaining primordial follicles [32].

During childhood and adolescence, AMH fluctuations are minimal and each girl maintains her relative level during pubertal transition [33, 34]. The stable AMH levels during the extensive loss of primordial follicles in childhood are probably balanced on one hand by an increased recruitment

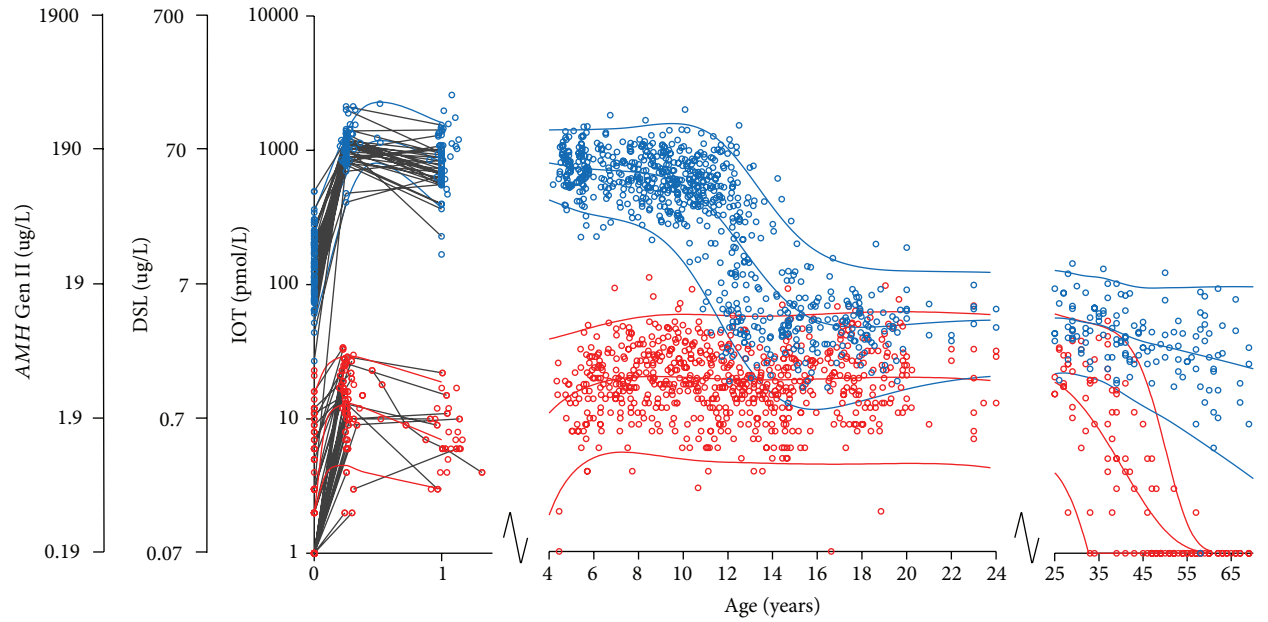

Figure 2: Serum AMH in 1953 healthy subjects (926 females and 1027 males) according to age. Females: red circles, males: blue circles. Longitudinal values during infancy are connected with grey lines. The red and blue curves represent the female and male reference ranges, respectively (median, ±2SD). The figure was redrawn from our previously published data using Immunotech Coulter enzyme immunometric assay in all subjects [11–13]. Please note the logarithmic y-axis. The y-axes for the DSL and Gen II assays were created using the following formulas: AMH (IOT) pmol/L = 2.0 × AMH (DSL) ug/L × 7.14 pmol/ug and AMH (IOT) pmol/L = 0.74 × AMH (Gen II) ug/L × 7.14 pmol/ug.

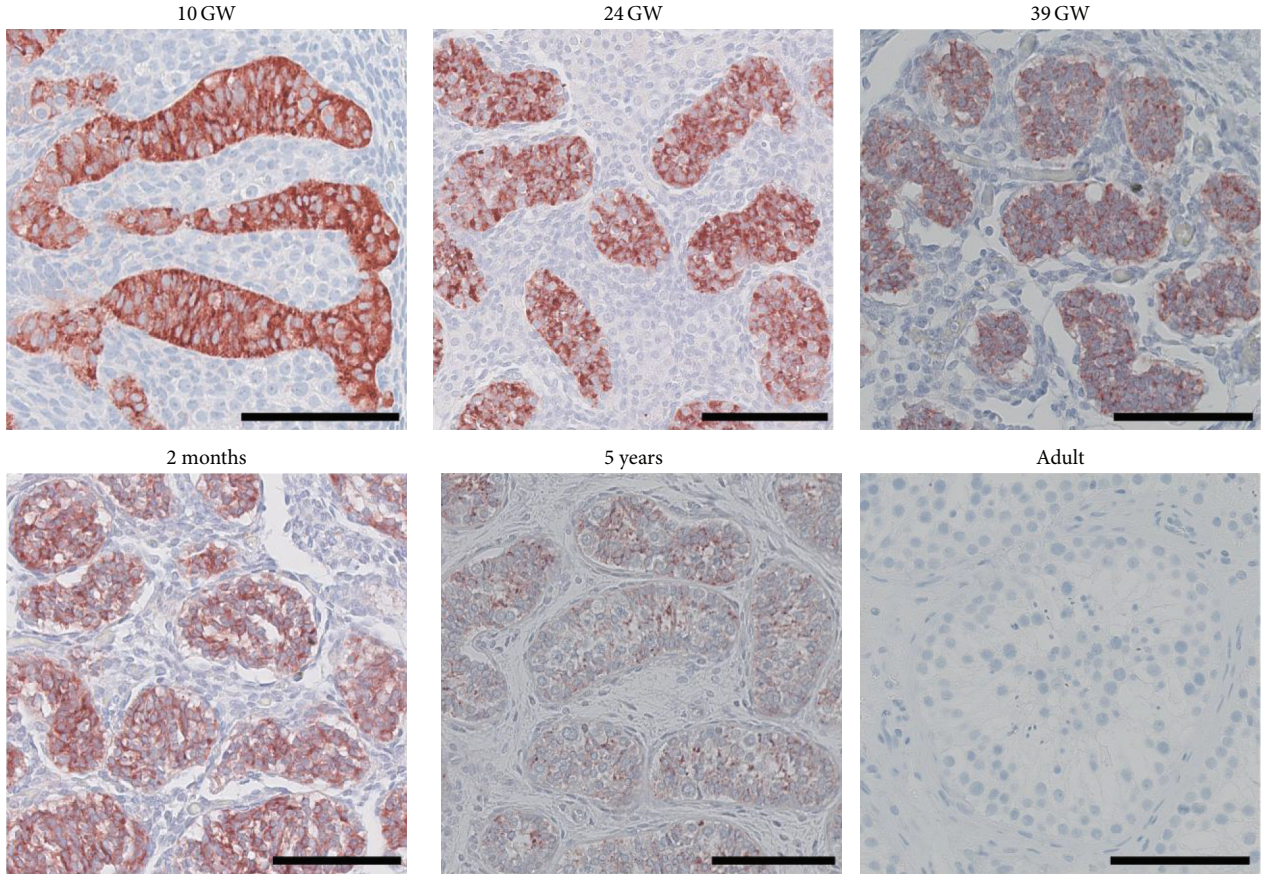

Figure 3: Immunohistochemical staining of AMH in testis tissue. GW: gestational week. Scale bar corresponds to 100 μm.

rate of primordial follicles during childhood (supplying AMH-producing follicles) and on the other hand by FSH-induced follicle growth at the time of pubertal onset (more follicles grow beyond AMH producing stages) [35].

In female mice, AMH inhibits primordial follicle recruitment and growth, which probably plays an important role in the maintenance of the follicle pool [36]. AMH furthermore plays a role in the regulation of ovarian steroidogenesis by inhibiting aromatase activity [37] and decreasing intrafollicular estradiol concentrations [38, 39].

5. AMH Assays

At present, three different AMH assays exist: Diagnostic Systems Lab (DSL by Diagnostic Systems Laboratories, Webster, TX, USA), Immunotech (IOT by Beckman Coulter, Inc., Brea, CA, USA), and the new AMH Generation II assay (Gen II by Beckman Coulter, Inc., Brea, CA, USA). The Gen II assay combines antibodies from the DSL and calibration standards from the IOT [40, 41]. Until the Gen II assay is ubiquitously implemented, there is a need for assay-specific reference ranges for clinicians to use. Few comparative studies between AMH assays have been performed, but Gen II levels appear to correlate well with both DSL and IOT, although the new assay produces higher values [41–45].

Previously, we reported age- and sex-specific reference ranges [13]; this figure has now been modified to make it assay-specific too, see Figure 2. It is important to note that we have not performed comparative studies with the testing of identical samples on the three different assays. The figure is merely a translation between assays based on current literature.

In Figure 2, the samples have been run with the IOT assay (Immunotech, Marseilles, France) [11, 12]. We use a conversion factor of 2.0 between DSL and IOT, which has previously been suggested and is widely accepted [11, 46, 47]. The units used by IOT and DSL are pmol/L and ug/L, respectively; it is therefore necessary to account for the molecular weight of AMH (140 kDa = 140 g/mol = 7.14 pmol/ug). Consider

$$\text{AMH (IOT) pmol/L} = 2.0 \times \text{AMH (DSL) ug/L} \times 7.14\ \text{pmol/ug}. \quad (1)$$

The y-axis for Gen II has been created based on a conversion factor of 0.74 as reached by Li et al. [42], which, to our knowledge, is the only study to date that has run identical samples on both the IOT and Gen II assays. Consider

$$\text{AMH (IOT) pmol/L} = 0.74 \times \text{AMH (Gen II) ug/L} \times 7.14\ \text{pmol/ug}. \quad (2)$$

The linearity of the relationship at higher values is questioned [42]. The clinical value of AMH measurements during childhood does, however, seem to be preserved despite the uncertainty of high-end values, as the value mostly lies in the distinguishing between low female and high male values in different clinical scenarios.

6. AMH and Its Clinical Use in Pediatric Patients

AMH has a wide clinical potential in pediatrics. Figure 2 provides a clinical tool where a specific AMH value can be interpreted in an age-, sex-, and assay-specific manner.

In the following section, we describe clinical conditions in which detectable versus undetectable AMH measurements are important for diagnosis.

6.1. Determination of the Presence of Testicular Tissue. When a child is born with ambiguous genitalia or bilaterally absent testes (as in Case 1), the determination of the presence or absence of testicular tissue is of utmost importance in terms of treatment and management options. A serum AMH concentration within the normal male reference range is highly indicative of testicular tissue [48–50]. In patients with ambiguous genitalia or cryptorchidism, a low or undetectable AMH concentration is conversely indicative of dysgenetic testicular tissue, anorchia (as seen in Case 1) [49–53] or ovarian tissue [48]. AMH can thus be used to distinguish between anorchia and cryptorchidism in patients without palpable testes.

In patients with ambiguous genitalia, an AMH value within the normal male reference range indicates intact Sertoli cell function. In the case of patients undergoing surgery, for example, a girl with an ovotestis, AMH can be used as a marker of testicular tissue before and after surgery [54].

The Relevance of Human Chorionic Gonadotropin (hCG) Tests in Patients with Cryptorchidism. hCG testing may not be necessary in determining the absence of testicular tissue; a simple AMH measurement has been suggested to have a higher predictive value than hCG stimulated testosterone levels (hCG stimulation may fail to increase serum testosterone in some patients with abdominal testes [48]), depending on whether or not hCG is given once or repetitively and on the patient's age [48, 50, 52].

In the case of a patient with undetectable levels of AMH and bilateral cryptorchidism, an hCG test should, however, be performed to asses Leydig cell function and to exclude the rare diagnosis of Persistent Müllerian Duct Syndrome [48, 55]. A lack of increased stimulated testosterone concludes the diagnosis of anorchia (see Case 1), whereas increased concentrations indicate the presence of testicular tissue, and that Persistent Müllerian Duct Syndrome (PMDS) should be considered (see below and Cases 2 and 3) [48].

AMH has furthermore been suggested as a marker used in the management of patients with an isolated microphallus or hypospadias to exclude the possibility of testicular dysgenesis. Few patients, however, have subnormal values indicating testicular dysgenesis as the underlying cause [50, 53, 56].

Hence, measuring serum AMH is mostly of great clinical value when dealing with patients with ambiguous genitalia or bilateral cryptorchidism.

6.2. Persistent Müllerian Duct Syndrome. Abnormal AMH secretion or action leads to the persistence of the Müllerian

ducts, that is, uterus, Fallopian tubes, and the upper part of the vagina. This can stem from a mutation in the gene encoding AMH or the AMH type II receptor (AMH-RII), known as PMDS, or it can be a sign of testicular dysgenesis. In the latter case, the phenotype will also be affected by disordered androgen secretion leading to external sexual ambiguity.

Patients with PMDS are referred to as either AMH-negative or -positive (with or without detectable serum concentrations of AMH, resp.), which gives clues to the underlying genetic defect [57]. AMH-negative PMDS is indicative of *AMH* gene mutations (as in Case 2, gene mutation illustrated in Figure 1), whereas AMH-positive PMDS leads to a suspicion of mutations in the *AMH-RII* gene (as in Case 3, gene mutation illustrated in Figure 1). In exceptional cases, however, AMH-positive PMDS may be due to a mutation in the *AMH* gene, which affects AMH bioactivity rather than secretion [58].

A fairly large group of PMDS patients (about 13–15% as reported by Josso et al. [57, 59]) remain without a genetic diagnosis, that is, no mutations found in neither the *AMH* nor the *AMH-RII* genes.

PMDS patients are phenotypically divided into two groups independent of the underlying genetic defect: patients born with bilateral cryptorchidism and patients born with one descended testis that drags the ipsilateral Fallopian tube into the inguinal canal creating a hernia and a contralateral abdominal testis [57, 60].

6.3. Virilized Females. AMH concentrations also indicate whether the virilization of a girl originates from testicular tissue or a granulosa cell tumor (AMH concentrations in the male reference range) or from adrenal androgens, that is, in the case of congenital adrenal hyperplasia (AMH concentrations in the female reference range). Values within the normal female reference range do not exclude the presence of abnormal gonadal tissue, but undetectable levels exclude the presence of testicular tissue in mildly virilized females [55, 61].

In the following section, we will describe clinical conditions in which the relative AMH concentration is important.

6.4. Low Serum Concentrations of AMH

Premature Ovarian Insufficiency. In pediatrics, Turner Syndrome patients experiencing accelerated loss of ovarian follicles and cancer patients receiving alkylating gonadotoxic treatment represent the majority of cases of premature ovarian insufficiency (POI). Low or undetectable AMH seems to be an excellent marker of POI in these patients [62–64].

6.5. Normal Serum Concentrations of AMH

6.5.1. Hypogonadotropic Hypogonadism. Hypogonadotropic hypogonadism (HH) includes an array of disorders all characterized by low or absent endogenous gonadotropins and consequently low or undetectable sex hormones in pediatric as well as adult patients.

In prepubertal and pubertal males with HH, AMH levels may be subnormal compared to the male reference range [65], whereas postpubertal levels may be high compared to normal male levels [66]. These phenomena could be explained by a lack of FSH stimulus prepubertally and a lack of intratesticular testosterone-mediated downregulation of AMH secretion during and after puberty, respectively [6, 22, 23, 67]. Pubertal and postpubertal levels may, however, also be low in patients with more severe HH. This is probably a consequence of the missing FSH stimulus and the subsequently smaller pool of Sertoli cells causing a deficient AMH secretion [68].

AMH has been proposed as a tool in differentiating between HH and constitutional pubertal delay but has not yet been proven clinically valuable [65], and some conclude that inhibin B seems to be the better marker of the two [68].

In females, AMH is low in the reference range due to the partial gonadotropin-dependent regulation of AMH [69]. Traditional endocrine evaluation (gonadotropins, inhibin B, and estradiol) remains as the preferred diagnostic tool for this condition, but AMH appears to be a promising marker of ovarian response in idiopathic HH patients with induced menstrual cycles [70]. This is important in fertility clinics but not as important in a pediatric setting.

Consequently, AMH is not yet of particular clinical use in pediatrics when suspecting HH in patients of either sex.

6.5.2. Klinefelter Syndrome (47,XXY). Klinefelter Syndrome (KS) is characterized by small testes, tall stature, and adult hypergonadotropic hypogonadism. Boys with KS have normal AMH concentrations until puberty [71–75]. After the expected pubertal decline, AMH values fall to subnormal concentrations [71, 72, 74, 76]. This may be explained by a progressive destruction of the testes seen in patients with 47,XXY [71]. AMH thus seems to be an excellent marker of testicular function in these patients.

6.6. High Serum Concentrations of AMH

6.6.1. Granulosa Cell Tumors. Granulosa cell tumors are sex cord tumors that make up 2–5% of ovarian neoplasms [77–80]. Only a few percent, however, are seen prepubertally [81]. In prepubertal girls, granulosa cell tumors may present as precocious puberty [82, 83]. In 1992, AMH was first identified as a granulosa tumor marker [84]. Serum AMH has been shown to be a fairly reliable and specific marker of granulosa cell tumors and their activity [83, 85–90]. In advanced stages, however, the large tumors may gradually lose their AMH expression, rendering AMH as a less reliable marker in these more advanced cases [5, 82]. One study directly found that tumor size was inversely related to AMH expression [91].

In a clinical pediatric setting, a prepubertal girl presenting with masculine AMH concentrations should be thoroughly examined for the presence of testes (i.e., in the complete type of androgen insensitivity syndrome) or of ovarian pathology (i.e., a granulosa cell tumor). A granulosa cell tumor cannot, however, be excluded in a girl presenting with AMH values within the reference range.

AMH, together with inhibin, has furthermore been proposed as a residual and relapse tumor marker postsurgically [85].

6.6.2. PCOS. Polycystic ovarian syndrome (PCOS) causes anovulatory infertility and hyperandrogenism along with elevated AMH in premenopausal women [4, 92–94]. AMH has not, however, been proven as a sensitive and specific diagnostic marker of neither PCOS nor of polycystic ovarian morphology [95]. Clear cutoffs do not exist in adolescence [96, 97], and several thresholds have been suggested in adults with varying degrees of sensitivity and specificity [4, 95, 98, 99].

Initially following menarche, anovulatory irregular menstrual bleedings are common and often resolve without diagnosis or treatment [100–102]. If an adolescent girl presents with a prolonged period of oligomenorrhea, an elevated serum AMH value may indicate underlying PCOS [92, 103, 104]. AMH is only mildly affected by the menstrual cycle and lowered by oral contraceptives [46, 105, 106], making it a useful marker for underlying pathology, namely, PCOS, in these patients when a high AMH concentration is found.

In conclusion, AMH is a very useful serum marker of gonadal differentiation and function in pediatric reproductive disorders. This paper has sought to simplify the correlation between the three AMH assays and review the usefulness in diagnosing and managing patients with various pediatric disorders of reproductive endocrinology.

7. Cases

7.1. Case 1: Boy Born with Anorchia. This boy was born with a hypoplastic scrotum, no palpable testes in the scrotum or inguinal canal, and an extreme micropenis. Laparoscopy at the age of 4 years was performed, and normal vasa deferentia were found along with normal testicular vessels. However, no testicles were located intra-abdominally. An hCG test at the age of 6 showed testosterone levels below 0.23 nmol/L. This concluded the diagnosis of anorchia, which was furthermore supported by undetectable AMH concentrations. Inhibin B was undetectable at all times. LH was immeasurable and FSH was between 1.37 and 1.63 U/L prior to puberty induction. Supplemental testosterone treatment was started at the age of 10.5 to induce puberty.

7.2. Case 2: AMH-Negative PMDS Patient. This patient was born at term with a left retractile testis and a right scrotal testis. At one month of age, a right-sided inguinal hernia appeared. The karyotype was that of a normal male, 46,XY. Inhibin B concentration was normal (402 pg/mL), and serum values of FSH, LH, and testosterone were all within the normal reference range for age. Herniotomy at the age of three months revealed an ovary-looking organ with a Fallopian tube attached bilaterally. Histology showed normal testicular tissue and orchidopexy was performed. Subsequently, laparoscopy was carried out and a uterus was found and removed including bilateral Fallopian tubes. Serum AMH was undetectable and, consequently, the *AMH* gene was sequenced. As illustrated in Figure 1, this resulted in the findings of two heterozygous missense mutations in exons 1 and 2 of *AMH*, respectively, that is, a substitution of the nonpolar alanine by the nonpolar proline (p.Ala120Pro) in exon 1 and a substitution of the polar tyrosine by the polar cysteine (p.Tyr167Cys) in exon 2.

One and a half year after the birth of this boy, a younger brother was born with a similar phenotype and the same *AMH* mutations were demonstrated.

7.3. Case 3: AMH-Positive PMDS Patient. This patient was born at term with nonpalpable bilateral cryptorchidism. The karyotype was that of a normal male, 46,XY. An hCG test at the age of 4.5 years resulted in rises in serum testosterone from <0.23 to 2.65 (at day 3) and 1.86 nmol/L (at day 4), respectively. Serum inhibin B was undetectable, and concentrations of FSH and LH levels were normal. Laparoscopy at the age of two years revealed the presence of intra-abdominal testes, a uterus, and bilateral Fallopian tubes; orchidopexy and hysterectomy were, thus, performed. Serum AMH was detectable in 5 of 5 samples varying from 17 to 75 pmol/L. Consequently, the *AMH-RII* gene was sequenced. As illustrated in Figure 1, a homozygous missense mutation in *AMH-RII* was confirmed, that is, a substitution of the polar aspartic acid by the polar tyrosine (p.Asp490Tyr) in exon 9.

Three years after the birth of this boy, a younger brother was born with a similar phenotype and the same *AMH-RII* mutation was demonstrated.

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Review Article

Potential Mechanisms for Racial and Ethnic Differences in Antimüllerian Hormone and Ovarian Reserve

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Accumulating evidence suggests that reproductive potential and function may be different across racial and ethnic groups. Racial differences have been demonstrated in pubertal timing, infertility, outcomes after assisted reproductive technology (ART) treatment, and reproductive aging. Recently, racial differences have also been described in serum antimüllerian hormone (AMH), a sensitive biomarker of ovarian reserve, supporting the notion that ovarian reserve differs between racial/ethnic groups. The existence of such racial/ethnic differences in ovarian reserve, as reflected by AMH, may have important clinical implications for reproductive endocrinologists. However, the mechanisms which may underlie such racial differences in ovarian reserve are unclear. Various genetic factors and environmental factors such as obesity, smoking, and vitamin D deficiency which have been shown to correlate with serum AMH levels and also display significant racial/ethnic variations are discussed in this review. Improving our understanding of racial differences in ovarian reserve and their underlying causes may be essential for infertility treatment in minority women and lead to better reproductive planning, improved treatment outcomes, and timely interventions which may prolong reproductive lifespan in these women.

1. Introduction

Accumulating evidence suggests that reproductive potential and function may be different across racial and ethnic groups. Differences have been demonstrated in pubertal timing, infertility, outcomes after assisted reproductive technology (ART) treatment, and reproductive aging. Black females are known to initiate puberty one year earlier and to achieve pubertal milestones earlier than white females [1, 2]. While infertility affects women of all races and ethnicities, US black and Hispanic women have disproportionately greater rates of infertility than whites [3], with recent evidence suggesting that these differences have been widening [4]. Moreover, a mounting body of evidence shows racial differences in ART treatment outcomes, with black, Hispanic, and Asian races associated with significantly lower pregnancy rates and live birthrates than whites [5–9]. While environmental, socioeconomic status, behavioral, and anatomic factors are likely contributors to racial disparities in ART outcomes, significant differences still remain even when these factors are controlled for [10, 11], suggesting that genetic factors may also play a role. Racial/ethnic differences have also been described in reproductive aging, as reflected by menopausal timing [12–14] and hormonal fluctuations [15–17]. Furthermore, race may also affect the prevalence of premature menopause, as differences have been noted between white, black, and Hispanic women [18]. Thus, the longevity of ovarian function may be influenced by race/ethnicity.

These racial/ethnic differences in reproductive potential and function suggest a racial difference in ovarian reserve. Antimüllerian hormone (AMH) is widely considered a highly sensitive marker of ovarian reserve. It is a member of the transforming growth factor-β superfamily. AMH suppresses the cyclical recruitment of primordial follicles at the pool of growing follicles and is primarily produced by the pool of small and large preantral and early antral follicles, which are believed to serve as a proxy for the number of primordial follicles in the ovary [19, 20]. It has been suggested that AMH may be the most accurate biomarker of ovarian aging and offer several advantages over traditional biomarkers of

ovarian reserve [19]. Compared with other hormonal markers of reproductive aging, AMH begins to gradually decline earlier in life [19, 21, 22], and its levels are not influenced by menstrual cycle timing, hormonal contraceptives, or pregnancy [19, 23–25].

Seifer et al. reported a significant difference in the mean level of AMH as a function of race or ethnicity [26]. They analyzed changes in AMH in a racially diverse, multicenter cohort study of HIV-infected women and high-risk seronegative women enrolled in the Women's Interagency HIV Study (WIHS). AMH levels were assessed at two time points in the study (median age: 37.5 years and 43.3 years). After controlling for age, BMI, smoking, and HIV status, black women demonstrated average AMH values that were 25.2% lower over time than those in whites ($P = 0.037$) [26]. In addition, AMH levels in Hispanic women were 24.6% lower over time than those in white women in the adjusted analysis, but this difference did not reach statistical significance ($P = 0.063$).This study provided the first biochemical evidence of racial differences in ovarian reserve, as measured by AMH. These findings have been recently corroborated by Gleicher et al. who reported that black women show a significantly greater age-related decline in AMH over time compared with white women [27]. Since the existence of racial/ethnic differences in ovarian reserve (as reflected by AMH) is expected to have important clinical implications for reproductive planning and infertility treatment of minority women, this review aims to shed light on and discuss possible underlying mechanisms for such racial and ethnic differences in AMH. While AMH is a sensitive marker of ovarian reserve, it is important to consider general limitations of currently available AMH assays when interpreting studies on AMH. Both the DSL and GenII AMH assays have been shown to exhibit significant within-subjects sample variability, likely related to instability of AMH under certain storage and assay conditions [28, 29]. A more robust AMH assay is expected to become available soon and should resolve these issues of AMH sample reproducibility in the future.

2. Genetic Factors

During fetal life, the primordial follicle pool is formed and increases initially to a peak of 6 to 7 million oocytes at 20 wk gestation [30]. Hereafter, the primordial follicle pool declines dramatically until there are approximately 1 million oocytes within the ovaries at birth [31]. Constant recruitment of primordial follicles into the growing follicle pool takes place throughout a woman's life, which is referred to as initial recruitment. By the time of puberty approximately 500,000 follicles remain, declining to 10,000–50,000 by the late 30s; during reproductive years, ongoing growth of follicles into antral stage and loss of follicles due to atresia lead to a gradual decrease in the number of oocytes, with eventual exhaustion of the follicle pool and menopause as the final result [30–32]. There is great variability in the quantity and quality of the oocyte pool, or ovarian reserve, among women. There is also wide variability in reproductive potential and the timing of reproductive events such as menarche and menopause, both of which have strong genetic heritability, based on several twin and family studies [33–35]. Ovarian aging, which leads to menopause with the exhaustion of the follicular pool, also seems to have a genetic component [36]. Therefore, it is likely that genetic factors play an important role in racial/ethnic differences in these reproductive traits and, specifically, ovarian reserve. However, very few studies have examined possible genetic associations between race/ethnicity and AMH.

In the first genome-wide association study (GWAS), to evaluate genetic associations with hormone markers of ovarian reserve, Schuh-Huerta et al. analyzed genetic variants associated with FSH and AMH, as surrogate measures of ovarian reserve, in a multiethnic fertile population of women [37]. Their study population included 232 Caucasian and 200 African American women who aged 25–45 and were prospectively enrolled in a community-based cohort. The authors found nominal genetic variants which were associated with FSH and AMH levels in both ethnic groups. Two genetic variants marginally associated with AMH were found in Caucasian women, located upstream of the JARID2 (jumonji, AT rich interactive domain 2) gene at 6p23 [37]. JARID2 is an ortholog of the mouse jumonji gene, which encodes a DNA-binding nuclear protein and is regulator of histone methyltransferases that negatively regulates cell growth and proliferation and is expressed in both human and mouse ovaries [38]. Therefore, JARID2 could play a role in cell growth within the developing ovary. In a related study, the same group investigated genetic variants associated with ovarian reserve as measured by antral follicular count (AFC) [39]. Interestingly, of the top 16 genetic variants associated with AFC in their study, 7 were associated with AMH levels. This is not surprising, as the number of preantral and antral follicles is thought to determine serum AMH level. Importantly, none of the associated genes in these studies have known roles in ovarian function and now represent an interesting group of candidate genes for further investigation. Future studies should also determine if any of these genetic variants may be responsible for racial/ethnic differences in AMH levels.

Gleicher et al. reported that the distribution of fragile X mental retardation (*FMR1*) genotypes correlates with serum AMH level [40]. Based on a normal range of 26–34 (median 30) CGG repeats, the authors used CGG counts on the two X chromosome alleles to define whether a genotype is normal (*norm*), heterozygous (*het*), or homozygous (*hom*). An individual was defined as *norm* when both alleles were within range, *het* by one allele outside, and *norm/low* or *norm/high*, depending on the abnormal count allele being above or below normal range. Both alleles outside range are defined as *hom*. It was found that AMH $\leq$ 0.8 ng/mL was significantly associated with the number of CGG repeats; every decrease by five CGG repeats in the het-norm/low group increased the likelihood of diminished ovarian reserve by 40%, while every increase by five CGG repeats in the het-norm/high group increased the risk by 50% [40]. Moreover, FMR1 genotype was found to be associated with specific ovarian aging patterns. Women with het-norm/low genotype showed high ovarian reserve when they were young, which rapidly declined with age. In contrast, the het-norm/high genotype was associated with low ovarian reserve at young age but relative preservation of ovarian reserve into older ages

[41, 42]. The same investigators reported that FMR1 genotypes vary between Caucasian, African, and Asian women [43], suggesting that FMR1 genotype may be linked to racial differences in AMH. In their study, African women showed a relatively high ovarian reserve at young age, characterized by the lowest FSH, the highest AMH, and the highest oocyte yield among races; yet, as they age they demonstrate the largest decline in AMH and oocyte yield and the poorest ovarian reserve compared to Caucasian and Asian women [27]. In contrast, Asian women showed a relatively low ovarian reserve in young age but the smallest decline in AMH and disproportional preservation of ovarian reserve at older ages compared to Caucasians and African women [27]. Remarkably, African women demonstrate a preponderance of the het-norm/low FMR1 genotype, while Asian women show a preponderance of the het-norm/high genotype [27]. Thus, FMR1 genotype may account for the observed racial differences in AMH and ovarian aging patterns.

BRCA1 and BRCA2 are crucial members of the DNA double-strand break repair family of genes, and mutations in the BRCA genes are associated with risk of breast, ovarian, and other cancers [44, 45]. Women who carry mutations in the BRCA1 gene show low response to ovarian stimulation and experience earlier menopause [46, 47]. Recently, it was reported that women who are BRCA mutation carriers display significantly lower serum AMH level than noncarriers (1.22±0.92 ng/mL versus 2.23±1.56 ng/mL; $P < 0.0001$) [48]. These observations support the possible role of DNA double-strand break repair in maintenance of human ovarian reserve and indicate that ovarian reserve is prematurely diminished in women with BRCA1 mutations. Several studies reported on variation in the prevalence of BRCA mutation carriers in women with breast cancer among various racial/ethnic groups. In a large US study which included 1727 breast cancer female patients younger than age 65 at diagnosis, estimates of BRCA1 prevalence were the highest in Hispanic patients (3.5%), followed by non-Hispanic whites (2.2%), African Americans (1.3%), and Asian Americans (0.5%) [49]. Prevalence was found to be particularly high in young (<35 years) African American patients (16.7%) [49]. Consistent with this study, similarly lower BRCA1 mutation prevalence rates in black patients compared with white patients were reported in 2 other population-based series of patients with breast cancer [50, 51]. In contrast to data on BRCA1 mutation frequency in the female breast cancer population, these data in the general population are very limited. Therefore, large population-based studies would be needed to establish the BRCA1 mutation frequency in the general population of different racial/ethnic groups of women and determine whether the association of BRCA1 mutation with diminished ovarian reserve and AMH may play a role in racial/ethnic differences in AMH (see Table 1).

3. Environmental Factors

Various environmental and lifestyle factors have been associated with serum AMH levels and may be implicated in racial/ethnic differences in AMH levels between women. Obesity is at epidemic proportions in the United States with recent data from the National Health and Nutrition Examination Survey showing that the combined prevalence of overweight and obesity was 64% in 2007-2008 among American women [52]. There is significant variation in obesity rates among American women according to race with blacks displaying the highest prevalence (49.6%), followed by Hispanics (43%) and whites (33%) [52]. Apart from being a known risk factor for diabetes, hypertension, cardiovascular disease, stroke, and certain cancers, overweight and obesity are also associated with poor ART outcomes in most studies [53–55], though the findings are not universal [56]. These observations suggest a possible association between obesity and lower ovarian reserve. Freeman et al. were the first to report an association between AMH levels and obesity [57]. In a cross-sectional study of AMH levels in late reproductive-age women, they found that AMH levels were 65% lower in obese women (BMI $\geq 30\,kg/m^2$) compared to nonobese women (BMI $\leq 30\,kg/m^2$) (0.016 ng/mL and 0.046 ng/mL, resp.). In their longitudinal analysis of a subgroup, obese women had significantly lower mean AMH levels over the 8-year interval compared to the nonobese women, corroborating the cross-sectional study results [57]. Consistent with these findings, in a study that was conducted to examine the impact of oral contraceptives on serum AMH levels by obesity status in reproductive-age women, it was found that AMH levels were 34% lower in the obese group compared to normal BMI women (2.9 ± 2.1 versus 4.4 ± 1.8 ng/mL, resp.) [58]. More recently, Buyuk et al. found a similar negative association between AMH and BMI among infertile women with diminished ovarian reserve, with mean random serum AMH levels being 33% lower in overweight and obese women compared with normal weight women [59]. However, in this study the investigators did not find an association between AMH and BMI in women with normal ovarian reserve [59].

The mechanisms by which obesity may influence ovarian function and AMH are unclear. One possible mechanism is lipotoxic effects on granulosa cells. It was recently demonstrated that mice fed a high-fat diet exhibit increased anovulation and decreased fertilization rates, concomitant with increased lipid accumulation, endoplasmic reticulum stress, mitochondrial dysfunction, and apoptosis in granulosa and cumulus cells [60]. In the same study, signs of lipotoxicity were observed in the follicular fluid of obese women undergoing controlled ovarian stimulation compared with normal weight women [60]. These findings support the notion that obesity has a detrimental effect on ovarian reserve, which is reflected by lower AMH. However, Su et al. reported that while AMH was lower in obese compared to normal weight late reproductive age women, no difference was found in antral follicle count, suggesting that the decrease in AMH level seen in obese women results from a physiologic process other than reduced ovarian reserve [61]. It is plausible that hormone metabolism, sequestration, or clearance may be altered in obese women. Consistent with this hypothesis, adiponectin, which is secreted from white adipose tissue and its serum levels are decreased in obese women [62], has been shown to modulate ovarian steroidogenesis in conjunction with insulin and gonadotropins [63]. Moreover, in a recent

Table 1: Potential factors and associated mechanisms underlying racial/ethnic differences in serum antimüllerian hormone (AMH) levels.

Factors	Nature of association with AMH	Potential mechanism/s
Genetic factors		
JARID2 gene	Marginal association with serum AMH level in genome-wide association studies [37, 39]	JARID2 negatively regulates cell growth and proliferation and is expressed in both human and mouse ovaries [38]
FMR1 genotype	AMH ≤ 0.8 ng/mL was significantly associated with the number of CGG repeats [40]	Theoretical altered FMR1 gene expression
BRCA1 mutation	BRCA1 mutation carriers display significantly lower serum AMH levels [48]	Loss of BRCA1 increases DNA double-strand breaks in human and mouse oocytes and is associated with reduced oocyte survival in mice [48]
Environmental factors		
Obesity	Inverse correlation between BMI and serum AMH [57–59, 61]	Lipotoxic effects on granulosa cells [60] Leptin decreases AMH gene expression in cumulus and granulosa cells [64] Adiponectin modulates ovarian steroidogenesis [63]
Smoking	Smoking is inversely correlated with AMH [37, 71, 72]	Polycyclic aromatic hydrocarbons cause oocyte destruction in mice [77, 78] Nicotine and/or its metabolites accumulate in granulosa cells and induce their apoptosis [79, 82] Cigarette smoke metabolites are associated with follicular oxidative stress [75]
Vitamin D deficiency	Decreased serum vitamin D levels are associated with lower serum AMH levels [65, 66]	Vitamin D-receptor complex binds the vitamin D response element on the AMH gene promoter resulting in upregulation of AMH gene expression [68]

JARID2: jumonji AT rich interactive domain 2; FMR1: fragile X mental retardation; BRCA1: breast cancer 1; AMH: antimüllerian hormone.

study by Merhi et al., the follicular fluid levels of leptin, another adipocytokine which is increased in obese women, were found to positively correlate with BMI and to suppress AMH and AMH receptor II gene expression in both cumulus and mural granulosa cells through the JAK2/STAT3 pathway [64]. Further studies are warranted to establish the mechanisms by which obesity decreases serum AMH levels and whether this reduction is reflective of reduced ovarian reserve or not.

Serum AMH concentration has been demonstrated to positively correlate with serum 25-hydroxyvitamin D [25(OH) D] levels [65, 66], suggesting that vitamin D deficiency is associated with lower ovarian reserve. In addition, Coney et al. found that vitamin D levels are lower in African American compared with white women [67]. In their study, black women had lower median levels of serum 25(OH)D compared with white women (27.3 nmol/L versus 52.4 nmol/L; $P < 0.001$), and 98% of black women had serum levels of 25(OH)D below 50 nmol/L compared with 45% of white women. Of note, the differences between the racial groups in the levels of 25(OH)D persisted despite adjustments for body weight, percentage body fat, and BMI [67]. These data suggest that vitamin D deficiency could account for racial differences in AMH between black and white women and that this effect is independent of BMI. Other than greater BMI, possible causes of lower vitamin D levels in black women include deeper skin pigmentation and decreased exposure to sunlight. Vitamin D may lead to increased AMH levels by a direct mechanism, as it was shown in a prostate cancer cell line that the AMH gene promoter has a vitamin D response element which binds the vitamin D-receptor complex, resulting in upregulation of AMH gene expression [68]. Further molecular investigations should elucidate the mechanism by which vitamin D may affect AMH production in granulosa cells. Given the global epidemic of vitamin D insufficiency, especially in black women [69], the observations of a relationship between AMH and vitamin D suggest that vitamin D supplementation may improve fertility outcome and minimize racial differences in ovarian reserve. Future studies are warranted to investigate these possibilities.

Multiple epidemiologic studies have reported that cigarette smoking leads to reduced ovarian function and fertility and an earlier age at menopause, suggesting that smoking impairs ovarian reserve [70]. Indeed, Sowers et al. recently demonstrated that women who were smokers had an earlier age at menopause and a more rapid decline in AMH levels, suggesting that smoking may lead to either fewer oocytes or an earlier decline in oocyte number [71]. In agreement with this study, Plante et al. found that active smoking but not former or passive smoking was associated with decreased AMH values in late reproductive age and perimenopausal women [72]. The authors suggested that smoking may directly cause depletion of antral follicles but not primordial follicles, such that smoking cessation may permit repopulation of the growing follicular pool and normalization of AMH. Similar association between smoking and AMH was also recently reported by Schuh-Huerta et al. [37]. It is well-known that smoking status varies across racial groups. In a study of women's health across the nation (SWAN) which examined vasomotor symptoms longitudinally in a multiethnic sample of US women undergoing the perimenopausal transition, African Americans had the highest rate of active smoking (24.6%),

followed by Hispanics (16.7%), whites (16.6%), Japanese (12.9%), and Chinese (1.6%) [73]. Variations in smoking status among racial/ethnic groups may thus be potentially responsible for racial differences noted in ovarian reserve, as reflected by AMH.

The mechanism of tobacco's toxic effect on the ovary is unclear but may be due to effects on oocyte quantity [74], oocyte quality, or disruption of endocrine function [75, 76]. Animal studies suggest that polycyclic aromatic hydrocarbons (known carcinogens in cigarette smoke) cause oocyte destruction in mice [77, 78]. In addition, increased levels of nicotine metabolites and cigarette carcinogens have been noted in ovarian follicular fluid of active and passive smokers, indicating that toxic constituents of cigarette smoke including nicotine and cadmium have access to the follicular environment and could affect ovarian function [79–81]. Specifically, cotinine, a major metabolite of nicotine, was shown to accumulate in the nucleus and cytoplasm of granulosa cells [79], and nicotine has been shown to induce granulosa cell apoptosis [82], providing a possible explanation for reduced AMH levels observed in smokers.

In summary, accumulating evidence suggests that significant racial differences exist in ovarian reserve and reproductive aging. While multiple genetic and environmental factors may underlie these observed racial differences in AMH, additional investigation is needed to determine their relative contribution to the time course of reproductive function and ovarian reserve. Further genome-wide association studies and well-designed longitudinal studies are expected to identify more underlying genetic factors and further increase our knowledge of the extent of reproductive aging differences across racial and ethnic groups. Improving our understanding of racial differences in ovarian reserve and their underlying causes may be essential for infertility treatment in minority women and lead to better reproductive planning, improved treatment outcomes, and timely interventions which may prolong reproductive lifespan in these women.

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Review Article

How Much Does AMH Really Vary in Normal Women?

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Anti-Mullerian Hormone (AMH) is an ovarian hormone expressed in growing follicles that have undergone recruitment from the primordial follicle pool but have not yet been selected for dominance. It is considered an accurate marker of ovarian reserve, able to reflect the size of the ovarian follicular pool of a woman of reproductive age. In comparison to other hormonal biomarkers such as serum FSH, low intra- and intermenstrual cycle variability have been proposed for AMH. This review summarizes the knowledge regarding within-subject variability, with particular attention on AMH intracycle variability. Moreover the impact of ethnicity, body mass index, and smoking behaviour on AMH interindividual variability will be reviewed. Finally changes in AMH serum levels in two conditions of ovarian quiescence, namely contraceptives use and pregnancy, will be discussed. The present review aims at guiding researchers and clinicians in interpreting AMH values and fluctuations in various research and clinical scenarios.

1. Introduction

Anti-Müllerian Hormone (AMH) is secreted into the circulation by small growing follicles in the ovary, until they have reached the size at which they may be selected for dominance (6–8 mm) [1]. Since the cohort of small growing follicles is in equilibrium with the total number primordial follicles, serum AMH levels reflect the ovarian follicular pool [2]. AMH is therefore considered an accurate marker of ovarian reserve [3, 4]. Moreover AMH levels vary less across different menstrual cycles as well as within one menstrual cycle as compared to other biomarkers of ovarian activity, such as FSH, which has a number of obvious clinical advantages [4–7]. Indeed, according to different studies, the measurement of AMH on a random basis throughout the menstrual cycle is associated with a very good accuracy when predicting ovarian response [8–10].

However while first studies reported a very low variability throughout the menstrual cycle [11–14], a number of more recent studies [15–17] indicate a reduction of circulating AMH in the luteal phase, hence raising the question if AMH should better be measured on a fixed day of the menstrual cycles to foster standardization and to allow better cross comparison between individual assessments.

In this review, we shed light on the partly controversial issue of AMH variability, with particular attention on AMH intracycle variability, that has been recently widely debated. Moreover we evaluate the impact of ethnicity, BMI, and smoking behaviour on AMH interindividual variability. Finally we discussed changes in AMH serum levels in two conditions of ovarian suppression, namely contraceptives use and pregnancy.

2. AMH Interindividual Variability

When talking about hormonal stability, two different types of variability should be considered: the interindividual and the intraindividual variability. The interindividual variability of AMH refers to variations in AMH levels between different subjects and is first of all secondary to a very high variability in the number of growing follicles within groups of women of similar age [18–20]. The high interindividual variability in AMH is not surprising, given the wide variability of ovarian reserve in women. Generally, high interindividual variability

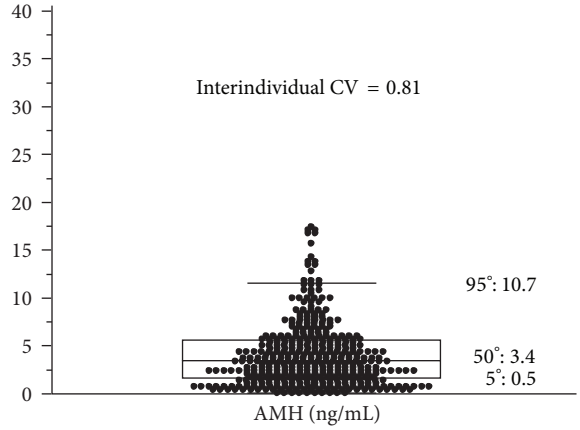

Figure 1: The interindividual variability of AMH refers to variations in AMH levels between different women. The coefficient of variability of AMH in a sample of 416 women aged 18–50 is 0.81. Generally, high interindividual variability is a good characteristic for a hormone when used as discriminatory biomarker in clinical setting (personal data).

is a good characteristic for a hormone when used as discriminatory biomarker in a clinical setting (Figure 1). Indeed the high interindividual variability of AMH makes it an ideal candidate biomarker with which to discriminate patients for diagnostic, prognostic, and other clinical purposes.

3. Effect of Ethnicity, BMI and Smoking on AMH Levels

In uni- and multi-variate analyses, black [21, 22] and Hispanic [21] women exhibit serum AMH levels 25% lower than those found in Caucasian women of similar age. Furthermore, an unexpectedly high number of black women has undetectable AMH levels despite relatively young age and regular menstrual cycles, hence indicating a potential discrepancy between actual ovarian reserve and what is indicated by AMH measurement in this population (Figure 2). More research on the underlying biological phenomena and consequences of this finding is clearly urgently needed. However, this finding indicates that care should be taken when using AMH reference values across different ethnicities.

Some papers, even if limited to small numbers of patients, indicated a negative relationship between BMI and serum AMH levels [23, 24]. However conflicting results have been reported by others [18, 25–28]. In a recent large study performed in a healthy general female population, AMH was negatively related to BMI, but the relationship was age dependent [27]. In other words, in women, AMH levels decreased and BMI increased with age; hence, the relationship between AMH and BMI was only secondary to the stronger relationship of the two variables with age.

There is clear evidence that smoking may directly accelerate ovarian follicular depletion, thereby reducing the age at menopause [29, 30]. Moreover, smoking has been shown to alter metabolic path for several hormones including estradiol. However contradictory results have been reported on the

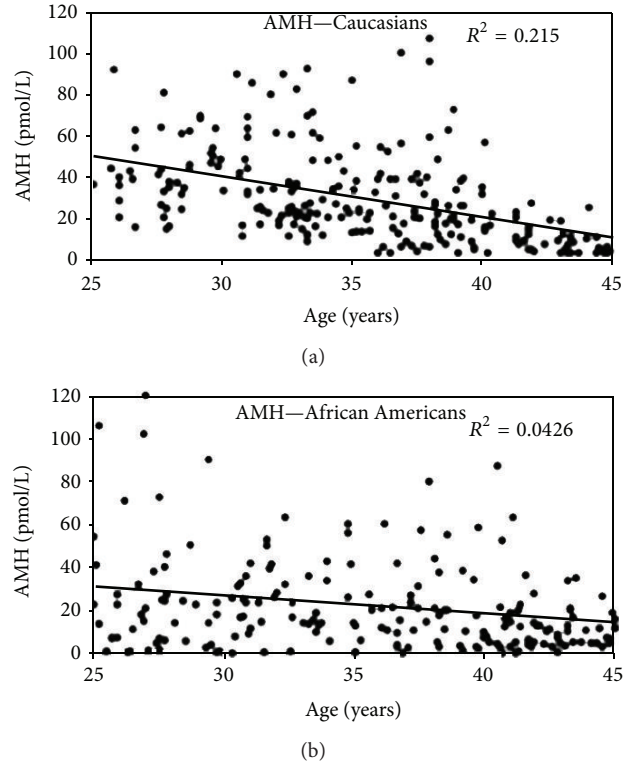

Figure 2: Measurements of AMH versus age in Caucasian (N = 232) and African-American women (N = 200). Total serum concentrations of AMH versus age indicate that AMH decreases with age but is highly variable between women and is more variable among African-American women. The corresponding correlation coefficients (R^2) and linear equations are shown. Please note how many African-American women had almost undetectable AMH levels although they were eumenorrhoic (reproduced with permission from Shuh-Huerta et al. [22]).

relationship between smoking and AMH, with some authors [31–33] reporting reduced AMH levels in smokers versus nonsmokers and others [18, 27, 34–36] reporting similar values in both groups of women. In a more recent study [27], AMH levels of 416 healthy women, including 99 smokers and 317 nonsmokers, were analyzed. As shown in Figure 3, at any age, the distribution of smokers was uniform in all quartiles of AMH distribution (Figure 3). In other words, in reference to a given age, a similar number of smoking women had high or low AMH levels, respectively. Accordingly, the debate on the impact of smoking on the follicular pool and the circulating AMH levels has not yet been settled. In conclusion, according to the published studies, it seems that the variability in ovarian reserve and secondly ethnicity may largely explain the high degree of interindividual variability in AMH levels.

4. AMH Intraindividual Variability: Long Term, Short Term, and Ultrashort Term

The intraindividual variability is indicative of variations in AMH levels in one single subject and may be secondary to

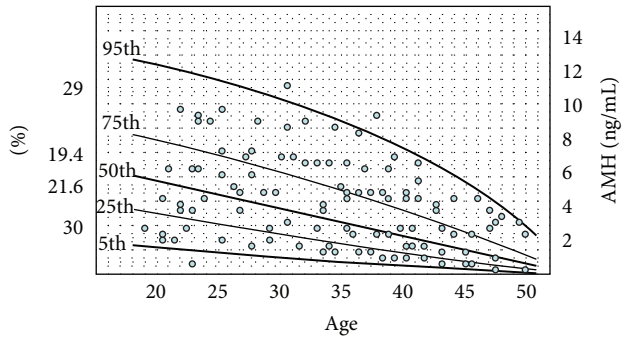

Figure 3: Indifferent distribution of serum AMH levels of smokers ($N = 99$) over quartiles of AMH distribution in the general female population ($n = 416$) (reproduced with permission from La Marca et al. [27]).

true biological variations in levels of circulating AMH in women.

We propose to distinguish among a long-term variability, a short-term variability and an ultra-short-term variability. The first refers to variations in AMH levels that occur year after year and are indicative of the decline in the ovarian reserve of a single woman. The second depends on the monthly physiologic variability in ovarian function; hence, the short-term variability may refer to intermenstrual cycle variability. The ultra-short-term variability indicates the day-by-day variability and refers to intramenstrual cycle variability.

In a recent prospective longitudinal study, serum AMH levels have been measured in healthy young prepubertal girls (6 to 13 years of age) every 6 months for 3 years and the mean intraindividual coefficient of variation (CV) for AMH was reported as 22%. This indicates that circulating AMH shows—on average—only minor fluctuations within a limited time span; thus, a random AMH measurement is likely to be representative indeed for a given girl [37]. The long, term variability in adult women has been mainly studied in cross-sectional studies, with some of them including as many as 10–15 thousand patients [18, 27, 38–41]. Overall, the studies are in good agreement that AMH declines with advancing age with a pattern that recalls the exponential decay of the primordial follicular pool [2, 42], which is best described by a quadratic equation [38].

The intermenstrual cycle variability has been analyzed in two well conducted prospective studies [14, 43]. Both studies calculated a similar intraclass coefficient (ICC) which was 0.89. The ICC is the ratio of the interindividual variability over the total variability. Hence the higher the ICC, the lower the intraindividual variability. Both studies concluded that 89% of the variation in AMH was due to between-subject variation, while only 11% of variability was secondary to individual fluctuation in AMH levels (Figure 4). Furthermore, a recent prospective study reported a correlation of 0.88 between AMH measurements performed on cycle day 2 or 3 in two subsequent cycles in women with regular spontaneous cycles [44]. AMH showed the highest between-cycle-correlation within an array of hormones assessed, including testosterone, FSH, E2, inhibin B, and LH.

A highly debated issue relates to whether AMH significantly varies or not throughout the menstrual cycle. Several studies have suggested that serum AMH levels fluctuate little during the menstrual cycle, as would be expected from the evidence that AMH is not secreted by the dominant follicle or corpus luteum [11–14] (Figure 5). AMH is unique among the known hormones produced by antral follicles, because its secretion seems to be only marginally influenced by gonadotropins and it is dramatically reduced as follicles reach the full gonadotropin sensitivity. As a consequence, AMH levels during the follicular phase do not reflect the activity of the developing large dominant follicle of the month, and conversely on any time point of the menstrual cycle AMH levels provide information on the number of small antral follicle present in the ovary which are available for cyclic follicular recruitment.

To study the intraindividual variability of AMH, Van Disseldorp et al. [14] calculated the intraindividual CV in a reanalyses of a previously published paper [11]. The authors reported that the intraindividual variability of AMH was only 13% and, most importantly, when dividing patients into quintiles according to basal AMH levels, the intraindividual fluctuations were shown to fall in the same quintile in 72% of the cases and to cross two quintiles in only 1% of the cases [14].

In contrast, some authors have noted significant fluctuations within one menstrual cycle [15–17]. A very recent study found serum AMH levels significantly lower in the luteal than follicular phase with a variation pattern similar to pituitary FSH, and the intraindividual variance of AMH was as high as 80% [17]. However the study was based on a very small group of subjects ($n = 12$), and some of them had as few as five blood samples throughout an entire menstrual cycle. Moreover when analyzing values for single patients, the proposed decline of AMH in the luteal phase was not evident in 25% of patients (Figure 6), hence raising the questions if the observed reduction of AMH in the luteal phase might be simply casual instead due to a biological reason indeed.

In another prospective study including 20 women, serum AMH levels were shown to fluctuate throughout the menstrual cycle [16]. In this case, the observed fluctuations were absolutely random throughout the cycle and not associated to typical gonadotropin or ovarian steroid patterns. Moreover, the amplitude was proportional to basal AMH levels: women with low AMH levels exhibited only minor fluctuations, whereas women with high basal AMH levels showed relatively higher fluctuations. The author speculated to categorize AMH pattern in "the ageing ovary pattern" and "the younger ovary pattern" [16]. In spite of the good quality of the study, where blood samples were collected from each woman daily along a whole menstrual cycle, some criticisms have been put on Roberts paper [45] for the lack of the calculation of the intraindividual coefficient of variation, which is considered the optimal analysis for hormonal variability. However, at bottom line, Sowers et al.'s study [16] indicates in a clear and convincing way that serum AMH levels vary throughout the menstrual cycle, that fluctuations may be relevant in those women with high basal levels, and most importantly that fluctuations are randomly distributed during the cycle.

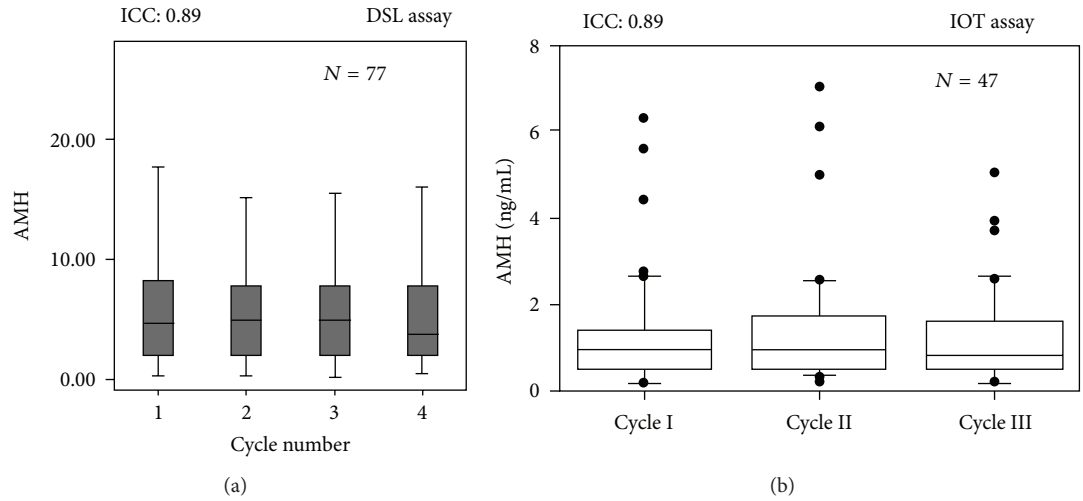

Figure 4: AMH intermenstrual cycle variability throughout several consecutive menstrual cycles. The reported intraclass coefficient (ICC) was 0.89 (reproduced with permission from van Sowers et al. [16] (a) and Fanchin et al. [43] (b)).

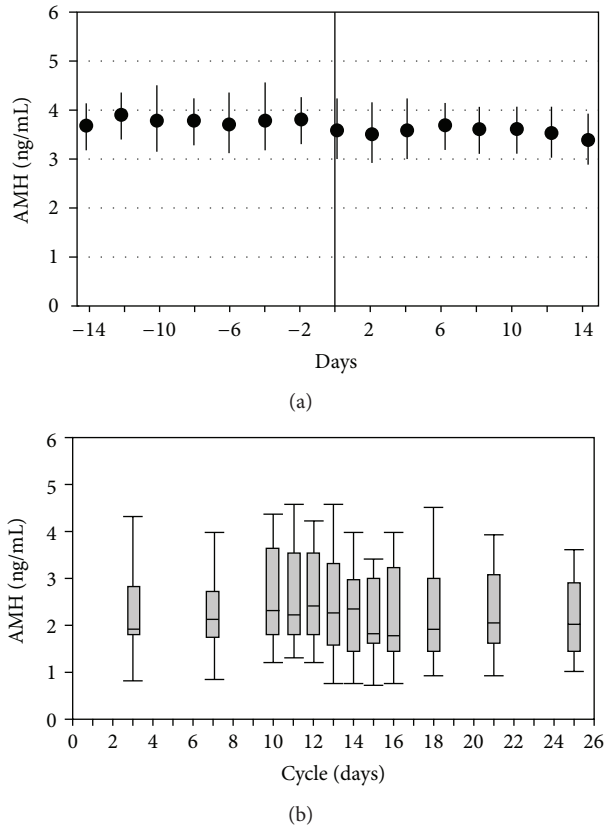

Figure 5: The AMH variability throughout the menstrual cycle. AMH appears to be stable (reproduced with permission from (a) La Marca et al. [12]; (b) Tsepelidis et al. [13]).

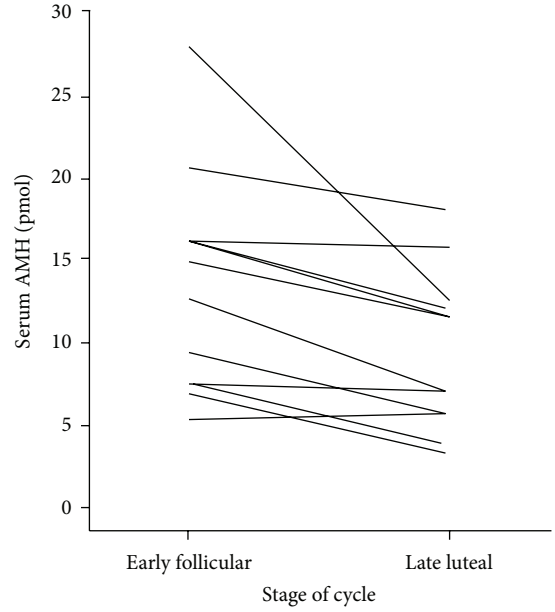

Figure 6: Changes in concentration of AMH for 12 women between the early follicular phase and late luteal phase of the cycle (reproduced with permission from Hadlow et al. [17]).

The random and noncyclic fluctuations in AMH indicate that measuring the hormone on a fixed day of the menstrual cycle would not yield any advantage of a random assessment, for example, on any day of the menstrual cycle.

In order to verify the effect of female age on the degree of AMH fluctuations, a recent study re-evaluated for the third time the data previously described by Hehenkamp et al. [11] and Van Disseldorp et al. [14]. In a group of 44 women between 25 and 46 years of age, the absolute intraindividual variation of AMH (deltaAMH), that is, the difference between maximum and minimum serum level throughout one cycle, was found to be significantly and negatively associated with age. In other words, younger women had significantly larger fluctuations in AMH levels than older women [28]. It may be concluded that in patients with low

ovarian reserve (usually aged women), AMH fluctuations have little clinical relevance, while in young patients with usually high ovarian reserve, fluctuations of AMH might indeed impact on the discriminatory capability of diagnostic and predictive tests, respectively [28].

The observed variability in AMH levels may have a limiting effect on the main current application of AMH as a predictive test in IVF practice. AMH is widely used to predict the ovarian response and to individualize the treatment according to this prediction [5, 9, 46, 47]. If AMH values cross the cut-off values proposed for the ovarian response categories because of true biological variability in AMH, this might lead to misclassification and erroneous treatment of patients. Hence the impact of the documented AMH variability needs to be tested in a clinical setting on a typical target population undergoing a clinically relevant predictive testing scenario.

As reported in detail in several reviews and metanalysis [5, 48–50], AMH is the best hormonal marker for the prediction of ovarian response in IVF. When using a random AMH measurement in order to prospectively predict ovarian response to exogenous FSH, correct categorization of 75% of patients in the three categories poor, normal, or hyperresponder can potentially be obtained [9]. Accordingly, although not as stable as thought before, AMH still remains the most "reliable" ovarian hormone and the best hormonal predictor of ovarian response to stimulation in IVF, with the important advantage of being randomly measurable.

5. AMH Serum Levels in Ovarian Quiescence Induced by OC Intake and Pregnancy

Since AMH production by antral follicles has been considered to be largely gonadotropin independent, a logical consequence is that pituitary suppression, as occurring with oral contraceptives (OC) or physiologically during pregnancy, should not be associated with relevant changes in serum levels.

A number of studies have been published on the effect of OC on AMH levels [24, 44, 51–57] and most of the available evaluations are not prospective. The very few prospective studies unfortunately limited the analysis to a few observational months (1 to 4 months) of OC treatment and are thus potentially and insufficiently informative. OC use has been reported either to insignificantly influence AMH concentration [44, 51, 52, 54, 55] or to reduce it significantly [53, 56–58].

A large cross-sectional study compared 180 and 76 twenty-year old OC users and nonusers, respectively, and found that long-term OC use was associated with a significant mean reduction in AMH levels by 13% [53]. Recently a cohort study based on 863 healthy women (228 OC users and 504 nonusers) reported that AMH serum levels were 29.8% lower in OC users than those in nonusers. The reduction in AMH was more pronounced with increasing duration of hormonal contraception. However no dose-response relation was found between the dose of ethinyl estradiol and the impact on serum AMH concentration [58].

In a well-conducted prospective study, AMH levels during OC pill intake in long-term OC users ($n = 25$) and 2 months after stopping the OC were assessed. Interestingly, a mean increase in AMH levels by 30% after cessation of the OC was found (from a mean of 2.0 ng/mL during OC to a mean of 2.6 ng/mL two months after the last pill, $P = 0.001$) [56].

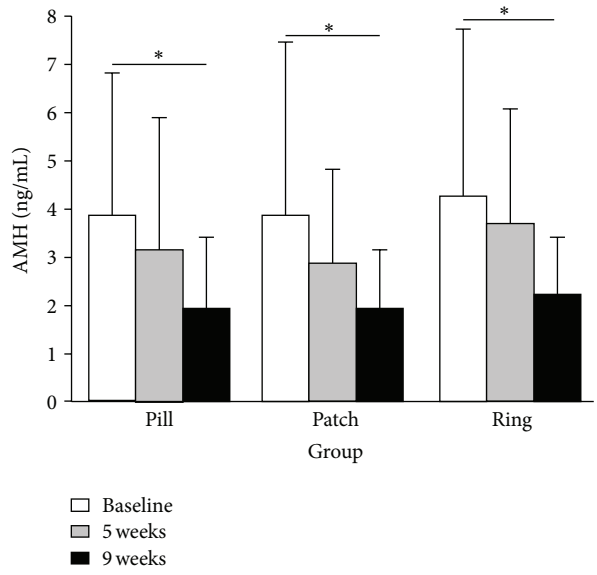

Figure 7: Serum AMH at baseline and after 5 and 9 weeks of administration of contraceptives (reproduced with permission from Kallio et al. [57]).

Finally, a small randomized trial recently confirmed largely these findings [57]. The study population consisted of 42 healthy women randomized to use for 9 weeks an OC in the form of either an oral pill ($n = 13$), a transdermal patch ($n = 15$), or a vaginal ring ($n = 14$). After 9 weeks of contraceptive use, serum AMH levels decreased significantly by almost 50% as compared to baseline in all treatment groups (Figure 7) [57].

This evidence is indicative of a suppressive effect of hormonal contraception on circulating AMH levels, at least when considering long-term use. Thus, serum AMH concentration may not retain its accuracy as predictors of the ovarian reserve in women using hormonal contraceptives for long time.

Pregnancy is a physiological condition associated with ovarian suppression because of suppressed endogenous gonadotropin release. According to the concept that AMH reflects the continuous FSH-independent noncyclic growth of small follicles in the ovary, it would be expected to find nonrelevant alteration in its levels during pregnancy. Indeed, an early small cross-sectional study reported unmodified AMH levels throughout pregnancy [59]. Subsequent studies reported contradictory results, with some confirming this finding [60, 61], while others describing a decrease in AMH levels during pregnancy [62, 63]. It has also been reported that the decline in AMH during pregnancy is evident when using the Beckman Coulter but not the DSL assay [63]. However, in the only longitudinal study available ($n = 60$), authors found a significant decrease in AMH levels in the

2nd and 3rd trimesters compared to the 1st trimester and the mean reduction at the end of pregnancy was of about 50% [64]. This study indicated that during pregnancy, there is a relative ovarian quiescence and reduced follicular maturation with a consequent decrease in the population of follicles secreting AMH. At the same time, at least part of the observed reduction in AMH levels during pregnancy could also be explained by the pregnancy-associated hemodilution and increased plasma-protein binding.

6. Conclusions

In conclusion, on top of the age related decline in AMH, significant fluctuations have been reported for a number of conditions and this has to be taken into account when interpreting values in clinical practice. Fluctuations in the menstrual cycle appear to be random and minor. This suggests that in clinical practice, AMH can be measured independently of the cycle phase. Prolonged ovarian suppression as induced by physiological or pharmacological interventions may reduce AMH levels, since the long and profound pituitary gonadotropin suppression is associated with a reduced number of antral follicles. The exact role of patients' characteristics, as ethnicity, and some habits, as smoking, on intra- and interindividual variability of AMH need to be investigated further.

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Clinical Study

Male Central Precocious Puberty: Serum Profile of Anti-Müllerian Hormone and Inhibin B before, during, and after Treatment with GnRH Analogue

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We aimed to describe the functional changes of Sertoli cells, based on the measurement of serum anti-Müllerian hormone (AMH) and inhibin B during treatment with GnRHa and after its withdrawal in boys with central precocious puberty. Six boys aged 0.8 to 5.5 yr were included. AMH was low at diagnosis in patients >1 yr but within the normal range in younger patients. AMH increased to normal prepubertal levels during treatment. After GnRHa withdrawal, AMH declined concomitantly with the rise in serum testosterone. At diagnosis, inhibin B was elevated and decreased throughout therapy, remaining in the upper normal prepubertal range. In patients with testicular volume above 4 mL AMH remained higher in spite of suppressed FSH. After treatment withdrawal, inhibin B rose towards normal pubertal levels. In conclusion, AMH did not decrease in patients <1 yr reflecting the lack of androgen receptor expression in Sertoli cells in early infancy. Serum inhibin B might result from the contribution of two sources: the mass of Sertoli cells and the stimulation exerted by FSH. Sertoli cell markers might provide additional tools for the diagnosis and treatment followup of boys with central precocious puberty.

1. Introduction

Precocious puberty in boys is defined as the appearance of the initial signs of sexual maturation at an age that is more than 2–2.5 SD below the population mean, which is 9 yr for boys [1]. Precocious puberty can be gonadotropin dependent due to the early activation of pulsatile gonadotropin secretion or independent of gonadotropins, as is the case with testotoxicosis or Leydig cell tumours [2]. The aetiology of an early activation of pulsatile gonadotropin secretion may be secondary to an organic lesion within the central nervous system (hydrocephaly, tumours, meningitis, etc.) or due to activating mutations of the genes encoding kisspeptin [3] or its receptor GPR54 [4]. However, in many cases the cause that triggers the onset of puberty cannot be identified. Gonadotropin-dependent precocious puberty is more prevalent in girls; nevertheless, the proportion of cases of precocious puberty secondary to organic lesions is higher in boys [2].

Gonadotropin-releasing hormone analogues (GnRHa) is the treatment of choice for gonadotropin-dependent precocious puberty of central origin. Therapy induces a reversible pituitary desensitization of GnRH receptors, thus suppressing the production of gonadotropins and subsequently that of sex steroids. This slows pubertal progression and decreases the rate of linear growth and skeletal maturation. Slowing the advancement of bone age is the primary goal of GnRHa therapy in order to improve final height [1, 2].

Monitoring treatment effectiveness during GnRHa therapy resides in multiple items. Growth velocity, secondary sexual characteristics, and bone maturation are the main clinical parameters. Serum LH and FSH measured by ultrasensitive assays are used for the assessment of pituitary desensitization

Table 1: Characteristics of boys with central precocious puberty included in this study.

	Age at diagnosis (years)	Bone age at diagnosis (years)	Peak LH at diagnosis (U/L)	Peak FSH at diagnosis (U/L)	Etiology	Age at GnRHa start (years)	Age at GnRHa end (years)
Patient 1	5.5	8.5	35.30	2.10	Idiopathic	5.6	8.9
Patient 2	4.3	8.5	6.90	0.70	Hypothalamic hamartoma	4.8	10.3
Patient 3	4.3	10.5	13.40	3.10	Hypothalamic hamartoma	4.3	9.8
Patient 4	1.7	3.6	37.00	2.30	Hypothalamic hamartoma	2.0	10.7
Patient 5	1.0	1.3	32.90	7.40	Idiopathic	2.2	N.A
Patient 6	0.8	2.0	84.90	1.70	Idiopathic	1.1	10.0

N.A: not applicable.

[5, 6], and serum testosterone levels are used to monitor testicular response to treatment.

Measurement of testicular volume is clinically utilized for the assessment of pubertal development in boys [7]; the increase in testicular volume beyond 4 mL is typically used to define the onset of puberty, either normal or precocious. The initial increase in testicular volume is due to Sertoli cell proliferation induced by FSH. Subsequently, testosterone provokes Sertoli cell maturation—characterized by Sertoli cell proliferation arrest—and triggers pubertal spermatogenesis in the seminiferous tubules, which mainly accounts for the dramatic enlargement of testis size to 15–25 mL [8]. Other early signs of pubertal Sertoli cell maturation, which occur even before serum testosterone rises, are the changes in serum levels of two specific Sertoli cell products: the decrease in anti-Müllerian hormone (AMH) [9–11] and the increase in inhibin B [12–14].

In the present study, our primary objective was to assess functional changes in Sertoli cells, based on the measurement of serum AMH and inhibin B during treatment with GnRHa and after its withdrawal in boys with central precocious puberty.

2. Methods

2.1. Patients. Boys diagnosed with central precocious puberty at the Division of Endocrinology of the Hospital de Niños Ricardo Gutiérrez, a tertiary paediatric public hospital in the city of Buenos Aires, Argentina, and treated with GnRHa between 1995 and 2013 were included in the study. Patients were excluded if history chart or results of serum hormone determinations (FSH, LH, testosterone, and AMH) were not complete or available. A clinical examination was performed at each visit to assess pubertal stage according to Marshall and Tanner [7] and to determine testicular volume by comparison with Prader's orchidometer [15]. The mean of the volume of both testes was reported. LH, FSH, testosterone, inhibin B, and AMH serum concentrations were assessed periodically before, throughout, and after treatment with GnRHa. The study was approved by the institutional review board. The need for informed consent was waivered owing to the observational design of the study, in which most of the procedures followed the standard care of patients with central precocious puberty, and because results of serum AMH and inhibin B levels were not considered for decision making.

2.2. Hormone Assays. AMH was determined using an ultrasensitive enzyme linked immunoassay specific for human AMH (Immunotech, Beckman-Coulter Co., Marseilles, France), following the manufacturer's instructions as previously published [16]. The analytical sensitivity of the assay, defined as the lowest AMH concentration significantly different from the calibrator zero, was 2.3 pmol/L. Intra- and interassay 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. Reference values were taken from our own data [16].

Serum inhibin B was measured using two-site enzyme-linked immunosorbent assays (Oxford Bio-Innovation Ltd, Oxon, UK) as previously described. Recombinant inhibin B (Genentech, San Francisco, CA, USA) was used as standard. The assay sensitivity was 15 pg/mL. Intra- and interassay coefficients of variation were below 10% for all assays. Reference values were taken from our own data [12].

Serum FSH and LH were measured by time-resolved immunofluorometric assays (IFMA, DELFIA; PerkinElmer, Inc. by Wallac Oy, Turku, Finland). The functional sensitivities were 0.05 and 0.10 IU/L, according to the 2nd WHO IS 80/552 for LH and IRP 94/632 for FSH, respectively. Intra- and interassay coefficients of variation for IFMA method were ≤3.2 and ≤7.3% for LH and ≤2.3 and ≤5.2% for FSH, as previously published [17].

2.3. Statistics. Hormone concentrations below the limit of the assay detection were assigned a concentration equivalent to the minimum detectable value in the respective assay. Data are expressed as median and range. Multiple regression analysis was performed to assess the association between serum inhibin B levels and testis volume during followup after GnRHa treatment. The level of significance was set at $P < 0.05$. Statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics (IBM Corporation, Somers, NY, USA).

3. Results

Six boys with central precocious puberty were included in the study (Table 1). Their median age at diagnosis was 3 years (range 0.8–5.5) with a bone age of 8.5 years (range 1.25–10.5).

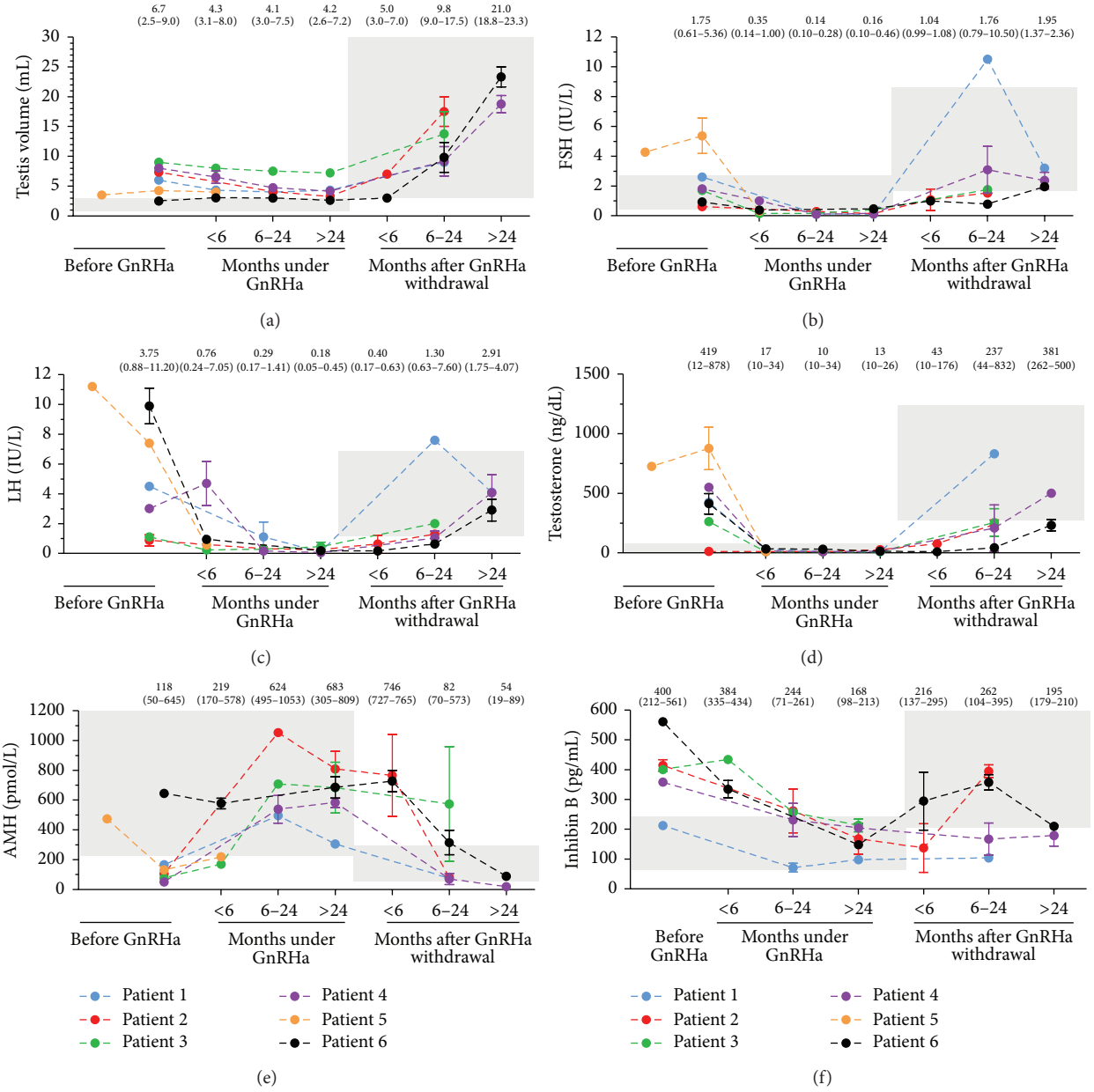

Figure 1: Testicular volume and hormone levels of boys with central precocious puberty before, during, and after withdrawal of GnRH analogue treatment. Medians and ranges are shown on the top of each figure for each time point.

In three cases, precocious puberty was due to a hypothalamic hamartoma and in the other three it was idiopathic. Patients were treated with triptorelin acetate every 28 days at a 110–190 μg/kg dose. GnRHa treatment was stopped at a median age of 10.1 (range 8.9–10.7). Patient 5 was still in treatment at the moment of the study.

At diagnosis, testicular volume was between 2 and 9 mL (Figure 1(a)), FSH was 1.75 IU/L (range 0.61–5.36) (Figure 1(b)), median basal LH was 3.75 IU/L (range 0.88–11.20) (Figure 1(c)), and testosterone was 419 ng/dL (range 12–878) (Figure 1(d)). Median peak LH after an acute GnRH test was 35.30 IU/L (range 6.90–84.90) (Table 1). Serum AMH was low in the four patients older than 1 year (median 67 pmol/L, range 50–165) but was within the normal range in the two patients aged ≤1 year (645 and 475 pmol/L) (Figure 1(e)). Interestingly, in Patient 5, who was diagnosed at 12 months of age, AMH (475 pmol/L) was within the normal prepubertal range. Due to social difficulties GnRHa treatment could not be started until the age of 2.2 yr when serum AMH had decreased to 131 pmol/L. Before treatment serum inhibin B was elevated (400 pg/mL, range 212–560) (Figure 1(f)).

Throughout treatment with GnRHa, testis volume decreased but remained moderately above the normal prepubertal size in most patients (Figure 1(a)). As expected, serum gonadotropins and testosterone showed a progressive decrement toward normal prepubertal levels and remained

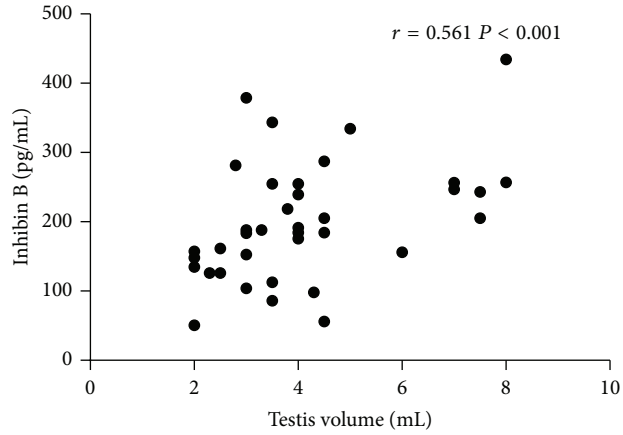

Figure 2: Correlation between testicular volume and serum inhibin B levels in boys with central precocious puberty during GnRH analogue treatment.

so until GnRHa withdrawal, when they increased again to pubertal levels (Figures 1(b)–1(d)). AMH increased progressively and remained within the normal prepubertal range during the whole treatment (Figure 1(e)). After GnRHa withdrawal, AMH progressively declined concomitantly with the rise in serum testosterone, as in normal puberty [16, 18].

Serum inhibin B levels decreased moderately throughout GnRHa therapy attaining its nadir after 24 months of treatment. Thereafter, they remained in the upper half of normal prepubertal age range (Figure 1(f)). A multiple regression analysis using patient and testis volume as the independent variables and inhibin B levels as the dependent variable showed a significant positive correlation between testicular volume and serum inhibin B levels during treatment ($r = 0.561$ $P < 0.001$) (Figure 2). Interestingly, during GnRHa treatment the lowest levels of inhibin B were observed in Patients 1, 2, and 6, in whom testicular volume achieved normal prepubertal range, whereas inhibin B remained higher in Patients 3 and 4, who maintained testicular volume above 4 mL in spite of suppressed FSH levels. After treatment withdrawal, inhibin B rose toward normal pubertal levels concomitantly with the reactivation of the gonadotropin axis.

4. Discussion

By means of serum AMH and inhibin B assessment, this work depicts the functional changes occurring in the seminiferous tubule compartment—and especially in Sertoli cells—in response to GnRHa treatment in boys with central precocious puberty. Our results show that the abnormally early maturation process suffered by Sertoli cells in boys with precocious puberty in response to testosterone is reversible when patients are efficaciously treated with GnRHa, most probably reflecting the decrease to prepubertal levels in intratesticular testosterone concentration. On the contrary, inhibin B levels do not always decrease to prepubertal values although FSH secretion is also curtailed by GnRH administration, most probably indicating that the increased mass of Sertoli cells is not fully reverted and continues secreting inhibin B independently of FSH stimulation.

Most of our patients had low serum AMH levels for their chronological age when central precocious puberty was diagnosed, showing the well-known inhibition exerted by androgens on Sertoli cell AMH production. The inhibitory action of androgens predominates over the stimulatory effect of FSH on Sertoli cell AMH expression [19, 20]. Conversely, FSH action on Sertoli cells is reflected by the initial increase in testicular volume and serum inhibin B. AMH downregulation and inhibin B upregulation also reflect the entry into meiosis of germ cells. In fact, the onset of meiosis is associated with a further decline in AMH expression, independently of testosterone, during pubertal development [21]. Concerning inhibin B production by the testis, the immature Sertoli cell synthesizes the α/βB dimer, whereas the pubertal Sertoli cell essentially produces the α subunit under FSH control; the βB subunit expression is mainly regulated by local factors produced by meiotic germ cells [22].

During GnRHa treatment AMH attained normal prepubertal levels, as previously described [9], which accounts for the usefulness of Sertoli cell serum markers to monitor the efficacy of GnRHa treatment in order to produce a sufficient decrease in gonadotropin production so as to lower intratesticular androgen action to prepubertal levels. The functional maturation of Sertoli cells induced at pubertal onset, reflected in AMH downregulation by androgens, seems to be a completely reversible process when treated soon before its establishment, as was the case in Patients 1–4 of our series and in previously published cases [9]. On the contrary, Sertoli cells do not revert to a fully immature state if the action of intratesticular androgens has persisted for years or decades, as shown in adult males receiving GnRHa treatment for prostate cancer [23].

A noteworthy observation in our study was that serum AMH remained in the normal prepubertal range in patients with central precocious puberty diagnosed within the first year of life, suggesting that the elevated androgen levels were unable to inhibit AMH production in young infants. Interestingly, testicular volume also remained within the prepubertal range in these patients, probably indicating that pubertal spermatogenesis had not been triggered. These observations are in line with the recent description that Sertoli cells are physiologically insensitive to the direct action of androgens in the first year after birth because they do not express the androgen receptor until later in life [24–26]. Concordantly, in our Patient 5, AMH was at prepubertal levels when diagnosed at 12 months of age and, because treatment could not be immediately installed, serum AMH decreased to pubertal levels at age of 2.2 years, supporting the concept of a gradual increase in androgen receptor expression within the Sertoli cell population.

At diagnosis, serum inhibin B levels were elevated in all patients, resembling what occurs under normal pubertal development at Tanner stages II to III. Treatment with GnRHa resulted in a reduction in serum inhibin B levels in all patients. However, the magnitude of this decrement

was variable amongst patients. In some patients, inhibin B remained in the upper normal range during treatment, in correlation with the persistence of moderately elevated testis volume, despite suppressed FSH secretion. This probably reflects that the increased mass of Sertoli cells induced by elevated FSH until diagnosis is not fully restored to the prepubertal stage and continues secreting inhibin B, independently of FSH stimulation [14]. We believe that serum levels of inhibin B reflects the addition of two pools: a basal one related to the mass effect of the Sertoli cell population independent of the gonadotropins and a second one related to the stimulation exerted by FSH. The mass effect is evidenced by the observation that testicular volume remains over the normal prepubertal volume in a subset of patients under GnRHa treatment, concomitantly with higher serum inhibin B levels. It could be argued that these elevated serum inhibin B levels result from an insufficient inhibition of the gonadotropin axis with GnRHa treatment. This seems unlikely, however, since serum levels of LH and testosterone were efficaciously suppressed and resulted in a normalization of serum AMH.

In summary, we describe the different functional changes observed in Sertoli cells of boys with central precocious puberty according to their age at the moment of diagnosis. Sertoli cell markers provide additional tools to diagnose central precocious puberty in boys older than 1 year and to monitor GnRHa treatment efficacy. The observation that serum AMH does not decrease in patients below 1 year confirms the lack of androgen receptor expression in Sertoli cells in early infancy. Finally, we hypothesize that serum levels of inhibin B represent the addition of two pools, one related to the stimulation exerted by gonadotropins and another one to the mass effect of the Sertoli cell population independent of the gonadotropins.

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Review Article

Serum AMH in Physiology and Pathology of Male Gonads

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AMH is secreted by immature Sertoli cells (SC) and is responsible for the regression of Müllerian ducts in the male fetus as part of the sexual differentiation process. AMH is also involved in testicular development and function. AMHs are at their lowest levels in the first days after birth but increase after the first week, likely reflecting active SC proliferation. AMH rises rapidly in concentration in boys during the first month, reaching a peak level at about 6 months of age, and then slowly declines during childhood, falling to low levels in puberty. Basal and FSH-stimulated levels of AMH, might become a useful predictive marker of the spermatogenic response to gonadotropic treatment in young patients with hypogonadotropic hypogonadism. After puberty, AMH is released preferentially by the apical pole of the SC towards the lumen of the seminiferous tubules, resulting in higher concentrations in the seminal plasma than in the serum. Defects in AMH production and insensitivity to AMH due to receptor defects result in the persistent Müllerian duct syndrome. A measurable value of AMH in a boy with bilateral cryptorchidism is predictive of undescended testes, while an undetectable value is highly suggestive of anorchia or ovaries, as would be the case in girls with female pseudohermaphroditism and pure gonadal dysgenesis. Lower serum AMH concentrations in otherwise healthy boys with cryptorchidism, who were compared with their age-matched counterparts with palpable testes, have been reported previously. AMH levels are higher in prepubertal patients with varicocele than in controls. This altered serum profile of AMH in boys with varicoceles may indicate an early abnormality in the regulation of the seminiferous epithelial function. Serum AMH is known to be valuable in assessing gonadal function. As compared to testing involving the administration of human chorionic gonadotropin, the measurement of AMH is more sensitive and equally specific. Measurement of AMH is very useful in young children, because serum gonadotropin concentrations in those who are agonadal are nondiagnostic in midchildhood and serum testosterone concentrations may fail to increase with provocative testing in children with abdominal testes.

1. Introduction

Anti-Müllerian hormone (AMH), also named Müllerian inhibiting substance (MIS), is a tissue-specific TGF-beta superfamily growth factor. AMH is secreted by immature Sertoli cells (SC) and is responsible for the regression of Müllerian ducts in the male fetus as part of the sexual differentiation process [1, 2]. AMH is also involved in testicular development and function [1, 2].

2. Physiology

2.1. Fetus. In the 7th week of gestation, the undifferentiated gonads differentiate into a testis in the XY embryo. Gonadal cells become segregated in two compartments: testicular cords and interstitial tissue. Testicular cords are composed by somatic SC and germ cells, surrounded by a basal membrane and peritubular cells. SC produce AMH and inhibin B. In early fetal life AMH expression is triggered by SOX9 gene, and enhanced by SF1 and WT1, independently of gonadotropic control [2, 3]. Later, FSH stimulates AMH production. In females, AMH is produced by the granulosa cells of primary and small antral follicles present in the ovaries from late fetal life throughout reproductive life [2].

Müllerian ducts regress in the male fetus during the 8th and 9th week of gestation through apoptosis and epithelial-mesenchymal transformation occurring in a cranial-to-caudal direction. By week 10, Müllerian ducts become insensitive to AMH [2, 4].

2.2. Neonate. The known transient increase of gonadotropins in the first hours after birth is followed by a sharp decrease as

of the second day of life. By the 7th day of live, gonadotropins level is high again. Leydig's cell testosterone (T) production follows the LH surge, with a certain delay. Testosterone level is high during whole neonatal life. SC-specific peptides inhibin B and AMH are at their lowest levels in the first days after birth but increase after the first week, likely reflecting active SC proliferation [5]. This AMH increase is probably related to FSH-induced SC proliferation, and also to activation of AMH gene transcription through a pathway mediated by cAMP [6, 7]. AMH rises rapidly in concentration in boys during the first month, reaching a peak level at about 6 months of age, and then slowly declines during childhood, falling to low levels in puberty [8, 9].

In humans, androgens both induce spermatogenesis and repress AMH. Androgen receptor (AR) protein is present in Leydig and peritubular cells of fetal and neonatal human testis, but not in SC. The absence of AR expression in SC of fetal and neonatal human testis contributes to the lack of germ cell maturation and of AMH repression despite strong testicular testosterone biosynthesis.

AMH is undetectable (54%) or very low (95% CI: <2–16 pmol/L) in female infants.

2.3. Puberty. The pubertal decline in AMH results from gradual activation of the hypothalamic-pituitary-gonadal axis, and subsequent increase in intratesticular testosterone, rather than from the interaction between SC and spermatogenic cells [10, 11]. A functional AR appears to be essential for intratesticular testosterone-mediated AMH repression to occur in human SC [12]. The early increase in intratesticular testosterone level is responsible for the inhibition of AMH expression [13, 14]. The AR expression is first observed in the nuclei of few SC at the age of 5 months, and progressively increases to high levels of AR expression in more than 90% of SC nuclei by the age of 8 years. The presence of AR in boys older than 8 years old explains the early pubertal decline of AMH induced by intratesticular testosterone rise, despite the increase in FSH secretion [15].

The close relationship between AMH and inhibin B suggests that inhibin B is an indirect indicator of AR-mediated SC maturation [10].

While AMH expression is downregulated by meiotic germ cells, the expression of inhibin B β-subunit is dependent on the coexistence of spermatogenesis [2, 16, 17]. In men, suppression of spermatogenesis due to the lack of testosterone could stimulate AMH and inhibit inhibin B expression [16].

AMH expression and secretion by SC is regulated by inhibitory paracrine actions of intratesticular testosterone and neighbouring germ cells and by a stimulating hormonal effect of FSH. The effect of FSH on testicular AMH production might be due to a direct effect on AMH expression in each individual SC, a proliferative effect on SC, or both. The prepubertal testis is mainly composed of SC, which represent more than 75% of gonadal mass [15, 18] and are active [15, 19]. AMH determination may also be used to explore the functional reserve in response to FSH, because the spermatogenic potential of the testis is dependent on SC function [15, 20]. Basal and FSH-stimulated levels of AMH, might become a useful predictive marker of the spermatogenic response to gonadotropic treatment in young patients with hypogonadotropic hypogonadism (HH) [15, 21].

2.4. Adult. In the human adult testis, spermatogenesis is under control of FSH and LH. FSH acts directly on SC, LH induces testosterone production after Leydig cell stimulation. Intratesticular testosterone acts via a paracrine mechanism on AR expressed by target cells situated in the seminiferous tubules [2, 3, 5]. In contrast to the neonatal phase, SC in the adult human testis do express ARs. In adulthood, the action of androgens on the seminiferous tubules is essential for full, quantitatively normal spermatogenesis and fertility. Most evidence suggests that this effect is mediated through an effect on SC. The number of SC is directly associated with sperm-producing capacity, since each of these somatic cells can nurture only a limited number of developing spermatogenetic cells [2, 3, 5]. In adults, AMH type 2 receptors have been detected in SC, suggesting an autocrine effect. Also, paracrine effects of AMH on Leydig cells and adult germ cells were found, as AMH directly inhibits Leydig cell differentiation and steroidogenesis and might be involved in sperm motility [22]. In cases of infertility, in the absence of the androgen-inhibitory effect, FSH is able to enhance testicular AMH secretion in man [15].

AMH in the testis is secreted by SC both apically into seminiferous tubules and basally towards the interstitium and circulation. After puberty, AMH is released preferentially by the apical pole of the SC towards the lumen of the seminiferous tubules, resulting in higher concentrations in the seminal plasma than in the serum [23]. A reduced fertility potential is characterized by reduction in sperm motility, sperm concentration, testicular volume, and decreased inhibin B level [24]. Also, AMH concentration in seminal plasma in azoospermia is lower than in normal men [25]. The assay of seminal AMH may be considered as a tool for prediction of gonadotropin therapy outcome in hypogonadotropic hypogonadism, since its early increase may be a marker of good spermatogenic response [23].

3. Pathology

3.1. Persistent Müllerian Duct Syndrome. Ambiguous genitalia due to impaired androgen secretion or action may be a result of various conditions with low, normal, or high AMH level. Defects in AMH production and insensitivity to AMH due to receptor defects result in the persistent Müllerian duct syndrome (PMDS) [26]. AMH deficiency due to mutations in AMH gene represents an early-onset fetal hypogonadism with SC-specific dysfunction. Patients are otherwise normally virilised, indicating normal Leydig cell function [2]. The persistence of the uterus and Fallopian tubes is an unpredicted finding at surgery for hernia or cryptorchidism in boys. AMH is undetectable but inhibin B serum level and androgens are in the normal male range [26–28]. In the absence of cryptorchidism, testes contain germ cells but fertility is frequent [29]. PMDS should not

be confused with testicular dysgenesis, where persistence of Müllerian derivatives is associated with external sexual ambiguity reflecting both AMH and androgen deficiency, that is, an early onset fetal hypogonadism with whole gonadal dysfunction [2].

3.2. Androgen Insensitivity Syndrome (AIS). In the newborn, defects of androgen signaling in target organs result in an anatomical phenotype of Leydig cell-specific hypogonadism causing androgen deficiency. In the complete form of androgen insensitivity syndrome, female external genitalia with short vagina and the absence of uterus and Fallopian tubes reflect the lack of androgen action together with the normal AMH production [2, 26, 30]. However, amenorrhoea is permanent due to Müllerian regression in fetal life. In partial forms of AIS, ambiguous genitalia and Wolffian duct underdevelopment of various degrees are observed. The diagnosis is made by normal to high testosterone and AMH, and the absence of Müllerian derivatives [2, 26, 31].

AMH signals through two membrane receptors: the type 2 receptor (AMHR2), which binds to AMH, and a type 1 receptor involved in signal transduction [27]. Mutations in the genes encoding AMHR2 and the androgen receptor are associated with the specific hormone-resistance syndromes [31]. PMDS due to mutations in the AMHR2 provoke insensitivity to AMH owing to disrupted ligand binding, signal transduction, or cellular transport [32]. The anatomic picture does not differ from that observed in patients with AMH gene mutations. Serum AMH makes the distinction since it is normal or elevated in patients with AMH insensitivity [26, 27].

3.3. Congenital Hypogonadotropic Hypogonadism (HH). Congenital HH affects the development of SC [10]. Severe gonadotropin deficiency leads to a decreased number of SC and therefore low AMH and inhibin B levels [10, 33, 34]. Circulating AMH provides a potentially useful tool for differentiating congenital HH from constitutional delay of growth and puberty in males with delayed puberty [10, 33, 35]. The assessment of AMH, might predict the clinical onset of puberty without the need for repeated clinical examination or GnRH testing [10, 33]. The early pubertal increase in inhibin B is tightly coupled to a decrease in AMH and therefore may reflect androgen-mediated differentiation of SC. The low AMH levels in patients with congenital HH and prepubertal testis volume may be of clinical value in early diagnosis of this condition, and the very low AMH level in boys with profound congenital HH suggests impaired development of the SC population [10, 33]. In untreated hypogonadotropic hypogonadal men, FSH induces a gradual increase in AMH levels, when hCG is added—AMH secretion is suppressed [15]. In contrast, Boukari et al. observed that AMH levels were not reduced in the two adult patients with mild androgen insensitivity syndrome (MAIS), who received gonadotropin treatment [12]. This AMH response pattern in MAIS is similar to that recently reported in two HH neonates [36] receiving a similar treatment, indicating that in both cases the failure to repress AMH is related to the absence of functional AR expression in SC.

3.4. Cryptorchidism. A measurable value of AMH in a boy with bilateral cryptorchidism is predictive of undescended testes, while an undetectable value is highly suggestive of anorchia or ovaries, as would be the case in girls with female pseudohermaphroditism and pure gonadal dysgenesis [37]. Values above the normal range for women remain diagnostic of the presence of testicular tissue, except in women with granulosa-cell tumors or sex-cord tumors that secrete AMH [38, 39].

The first phase of typical testicular descent takes place between the 10th and 15th week of human gestation [40]. This occurrence is independent of androgen levels, as the process has been found to transpire in patients with complete androgen insensitivity, and may also be controlled by AMH and insulin-like hormone 3 (INSL3) [24, 41, 42]. INSL3 is secreted by the Leydig cells shortly after the onset of testicular development, and controls the thickening of the gubernaculum anchoring the testis to the inguinal region [43]. Disruption of the INSL3 gene in mice results in bilateral intra-abdominal testes [44, 45]. In humans on the other hand, it was found that only 1,9% of the cases of cryptorchidism were caused by INSL3 gene mutations, and that the mutations of the INSL3 receptor on the whole were uncommon [46, 47]. The second or inguinoscrotal phase of testicular descent occurs between 26th and 40th weeks of gestation [40]. During this phase, the testis migrates through the inguinal canal and across the pubic region to the scrotum. There is much clinical evidence, that shows reduced androgen action to be associated with undescended testes [24, 40–45].

Unilateral cryptorchidism carries an increased risk of infertility in adulthood. Up to 30% of men operated on in childhood for unilateral cryptorchidism are likely to be subfertile in later life [48–51]. Men who undergo an operation for bilateral cryptorchidism are more affected—up to 54% are infertile according to their semen and hormonal analysis [49, 52].

Lower serum AMH concentrations in otherwise healthy boys with cryptorchidism, who were compared with their age-matched counterparts with palpable testes, have been reported in several studies [53–55]. Some authors observed upward trend in AMH concentration one year after orchidopexy, but it was statistically insignificant [56]. In contrast, Aksglaede et al. did not find the difference in AMH concentrations between patients with Klinefelter Syndrome, with or without a history of cryptorchidism. The exception to this was noted in untreated patients, 10–14 years old, in whom the expected puberty decline in AMH tended to occur later than in the noncryptorchid patients of the same age [57].

3.5. Varicocele. In a large study of 124 boys with varicocele Trigo et al. showed that AMH levels were higher in prepubertal patients with varicocele than in controls. Similarly, inhibin B levels were higher in pubertal boys with varicoceles than in the controls [58]. This altered serum profile of gonadal hormones in boys with varicoceles may indicate an early

abnormality in the regulation of the seminiferous epithelial function [58].

In another study, Goulis et al. measured peripheral vein and spermatic vein inhibin B and AMH concentrations. In peripheral vein inhibin B, concentrations in men with varicocele were lower as compared to controls, but there was no difference in AMH concentrations. Spermatic vein inhibin B concentrations in men with varicocele were higher compared to those of peripheral vein. On the contrary, spermatic vein AMH concentrations were lower compared to those from peripheral vein [59]. The clinical significance of AMH concentrations in peripheral and spermatic vein remains to be elucidated.

4. AMH as a Marker Evaluating Gonadal Function

Serum AMH is known to be valuable in assessing gonadal function [33, 60, 61]. As compared with testing involving the administration of human chorionic gonadotropin, the measurement of AMH is more sensitive and equally specific. Measurement of AMH is very useful in young children, because serum gonadotropin concentrations in those who are agonadal are nondiagnostic in midchildhood and serum testosterone concentrations may fail to increase with provocative testing in children with abdominal testes [33, 37, 62]. Testicular size and sperm density in adult men are positively correlated to germ-cell status in the testes in childhood [63, 64]. In prepubertal boys, low serum AMH correlates with small testes [47]. Early postnatal admission of recombinant human FSH is resulting in an increase of testicular size and elevation of serum AMH level [40].

5. Conclusion

AMH is one of the key factors conditioning the normal development of male genitals. Serum AMH determination is clinically valuable in assessing gonadal function. Basal and FSH-stimulated levels of AMH, might become a useful predictive marker of the spermatogenic response to gonadotropic treatment in young patients with hypogonadotropic hypogonadism. A measurable value of AMH in a boy with bilateral cryptorchidism is predictive of undescended testes, while an undetectable value is highly suggestive of anorchia or ovaries, as would be the case in girls with female pseudohermaphroditism and pure gonadal dysgenesis. AMH levels are higher in prepubertal patients with varicocele than in controls, which indicate an early abnormality in the regulation of the seminiferous epithelial function.

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Research Article

Serum Anti-Müllerian Hormone Levels in Patients with Epithelial Ovarian Cancer

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Objectives. The aim of our study was to examine serum anti-Müllerian hormone (AMH) concentration in ovarian cancer patients in relation to clinicopathological features, such as a pathological subtype of the tumor, (FIGO) stage, grading, and overall 5-year survival. *Material and Methods*. We enrolled 72 epithelial ovarian cancer patients in our study, aged 45–79 years, who underwent optimal cytoreductive surgery. In all patients, serum AMH concentration was measured using a two-step sandwich type enzyme immunoassay before surgery. As a reference value for women over 45 years we accepted anti-Müllerian hormone concentration below 1 ng/mL. *Results*. In the whole group of patients with ovarian cancer, median serum concentration of AMH was 0.07 (0.0–0.37) ng/mL, whereas in the group of those with positive AMH values (≥0.14 ng/mL) it was 0.31 (0.15–0.73) ng/mL. No significant correlation was found between serum AMH levels and FIGO stage, histological subtype, or grading ($P > 0.05$). The analysis of five-year survival rate related to AMH levels showed no statistically significant differences. There were no differences in survival rates between patients with positive or negative serum AMH levels. *Conclusion.* Measurement of serum anti-Müllerian hormone levels was not useful in predicting clinicopathological features and survival in patients with ovarian cancer.

1. Introduction

Among cancers of female reproductive system, ovarian cancer became the second most common and is the fifth ranked cause of cancer-related mortalities in women in Europe and the United States [1]. Although understanding of ovarian cancer has improved substantially, the etiology of the disease remains unknown. Moreover, even though surgical procedures made a great progress and new protocols of chemotherapy were introduced, the 5-year survival rate does not exceed 45%. The main reason for this situation is that the majority of women with ovarian cancer turn to gynecologist for help at the late stage of the disease and that despite introducing new markers for the detection of ovarian cancer, their low diagnostic sensitivity does not permit to use them as screening [2, 3].

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), belongs to a larger family of transforming growth factor-β (TGF-β). AMH signals through two transmembrane receptors, type II which is specific (present in Müllerian duct and gonads) and type I receptors, shared with the bone morphogenetic proteins family [4, 5].

AMH is expressed in the Sertoli cells of fetal testis from the seventh week of pregnancy [6], and its secretion is fundamental in regression of the Müllerian ducts [7].

In women AMH is produced by granulosa cells, from preantral and antral follicles. Serum AMH levels are undetectable in newborns, increase during childhood and adolescence reaching its peak in the early 20s, and remain stable throughout reproductive period only to decrease during menopausal transition [8, 9]. During menstrual cycle serum

AMH maintains its level and lowers slightly in early secretory phase [10, 11].

Studies on mice had shown that AMH inhibits the transition from the primordial to the primary follicular stage. AMH paracrine signaling inhibits FSH-related follicle growth leading to selection of primary follicle. FSH and estradiol decrease AMH gene expression in granulosa cells [12]. Recent in vitro studies confirmed that increased expression of AMH is caused by bone morphogenetic protein (BMP-6) and also that AMH inhibits recruitment of primordial follicles in order to keep ovarian function in balance [13].

The number of primordial follicles decreases with age of women. Lower serum AMH levels precede the increase of FSH and inhibin B levels and thus are the most accurate parameter of ovarian reserve in clinical practice [14–16]. AMH possesses high prognostic value in prediction of number of obtained oocytes [17, 18]. Besides, ovarian hyperstimulation syndrome seems to be associated with significantly higher basal AMH levels [19].

In women undergoing oncologic treatment AMH is considered a useful marker of damage to the ovarian reserve [20, 21].

The fact that AMH expression is restricted to ovarian granulosa cells in women led to establishing AMH levels as a serum marker of granulosa cell tumours (GTCs). Recent studies showed an increased serum AMH concentration in 76–93% of women with both primary and recurrent GTCs [22, 23].

As anti-Müllerian hormone belongs to transforming growth factor-β family and its representatives play an important role in ovarian cancer carcinogenesis, the aim of this study was to examine AMH concentration in ovarian cancer patients in relation to clinicopathological features, such as a pathological subtype of the tumor, FIGO stage, grading, and overall 5-year survival.

2. Patients, Materials, and Methods

2.1. Patients. We enrolled 72 epithelial ovarian cancer patients aged 45–79 years (mean 58.5 ± 10 years) treated in Department of Gynecology and Obstetrics, Ludwik Rydygier Collegium Medicum in Bydgoszcz in the period of 2005-2006.

Only women who underwent optimal cytoreductive surgery were considered for further analysis, all of them with residual cancerous focuses smaller than 1 cm in diameter. All patients were operated by experienced gynecological oncologist. The standard surgical protocol included tumorectomy, hysterectomy with bilateral salpingoovariectomy, pelvic lymphadenectomy, omentectomy, and appendectomy.

Women who had ever used hormonal replacement therapy or had undergone ovulation induction were excluded. All patients were Caucasian, after menopause with BMI range from 19 to 30 kg/m^2.

International Federation of Gynecology and Obstetrics (FIGO) ovarian cancer staging system was used to assess clinical stage of the disease. Early stages were confirmed in 16 patients (FIGO I in 9 women, II in 7) and advanced disease in 56 patients (FIGO III—50, IV—6). All ovarian cancer cases were with histological confirmation, of which 59 (82%) serous, 5 (7%) mucinous, and 8 (11%) endometrioid. Histological examination was performed in the Department of Pathology, Antoni Jurasz University Hospital in Bydgoszcz, and histological grading was determined (G1 in 9, G2 in 23, and G3 in 40 patients).

Table 1: Baseline characteristics of the study group.

Parameter	Patients with ovarian cancer $n = 72$
Age (years)	58.5 ± 10.4
BMI (kg/m^2)	22 ± 2.2
FIGO stage *n* (%)	
I	9 (12.5%)
II	7 (10%)
III	50 (69.5%)
IV	6 (8%)
Histological subtype *n* (%)	
Serosum	59 (82%)
Mucinous	5 (7%)
Endometrioid	8 (11%)
Histological grading *n* (%)	
G1	9 (12.5%)
G2	23 (32%)
G3	40 (55.5%)

After optimal cytoreductive surgery, all women underwent 6 courses of chemotherapy based on carboplatin and paclitaxel. Baseline characteristics of the study participants are shown in Table 1.

The Bioethical Committee at the Ludwik Rydygier Collegium Medicum, Nicolaus Copernicus University of Torun, have reviewed and approved this study. All participants have provided informed consent.

2.2. Methods. Blood samples were collected (10 mL) after admission to the hospital, on the day before surgery. After centrifugation in standard conditions serum was obtained, aliquoted, and stored at −70°C until assayed.

Serum AMH concentration was measured using a two-step sandwich type enzyme immunoassay (Immunotech AMH/MIS ELISA kit, Beckman Coulter). The imprecision of the assay was 12.3% at 0.2 ng/mL and 5.1% at 15.8 ng/mL. The lowest AMH concentration in a sample which could be detected with a 95% probability was 0.08 ng/mL (lower detection limit). Concentration of AMH below 0.14 ng/mL has been accepted as negative.

Reference values for anti-Müllerian hormone for women over 45 years were less than 1 ng/mL [24].

The Kolmogorov-Smirnov test was used to assess normality of distribution of investigated parameters. Data were expressed as mean ± standard deviation and median with 25th–75th percentiles. Comparison between the groups was performed by using the Mann-Whitney U test and the

Table 2: AMH concentration in relation to FIGO stage.

	FIGO stage	Statistical characteristics							Mann-Whitney U test
		Mean	SD	Min	Q1	Q2	Q3	Max	
AMH/MIS (ng/mL)	I/II	0.39	0.65	0.00	0.00	0.1	0.34	1.75	0.702
	III/IV	0.34	0.79	0.00	0.00	0.08	0.33	3.26	

AMH/MIS: anti-Müllerian hormone/Müllerian inhibiting substance; FIGO: Federation Internationale de Gynecologie et d'Obstetrique; Max: maximum value; Min: minimum value; Q1: lower quartile; Q2: median; Q3: upper quartile; SD: standard deviation.

Table 3: AMH concentration in relation to grading.

	Grading	Statistical characteristics							P value Kruskal-Wallis test
		Mean	SD	Min	Q1	Q2	Q3	Max	
AMH/MIS (ng/mL)	G1	0.17	0.21	0.00	0.00	0.1	0.34	0.58	0.875
	G2	0.37	0.75	0.00	0.00	0.04	0.37	3.26	
	G3	0.3	0.62	0.00	0.00	0.01	0.31	2.95	

AMH/MIS: anti-Müllerian hormone/Müllerian inhibiting substance; Max: maximum value; Min: minimum value; Q1: lower quartile; Q2: median; Q3: upper quartile; SD: standard deviation.

Table 4: AMH concentration in relation to histological type of cancer.

	Histological type	Statistical characteristics							P value Mann-Whitney U test
		Mean	SD	Min	Q1	Q2	Q3	Max	
AMH/MIS (ng/mL)	Serosum	0.23	0.49	0.00	0.00	0.04	0.3	3.26	0.653
	Others	0.68	1.04	0.00	0.00	0.02	1.44	2.95	

AMH/MIS: anti-Müllerian hormone/Müllerian inhibiting substance; Max: maximum value; Min: minimum value; Q1: lower quartile; Q2: median; Q3: upper quartile; SD: standard deviation.

Table 5: Prognostic factors for overall survival selected by Cox's univariate analysis.

	Statistical characteristics					
	Parameter evaluation	Chi-squared	P value	HR	95% CI HR lower endpoint	95% CI HR upper endpoint
Age	0,02	3,66	0,06	1,03	0,99	1,05
AMH (ng/mL)	−0,01	0,66	0,41	0,98	0,95	1,02
Histo-Pat (serosum)	0,05	0,07	0,79	1,1	0,53	2,27
Grading (G2 + G3)	−1,04	4,24	0,04	0,12	0,02	0,9
FIGO (III/IV)	−0,83	10,04	0,001	0,19	0,06	0,53

AMH/MIS: anti-Müllerian hormone/Müllerian inhibiting substance; CI: confidence interval; FIGO: Federation Internationale de Gynecologie et d'Obstetrique; HR: hazard ratio.

Table 6: Prognostic factors for overall survival selected by Cox's multivariate analysis.

	Statistical characteristics				
	Parameter evaluation	P value	HR	95% CI HR lower endpoint	95% CI HR upper endpoint
Age	0,02	0,38	1,02	0,98	1,06
AMH (ng/mL)	−0,02	0,22	0,97	0,94	1,01
Histo-Pat (serosum)	0,08	0,7	1,17	0,51	2,67
Grading (G2 + G3)	−0,67	0,2	0,26	0,03	2,01
FIGO (III/IV)	−0,73	0,01	0,23	0,08	0,68

AMH/MIS: anti-Müllerian hormone/Müllerian inhibiting substance; CI: confidence interval; FIGO: Federation Internationale de Gynecologie et d'Obstetrique; HR: hazard ratio.

Kruskal-Wallis test for non-Gaussian distributed variables. P value < 0.05 was considered statistically significant.

Overall survival rate was examined for significance using log-rank test and Kaplan-Meier curves.

Univariate and multivariate Cox regressions were performed. For the analysis, a forward selection with a P value of less than 0.05 for entry was applied. The effects of the variables were expressed as hazard ratios per 1 SD change to allow for a better comparability between the effect sizes of the different tested variables.

All statistical analyses were performed using Statistica for Windows Statsoft 10.0.

3. Results

In the group of patients with ovarian cancer median serum concentration of AMH was 0.07 (0.0–0.37) ng/mL, whereas median concentration in the patients with positive AMH values (≥0.14 ng/mL) was 0.31 (0.15–0.73). Values equal to/above 0.14 ng/mL were found in 44 women (61%).

Median concentrations of serum AMH in relation to FIGO stage did not differ significantly (Table 2).

No significant correlations were found between serum AMH concentration and histopathological subtype or grading (Tables 3 and 4).

The results of Cox regression of the predictive power of variables are shown in Tables 5 and 6. In the univariate analysis grading and FIGO were significantly correlated with survival time in women with ovarian cancer. In contrast, in the multivariate analysis only FIGO stage had a statistically significant effect on survival time.

Overall survival rate was also examined in relation to AMH level. Kaplan-Meier analysis was performed in two groups of ovarian cancer patients: first group ($N = 28$) of women with serum concentration below the detection limit and another ($N = 44$) who displayed values over 0.14 ng/mL (Figure 1). The long-rank test showed no statistical significance ($P = 0.98$). The analysis of five-year survival rate related to AMH levels showed no statistically significant differences; there were no differences in survival rates between patients with positive or negative AMH values (Figure 1).

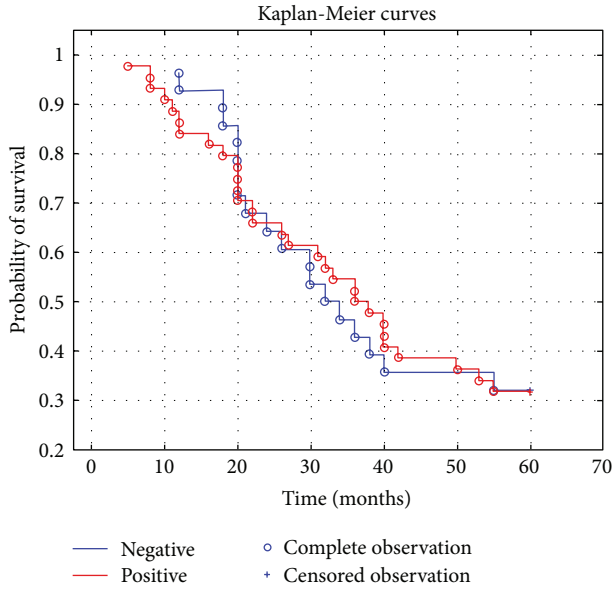

Figure 1: Survival curves in relation to AMH levels.

4. Discussion

Although it was commonly believed that the cells of epithelial ovarian cancer come from the epithelium covering the surface of the ovary, the current theory states that cancer arises from the cells of the Fallopian tube. Most recent studies indicate that the vast majority of ovarian tumors derive from the fimbriae of Fallopian tube and other components developed from the Müllerian ducts [25]. Anti-Müllerian hormone (AMH) initiates the process of regression of Müllerian ducts. Based on these facts some authors suggest that the determination of AMH could be important in the diagnosis and treatment of epithelial ovarian cancer [26, 27].

The median concentration of AMH in epithelial ovarian cancer patients was 0.07 ng/mL taking into account both positive and negative AMH results. However, in the group of women with AMH levels above 0.14 ng/mL, the median was 0.31 (0.15–0.73). The results observed in our study were similar to those of age-specific AMH values found by others but in healthy women [24].

Despite the lack of statistically significant differences, women with FIGO classifications I and II had lower AMH levels than women with FIGO stages III and IV. On the other hand, according to histological grading, highest AMH values were observed in women with G1 staging.

Detailed analysis of concentrations of anti-Müllerian hormone has revealed no differences in the levels of AMH, depending on the type of cancer, clinical stage, and histological grade. There was no correlation between serum AMH and the five-year survival rate. There were no differences in years of survival of patients with AMH in serum compared to patients who were negative for anti-Müllerian hormone. In our study only advanced clinical stage according to FIGO was an independent poor prognostic factor.

To the best of our knowledge there are no available studies demonstrating the usefulness of determination of AMH concentrations in the serum of patients with epithelial ovarian cancer.

The search for alternative therapies in the treatment of ovarian cancer has led to research on biologically active substances which might inhibit the proliferation of the tumor. Masiakos et al. hypothesized that the anti-Müllerian hormone may serve as such factor because it causes apoptosis and the regression of the Müllerian ducts in embryos, by variation of ovarian tumor cell biology [26]. Further studies showed the presence of type II receptor for AMH in the ovary and in ovarian cancer cells, and its inhibitory effect was confirmed in transgenic mice. In addition, tests were performed on cells obtained from peritoneal fluid from patients with ovarian cancer at FIGO stages III/IV. Masiakos et al. are of the opinion that the determination of AMH and its receptor by flow

cytometry could be used in selection of patients with poor prognosis, but this fact has not been confirmed in subsequent studies [26].

In conclusion, AMH belongs to the family of transforming growth factor, which plays an important role in ovarian carcinogenesis. However, none of the limited studies could demonstrate the role of the anti-Müllerian hormone in the serum of patients with ovarian cancer. Similarly, in our study, we failed to show any benefits from the determination of serum AMH in women with ovarian cancer.

Despite our findings concerning epithelial ovarian cancer, serum AMH levels remain well-established marker in granulosa-theca cell tumors. It is necessary to perform further studies to determine the tissue expression of AMH and type II receptor for AMH in ovarian cancer tissue and the potential usefulness of monoclonal antibodies against AMH and its receptor in the diagnosis and treatment of women with ovarian cancer.

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