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Comments
At the time of publication, Mary Lee was not yet affiliated with the University of Massachusetts Medical School.

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The Role of Müllerian Inhibiting Substance in the Evaluation of Phenotypic Female Patients with Mild Degrees of Virilization

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Müllerian inhibiting substance (MIS) is a sexually dimorphic gonadal hormone with proven efficacy in the evaluation of boys with cryptorchidism and children with intersex conditions. We examined the role of MIS determination in the evaluation of 65 phenotypic females with mild virilization. Among the 28 subjects with MIS values elevated above the normal female range, all had abnormal gonadal tissue: ovotestes in 11, testes in 7, dysgenetic gonads in 7, and MIS-secreting ovarian tumors in 3. Among the 37 children with serum MIS in the normal female range, 19 had detectable MIS and 18 had unmeasurable MIS. In the former group with measurable but normal female MIS values, 16 subjects had ovaries, 1 had an ovotestis, and 1 had dysgenetic gonads containing testicular elements. Of 18 children with undetectable MIS values, 16 had ovaries and 2 had ovarian dysgenesis. In this study, elevation of serum MIS above the normal female range was consistently associated with the presence of testicular tissue or MIS-secreting tumors, mandating additional evaluation and surgical exploration. A value within the normal female range in a virilized patient did not exclude dysgenetic testicular tissue or ovotestis, whereas undetectable values were consistent with the absence of testicular tissue. (J Clin Endocrinol Metab 88: 787–792, 2003)

MULLERIAN INHIBITING SUBSTANCE (MIS) also known as anti-Müllerian hormone (AMH) (1) promotes involution of the Müllerian ducts during male sexual differentiation and also exerts paracrine actions on postnatal ovarian and testicular development (2–7). MIS is a member of the TGF-β family of peptide hormones that regulates cellular growth and differentiation (1, 8–11). The expression of MIS is sexually dimorphic in Sertoli cells of the testis and granulosa cells of the ovary. Consequently, mean serum MIS values are much higher in males than females, particularly in prepubertal children. In girls, the expression of MIS is low or undetectable at birth, then increases slightly, but remains distinctly lower than the male range until 8–10 yr of age (12, 13). At puberty, MIS values in females and males start to overlap (12, 13). Serum MIS in women varies across the menstrual cycle within a narrow range (mean values of 1.4–1.7 ng/ml) (14). In boys, serum MIS rises rapidly after birth, peaks in late infancy, then decreases by puberty to low adult values (12, 15–19). The abundant expression of MIS in the prepubertal testis has proven useful for the evaluation of boys with cryptorchidism and children with intersex conditions (19–24). In boys with cryptorchidism, a measurable serum MIS is highly predictive of testicular tissue, whereas an undetectable value is consistent with anorchia (21–24). In children with intersex conditions, MIS not only distinguishes between testes and ovaries but also helps define the extent of testicular parenchymal tissue (19–23). Because serum MIS in prepubertal girls is near the detection limit of the MIS assays, its utility for the evaluation of gonadal disorders in phenotypic females has not been systematically explored.

Although the role of MIS in the regression of Müllerian ducts during male sexual differentiation is well understood, its role in females is less clear. MIS induces follicular growth and differentiation (4, 5, 7) and inhibits recruitment of primordial follicles (25) in animal studies. In women, MIS is measurable in ovarian follicular fluid (26) and its concentration in serum fluctuates across the menstrual cycle, peaking in the late follicular phase (13, 14). Women with polycystic ovarian syndrome have higher serum MIS values, which correlate inversely with serum estradiol, indicating that MIS may play a role in regulating estradiol synthesis as well as in the disordered folliculogenesis of polycystic ovarian syndrome (26).

Because MIS is a hormone restricted in expression to the gonads and expressed at much higher levels in testicular than ovarian tissue, we reasoned that MIS determination would help differentiate between gonadal and nongonadal causes of mild virilization. MIS values would be low in female infants with a 46,XX karyotype and normal ovarian tissue who are virilized from in utero exposure to maternal androgens or elevated adrenal androgens as with congenital adrenal hyperplasia (CAH). On the other hand, serum MIS would be elevated above the normal female range if the virilization is caused by testicular androgens in a child with a 46,XY karyotype and male pseudohermaphroditism. With acquired virilization, MIS determination might help distinguish between gonadal androgens and those from other sources. For example, androgen-secreting ovarian tumors such as Sertoli-Leydig cell tumors and selected virilizing granulosa-theca cell tumors (27, 28) might express high levels...
of MIS (29, 30). Therefore, we examined serum MIS concentrations in relation to gonadal histology and cytogenetic findings in phenotypic girls with mild virilization (nonpalpable gonads, clitoromegaly, and partial labial fusion).

Patients and Methods

Patient selection

We identified 65 phenotypic females who presented with virilization between 1 d and 17 yr of age and had MIS measurements from December 1995 to April 2000. These patients were evaluated initially in the Pediatric Endocrine Unit or the Pediatric Surgical Unit of the Massachusetts General Hospital for Children (n = 20), or at other institutions (n = 45). At Massachusetts General Hospital, the parents gave verbal consent for MIS determination, in accordance with approved institutional guidelines. However, because MIS determination is available as a standard diagnostic test, this was not uniformly obtained at other institutions. All patients underwent surgical exploration, gonadal biopsy, or diagnostic imaging. Clinical findings, cytogenetic analysis, operative details, and pathologic details of the gonadal biopsies were obtained from chart review and communications with the patients’ physicians.

Serum MIS assay

Serum MIS was measured by an ELISA as described in earlier studies (12, 16, 22). This assay does not cross-react with other members of this gene family, and is specific for primate MIS. The intraassay coefficient of variation for the assay is 9%, and the interassay coefficient of variation is 15%. The limit of sensitivity is 0.3 ng/ml. MIS values that fell below this (<0.3 ng/ml) were considered undetectable. Values of MIS that fell within the range for age-matched females were designated as normal, and MIS values above the female range were designated as elevated. We previously reported mean serum MIS values of 0.7 ng/ml (range, 0.2–1.9 ng/ml) in female infants, ages 2–12 months (12). By 18 months, MIS rises minimally to prepubertal values of 0.9 ng/ml (range, 0.2–3.9 ng/ml), then increases further to pubertal and adult values of 2.9 ng/ml (range, 0.2–8.9 ng/ml) (12). All MIS measurements were made on specimens that were freshly collected or stored for less than 2 yr.

Statistical analysis

The Student’s t test was used to compare means between groups. Sensitivity, specificity, and positive and negative predictive values with 95% confidence intervals were calculated using two-way contingency table analysis of Javastat, based on methods described by Fleiss (31), and the program Matlab (binofit.m) produced by The Mathworks Inc. (Newton, MA), which uses methods described by Johnson et al. (32).

Table 1. Gonadal status, mean MIS, and karyotype of study population (n = 65)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diagnosis</th>
<th>n</th>
<th>Gonadal status (n)</th>
<th>MIS (ng/ml)</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Congenital adrenal hyperplasia</td>
<td>13</td>
<td>Ovaries</td>
<td>0.8 ± 0.3</td>
<td>All 46,XX</td>
</tr>
<tr>
<td>2</td>
<td>Idiopathic virilization</td>
<td>20</td>
<td>Ovaries</td>
<td>0.6 ± 0.1</td>
<td>All 46,XX</td>
</tr>
<tr>
<td>3</td>
<td>True hermaphrodite</td>
<td>14</td>
<td>Bilateral ovotestes (6)</td>
<td>10.5 ± 2.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ovotestis and ovary (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Testis and ovary (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Gonadal dysgenesis</td>
<td>10</td>
<td>Dysgenetic</td>
<td>5.2 ± 1.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Partial androgen insensitivity</td>
<td>5</td>
<td>Bilateral testes</td>
<td>25.3 ± 15.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>All 46,XY</td>
</tr>
<tr>
<td>6</td>
<td>Virilizing ovarian tumor</td>
<td>3</td>
<td>Sertoli-Leydig cell tumor</td>
<td>69.0 ± 25.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>All 46,XX</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly greater than group 1. Groups 3, 4, and 5, P ≤ 0.01; group 6, P = 0.0001.

<sup>b</sup> Significantly greater than group 2. Groups 3 and 4, P ≤ 0.001; group 5, P ≤ 0.01; group 6, P ≤ 0.0001.

<sup>c</sup> Significantly lower than group 6. Groups 3 and 4, P ≤ 0.001.

Results

Patient characteristics

The subjects included 65 phenotypic females with mild degrees of virilization (Prader stages 1 and 2). All subjects were considered to have clitoromegaly by the examining physician. Some also had posterior labial fusion. The diagnoses, gonadal histology, karyotype, and MIS values are elaborated in Table 1. Mean age was 2.4 ± 0.5 yr (range of 1 d to 17 yr), and most were prepubertal (61/65).

Of the 65 subjects studied, 28 had elevated MIS values with a mean ± sem of 18.9 ± 5.1 ng/ml and individual values of 5.0 to 92.5 ng/ml (Table 2). Thirty-seven subjects had MIS in the female range with a mean ± sem of 0.6 ± 0.1 ng/ml and individual values ranging from undetectable to 3.3 ng/ml. Of the latter group, MIS was measurable in 19 and undetectable in 18 subjects. Cytogenetic analysis was available in all but two patients, and all subjects underwent surgical exploration, gonadal biopsy, and/or imaging studies. Gonads were not biopsied in thirteen 46,XX girls diagnosed with CAH and in twenty 46,XX Prader 1 girls with low or undetectable testosterone and normal imaging.

MIS and gonadal histology

All subjects with ovarian tissue had individual MIS values that fell within the normal female range. The lowest mean MIS value was found in this gonadal category (Fig. 1). The highest MIS values were found in 46,XX females with MIS-secreting ovarian tumors, followed by patients with bilateral testes. The individual MIS values in both of these categories were clearly above the female range but within the normal male range (Fig. 2). Subjects with bilateral or unilateral testes (n = 7) had higher MIS values than those with dysgenetic gonads or ovotestes (n = 22) (23.6 ± 11.1 ng/ml vs. 7.3 ± 1.6 ng/ml, P < 0.05, Fig. 1). In this latter group with abnormal gonads, a number of individual MIS values fell within the normal female range, although the mean value was significantly higher than that of girls with ovaries (7.3 ± 1.6 ng/ml vs. 0.7 ± 0.1 ng/ml, P < 0.0001). Among those with an
ovotestis, the presence or absence of Y chromosomal material did not alter the mean MIS value (7.5 ± 1.4 ng/ml vs. 8.1 ± 3.2 ng/ml).

**MIS and karyotype**

All 28 subjects with elevated serum MIS had gonads containing testicular tissue or MIS-secreting ovarian tumors (Table 2). Five of the seven subjects with testes were subsequently diagnosed with partial androgen insensitivity. The other two had 46,XX true hermaphroditism with unilateral testes. Eleven subjects had ovotestes, defined by the presence of both ovarian follicles and seminiferous tubules containing germ cells. Seven of these children had a 46,XX karyotype. The karyotype was unknown in one. The other three had the following karyotypes: 46,XY, 46,XX/46,XY, and 45,X/46,XY. The presence of ovarian follicles was unexpected in the patient with a 45,X/46,XY karyotype, raising the possibility of a gonadal 46,XX cell line. Seven subjects had dysgenetic testes, defined by the presence of dense fibrosis between tubules and absence of germ cells. Of these, the karyotype was 46,XY in three, 45,X/46,XY in three, and unknown in one. All three subjects with virilizing ovarian tumors had 46,XX karyotypes.

Among the 37 virilized subjects with MIS values in the normal female range, 19 had measurable values, whereas 18 had undetectable serum MIS (Table 2). Among those with measurable MIS values, one 8 yr old with a 46,XX karyotype and an MIS value of 0.7 ng/ml had an ovotestis, and a 7 month old with a 45,X/46,XY karyotype and low MIS of 0.3 ng/ml had dysgenetic testicular tissue. The rest had presumably normal ovaries. None of the subjects in the group with undetectable MIS values had testicular tissue or dysgenetic gonads with testicular elements. Sixteen had ovaries, whereas two patients aged 2.5 and 4 yr with 45,X/46,XX karyotypes and clitoromegaly had ovarian dysgenesis without any testicular elements identified.

| TABLE 2. Gonadal status and karyotype according to elevated or normal MIS values |
|--------------------------|--------------------------|---------------------|---------------------|---------------------|
| **MIS** | **Gonadal status** | **Karyotype** | **1** | **2** | **3** |
| Elevated (n = 28) | Testis | 2 (U/L) | 5 (B/L) | 2 (45,X/46,XY) | 1 |
| | Ovotestis | 7 | 1 | (46,XX/46,XY) | |
| | Dysgenetic gonad | | | | |
| | Testicular elements + | | | | |
| | Testicular elements – | | | | |
| | MIS-secreting ovarian tumor | 3 | 3 | (45,X/46,XY) | 1 |
| Normal (n = 37) | Ovotestis | 1 | | |
| Detectable (n = 19) | Dysgenetic gonad | | | |
| | Testicular elements + | | | |
| | Testicular elements – | | | |
| | Ovaries | 17 | | |
| Undetectable (n = 18) | Dysgenetic gonad | | | |
| | Testicular elements + | | | |
| | Testicular elements – | | | |
| | Ovaries | 16 | | |
| U/L Unilateral; B/L bilateral. | | |

FIG. 1. Mean serum MIS concentrations in 65 mildly virilized phenotypic females according to gonadal status. The groups of subjects with testes, ovotestes, dysgenetic gonads, or MIS-secreting ovarian tumors all had significantly higher MIS values than the group of girls with ovaries (*, P < 0.0001).

FIG. 2. Individual MIS values by gonadal status in relation to normal female MIS values for age. Normal MIS values in girls (broken black lines) and boys (solid black lines) with the mean and lower and upper limits depicted. Individual serum MIS concentrations of girls are plotted according to gonadal status. Subjects with testes, ovotestes, or MIS-secreting ovarian tumors had values above the female range, whereas those with dysgenetic testes (open triangles) had MIS values that ranged from the low normal female to above the normal range. All subjects with ovaries had values within the normal female range for age.
All 33 subjects with presumptive ovarian tissue (no testicular elements or dysgenetic gonads detected by imaging studies) had 46,XX karyotypes. Thirteen of these subjects were confirmed to have 46,XX CAH, whereas the remaining 20 girls had mild idiopathic virilization of unidentified etiology and low neonatal testosterone values. Further work-up was advised in these 20 girls, all of whom were classified as Prader 1 with clitoromegaly ± minimal posterior labial fusion.

Comparison of MIS and karyotype determination

Testicular tissue was found in all subjects in this series with Y chromosomal material (nine with a 46,XY karyotype and six with sex chromosome mosaicism). As anticipated from previous clinical observations, the presence of an Y chromosome was associated with the presence of testicular tissue (33, 34). The absence of Y chromosomal material in the peripheral karyotype, however, was not predictive of absent testicular tissue. In this series, 10 subjects with a 46,XX karyotype had testes or ovotestes. Therefore, MIS determination was compared with cytogenetic analysis for discerning the presence of testicular tissue (Table 3). This analysis indicated that an elevated MIS value and Y chromosomal material were both highly specific and predictive for testicular tissue (Table 3). However, the absence of Y chromosomal material was less sensitive and predictive for the absence of testicular tissue than MIS determination (negative predictive value of 72.9% vs. 94.6% (Table 3).

Discussion

We have shown that MIS determination distinguishes gonadal from nongonadal causes of mild virilization in phenotypic females. If Y chromosomal material is present in a virilized child, testicular tissue is likely, and MIS would likewise be elevated. In the absence of Y chromosomal material, an elevated MIS suggests that either testicular tissue or a virilizing ovarian tissue is present. In this study, all of the 18 subjects with undetectable MIS values had apparently normal or dysgenetic ovaries: no testicular tissue or gonadal tumors were identified. In 16 of these 18 cases, the karyotype was 46,XX. The most frequent diagnosis was CAH with virilized child, testicular tissue is likely, and MIS would likewise be elevated. In the absence of Y chromosomal material was less sensitive and predictive for the absence of testicular tissue than MIS determination (negative predictive value of 72.9% vs. 94.6% (Table 3).

Across species, the expression of MIS is limited to the gonads, making it a highly specific marker of gonadal tissue. During early childhood, MIS expression is much higher in the testes than the ovary, with no overlap in mean serum concentrations (12). These characteristics of MIS expression, i.e. its sexual dimorphism and specificity for gonadal tissue, have facilitated the use of the MIS assay for evaluating children with diverse gonadal disorders (19–24, 35). In boys with cryptorchidism, MIS determination reliably distinguishes between anorchia and undescended testes (22, 24), with the rare exception of a patient with the persistent Mullerian duct syndrome caused by an MIS gene defect (36–38). In children with intersex conditions, MIS determination helps ascertain the presence of testicular tissue and aids differential diagnosis (19, 21, 23, 35). This study extends these findings by focusing on a subgroup of patients with lesser degrees of genital ambiguity who were deemed to be phenotypic females with virilization.

The evaluation of virilized girls can be challenging, particularly in the prepubertal years when gonadotropins and sex steroids are nondiagnostic (39, 40). Cytogenetic analysis, although not indicative of a specific condition, can help direct further evaluation. If Y chromosomal material is detected, quantitative assessment of MIS secretion may help distinguish between conditions primarily affecting androgen synthesis or action (such as androgen insensitivity) from those due to abnormal formation and structure of the gonad such as a dysgenetic testes or anorchia (19, 21, 23). A normal or high normal MIS value is consistent with a structurally normal testis, whereas a low value may indicate an abnormality of testicular formation. One caveat must be kept in mind, however. In children with dysgenetic testes, serum MIS can fall either within the normal female range or above the female norms to the low normal male range (19, 22). Therefore, an MIS value within the normal female range in a virilized female does not exclude dysgenetic testicular tissue or ovotestis and cannot be used as confirmation of normal ovaries. In this situation, more extensive evaluation including assessment of basal or stimulated testosterone levels or imaging studies may help establish the diagnosis.

In virilized girls, it is not uncommon to find a 46,XX karyotype or a mosaic karyotype without Y chromosomal material. Only 15 of our subjects had Y material detected, whereas 48 had a 46,XX karyotype and 2 had mosaicism (45,X/46,XX). In this study, 13 of the 48 virilized patients with a 46,XX

### TABLE 3. Test parameters for MIS determination vs. cytogenetic analysis (n = 63)\(^a\)

<table>
<thead>
<tr>
<th>Testicular tissue</th>
<th>Karyotype</th>
<th>Testicular tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated MIS</td>
<td>Present</td>
<td>Y material present</td>
</tr>
<tr>
<td>Normal MIS</td>
<td>Absent</td>
<td>Y material absent</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MIS</th>
<th>Y material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>92.9% (76.5—99.1%)</td>
</tr>
<tr>
<td>Specificity</td>
<td>100.0% (90.0—100.0%)</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>100.0% (86.8—100.0%)</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>94.6% (81.8—99.3%)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent confidence intervals.  
\(^a\) Karyotype was unknown in two subjects.
karyotype had a diagnosis of CAH, the predominant cause of virilization in 46,XX individuals. For this subset of patients, MIS determination would not provide additional useful information. In the remaining subjects, cytogenetic analysis is not discriminatory for gonadal pathology; for example, a 46,XX karyotype can be associated with normal ovaries, true hermaphroditism (41) or even 46,XX sex reversal (males with intact testes) (42). In a virilized patient with no Y sequences detected peripherally, however, MIS determination would help identify testicular tissue or ovarian tumors. Of the 12 patients with a 46,XX karyotype and elevated MIS in this study, 9 had either testes or ovotestes (all with diagnoses of true hermaphroditism) and 3 had ovarian tumors. In these subjects, the cytogenetic analysis was nondiagnostic, whereas MIS determination reliably predicted testicular tissue or a virilizing tumor.

Our data demonstrate that MIS has a high sensitivity, specificity, and positive and negative predictive values for detecting testicular tissue or an MIS-secreting virilizing ovarian tumor in phenotypic females with virilization. MIS determination is especially valuable during late infancy and the prepubertal years when gonadotropins and testosterone are nondiagnostic (42, 43). MIS values that are elevated to the male range are consistent with the presence of testicular tissue or tumor. MIS can then be measured postoperatively to document complete removal of any tumor in phenotypic females with virilization. Our study confirms the value of MIS determination in conjunction with cytogenetic, steroid, and radiologic studies in the evaluation of phenotypic females with virilization.

Acknowledgments

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