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Assay Reproducibility and Within-Person Variation of Mullerian Inhibiting Substance

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Abstract

Objectives—To assess reproducibility of a commercial mullerian inhibiting substance (MIS) assay and evaluate within-person variation in serum MIS levels.

Design—Assay reproducibility was evaluated by measuring MIS in multiple serum aliquots from the same blood collection. Within-person variation was assessed by measuring MIS in serum collected twice from the same individuals.

Setting—Fox Chase Cancer Center, Philadelphia, PA

Patient(s)—Assay reproducibility was evaluated using serum from 5 volunteers with regular menstrual cycles. Within-person variation was evaluated in serum from 20 premenopausal women who donated blood twice at least 1 year apart.

Intervention(s)—For both studies, samples were randomly ordered in batches and laboratory personnel were blinded to which aliquots were from the same subject.

Main Outcome Measure(s)—MIS was measured using an enzyme-linked immunosorbent assay.

Results—Within- and between-batch coefficients of variation (CVs) of the assay were 7.9% and 12.3%, respectively. After deleting one subject with extreme values, these CVs decreased to 7.6% and 7.7%, respectively. Within- and between-subject variance in MIS measurements were 2.19 and 0.31, respectively, and the intraclass correlation coefficient was .88 (95% confidence interval = .77 – .98).

Conclusion(s)—MIS serum concentration is relatively stable over one year in premenopausal women and can be measured with good reproducibility using a commercial kit.

Key Words and Phrases

Mullerian inhibiting substance (MIS); anti-mullerian hormone (AMH); assay reproducibility; coefficient of variation (CV); within-person variation; intraclass correlation coefficient (ICC)

Introduction

Mullerian inhibiting substance (MIS) is primarily known for its role in regulating the in-utero sexual differentiation of males (1). In adults, MIS is secreted by both the testes and ovaries where it regulates steroidogenesis and germ cell development. In females, MIS is undetectable in serum until the prepubertal period, rises during puberty to an average of 2–5 ng/ml and remains at this level through the reproductive years, declining to undetectable levels again after menopause (2–4). MIS serum levels correlate with the number of antral follicles and are a marker of ovarian aging (3,5,6), which is characterized by a progressive loss of follicles. Because serum MIS reflects ovarian reserve, it has been evaluated and shows potential as a predictor of response to controlled ovarian stimulation (7). Ovarian follicle maturation is disrupted in polycystic ovary syndrome (PCOS) resulting in elevated serum MIS, and MIS is a promising biomarker for the diagnosis and management of PCOS patients (8,9). Serum MIS also is elevated in women with ovarian granulosa tumors and is used in the diagnosis and management of women with this cancer (10). Some data also suggest possible roles for MIS in epithelial ovarian cancer and breast cancer (1), although more research is needed. Because serum MIS appears to be a promising biomarker of ovarian function, we conducted a pilot study to determine the reproducibility of a commercially available assay to measure MIS in serum and to evaluate the within-person variation in serum MIS levels over one year.

Material and Methods

Assay reproducibility was assessed using serum collected from 5 healthy volunteers who were 31–42 years old with regular menstrual cycles. Serum from a single blood collection from each subject was separated and aliquoted into 0.1 ml samples. Each aliquot was labeled with a unique ID. Between 4 and 5 samples from each of the 5 subjects were measured in 4 separate batches on different days. The protocol was approved by the Fox Chase Cancer Center Institutional Review Board (IRB) and all participants gave written informed consent.

Within-person variation was assessed using serum from 20 premenopausal women in the Columbia, Missouri (MO) Serum Bank who donated blood on 2 or more occasions at least 1 year apart from 1977–1981. Samples from the same participant were collected during the same menstrual cycle phase on both occasions; 9 participants donated samples during the follicular phase and 11 during the luteal phase. Serum vials were labeled with a vial ID that was unique for each subject at each blood collection and stored at -70°C . MIS in serum from the 2 blood collections for each subject were measured in the same assay batch. Columbia, MO Serum Bank protocols were approved by the University of Missouri Hospitals and Clinics and National Cancer Institute IRBs and all participants gave written informed consent for use of their samples in future research. The protocol for evaluation of within-person variation was approved by the Fox Chase Cancer Center IRB.

For both the reproducibility and within-person variation studies, serum samples were randomly ordered in batches and laboratory personnel were blinded to which aliquots were from the same subject. All assays were performed in the Cancer Prevention Biomarker and Genotyping Facility at the Fox Chase Cancer Center, Philadelphia, PA. MIS was measured in duplicate using a commercially available enzyme-linked immunosorbent (ELISA) kit (Diagnostic Systems Laboratories Inc, Webster TX) according to manufacturer's instructions. The assay limit of detection is .06 ng/ml.

Variability in laboratory MIS measurements was evaluated by the coefficient of variation (CV), which is the standard deviation of measurements divided by the mean (11). A nested components of variance model was used to obtain restricted maximum likelihood estimates of the variances for subject (σ_a^2), batch (σ_b^2), and replicate within-batch (σ^2).

For the MIS assay to be informative, between-person variation in measurements needs to be large compared to assay variation. When this is true, the variance in MIS measurements due to subjects will be large relative to the total variance in MIS measurements. We, therefore, estimated the intraclass correlation coefficient (ρ_I) from the components of variance as the proportion of the total variance that was due to subject using the formula

$\rho_I = \sigma_a^2 / (\sigma_a^2 + \sigma_b^2 + \sigma^2)$ (12). An intraclass correlation coefficient close to 1 indicates that assay variability is small relative to between-person variability and the assay can adequately discriminate individuals.

Within-person variation in MIS measurements over time relative to total variation (within-person plus between-person variation) also was assessed by estimating the intraclass correlation coefficient (ρ_I). Random effects ANOVA was used to estimate between-subject variance (σ_a^2) in MIS measurements and within-subject variance in these measurements (σ^2). The intraclass correlation coefficient was then calculated as $\rho_I = \sigma_a^2 / (\sigma_a^2 + \sigma^2)$ (12) (12).

All analysis was performed in either SAS 9.1 or STATA 10.

Results

Results of the evaluation of MIS assay reproducibility are shown in Figure 1 where MIS levels in individual serum aliquots from the same blood collection from each of 5 subjects are plotted. The subjects' mean ($\pm$ SD) MIS concentrations ranged from 0.6 ± 0.18 ng/ml to 11.5 ± 1.05 ng/ml, while the grand mean ($\pm$ SD) of all MIS measurements was 3.5 ± 4.08 ng/ml. Within- and between-batch variances were .077 and .188, respectively. The calculated within-batch CV was 7.9%, the between-batch CV was 12.3%, and the total CV was 14.6%. After deleting one subject with extreme MIS values that had a mean ($\pm$ SD) of 11.5 ± 1.05 ng/ml, the within-batch CV was 7.6%, the between-batch CV was 7.7% and the total CV was 10.8%. Including all subjects, the intraclass correlation coefficient was 98.7%. After deleting the subject with extreme values, the ICC was still excellent at 93.6%.

Results of the evaluation of within-person variation in MIS serum levels over time are shown in Figure 2 where MIS concentrations in serum samples from 20 subjects collected on two occasions are plotted. The median time between first and second blood collections was 1.1 years (range = 1.0 – 1.3 years), and the median age of subjects at the time of the first blood collection was 41 years (range = 36–44 years). All but one subject had at least one full-term pregnancy and their mean ($\pm$ SD) body mass indexes (BMIs) at the first and second blood collection were 25.4 ± 6.27 and 25.5 ± 6.13 , respectively. The between-subject variance in MIS levels was 2.19, which was large relative to the within-subject variance of 0.31. Moreover, the intraclass correlation coefficient was .88 (95% CI = .77 – .98). Analysis on the log scale decreased the magnitude of the intraclass correlation coefficient to .78 (95% CI = .60 – .95). Results indicate that women's MIS serum levels are relatively stable, at least over a one year period.

Discussion

Results of this pilot study indicate that a commercially available MIS ELISA kit yields reproducible results and that women's serum MIS levels are relatively stable over a 1 year period. Thus serum MIS could be an informative biomarker of ovarian function. MIS assay reproducibility was evaluated in serum collected close in time to the conduct of the assays, while within-person variation in serum MIS concentration was evaluated in samples collected approximately 30 years earlier. We are not aware of any reports of long-term stability of MIS in stored serum. However, serum was stored continuously at -70°C or colder. Moreover, MIS

concentrations in our stored samples ranged from $<.06 - 5.5$ ng/ml, which is consistent with concentrations for healthy premenopausal women reported in the literature (2,5,6,14–17).

Our results on characteristics of the Diagnostic Systems Laboratories' MIS ELISA assay are in general agreement with results reported previously by England et al (13). In their replicate analysis of kit controls, inter-assay CV's were 5.7% and 4.6% at MIS concentrations of 1.0 and 4.2 ng/ml, respectively. Furthermore, assay accuracy as assessed by recovery of MIS spiked into patient sera at 1 and 4 ng/ml averaged 85.5% (range = 81 – 94%). Thus, reproducibility and accuracy of the ELISA kit appear to be acceptable for research purposes.

Our results on within-person variation of serum MIS levels over time in premenopausal women concur with three previous reports. Fanchin et al (18) evaluated the short term reproducibility of serum MIS concentrations in 47 normo-ovulatory infertile women 25 to 40 years old. The intraclass correlation coefficient of MIS in serum samples collected from these women during 3 consecutive menstrual cycles was .89 (95% confidence interval = .83 – .94). In a study more similar to ours, van Rooij et al (4) evaluated longer term reproducibility of serum MIS concentrations in blood samples collected 4 years apart from 81 healthy volunteers. The women averaged 39.6 and 43.6 years old at times one and two, respectively. All had regular menstrual cycles at time one and 67 still had regular cycles at time two, whereas 14 had entered the menopausal transition. The age adjusted correlation coefficient for MIS concentration in serum collected at the two time points was .66 ($p < .01$). Thus, although serum MIS concentrations decline with advancing age (3,4), women's relative concentrations appear to track over time, at least until menopause when MIS is no longer measurable in serum (3).

One of the difficulties studying ovarian hormones is that many vary in concentration dramatically across the menstrual cycle. Although we did not evaluate variation in MIS concentrations over the menstrual cycle in this study, MIS has been reported not to vary markedly by day of the cycle. La Marca et al (19) measured MIS in serum collected every other day during one complete menstrual cycle in 12 healthy women and found that mean concentrations varied non-significantly between 3.4 and 3.9 ng/ml. Although Cook et al (15) reported significant differences in serum MIS concentrations during the early follicular, peri-ovulatory and luteal phases of the menstrual cycle in 20 healthy women with normal cycles, the magnitude of the differences was fairly small; mean concentrations ranged from 1.4 to 1.7 ng/ml with the highest levels obtained during the peri-ovulatory phase. Hehenkamp et al (16) similarly reported that MIS levels were highest during the peri-ovulatory phase of the cycle, although levels only varied within 10 percent of the mean across the cycle. This is in contrast to serum estradiol, which varied by close to 100 percent. This relative constancy of serum MIS concentrations across the menstrual cycle yields several advantages for research including simplified study designs, greater power to detect associations, and improved interpretability of results.

In conclusion, MIS is a biomarker of ovarian function that can be measured with good precision using a reproducible assay kit that is commercially available. Its constancy over the menstrual cycle and relative stability over time contribute to its emerging utility. MIS is best understood in controlling apoptosis in the Mullerian ducts, the function for which it is named, but this is certainly not its limit as a growth and development mediator. Its value in the clinical arena is just beginning to be understood and we should be encouraged to continue to pursue its clinical applications in the future.

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Figure 1. MIS Assay Reproducibility. Each symbol represents the MIS concentration in an aliquot of serum from a single blood collection.

Figure 2. MIS Concentrations in Serum Samples Collected One Year Apart. Each dot represents the MIS concentration in serum from a single subject. The line is from the regression of the second MIS measurement on the first MIS measurement. Spearman correlation is reported.