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Müllerian inhibiting substance levels at the time of HCG administration in IVF cycles predict both ovarian reserve and embryo morphology

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BACKGROUND: Pre-antral and early antral follicles secrete Müllerian inhibiting substance (MIS), suggesting that MIS may directly reflect ovarian reserve. Since little is known about how ovarian reserve affects oocyte quality, we attempt here to assess the predictive value of MIS on embryo morphology and IVF outcome. To do so, we measured MIS at the time of HCG administration 36 h prior to oocyte retrieval. METHODS: A total of 257 patients undergoing IVF were prospectively recruited. We measured MIS levels by enzyme-linked immunosorbent assay at the time of HCG, and compared the MIS values to day 3 FSH levels in the prediction of embryo morphology and IVF outcome. RESULTS: The distribution of MIS levels was skewed, with a median of 2.7 ng/ml (range 0 to 28.5 ng/ml). MIS values at the time of HCG administration inversely correlated with basal FSH levels ($P = 0.002$), and both correlated significantly with patient age, number of mature follicles, number of oocytes retrieved and serum estradiol levels. MIS levels correlated significantly with a greater number of 6-cell embryos and better embryo morphology score, while basal FSH levels did not correlate with these outcome variables. MIS levels ⩾2.7 ng/ml portended improved oocyte quality as reflected in a higher implantation rate ($P = 0.001$) and a trend toward a better clinical pregnancy rate ($P = 0.084$). CONCLUSIONS: MIS levels seem to predict not only ovarian reserve, but also embryo morphology. Measurement of MIS at the time of HCG administration may, therefore, in the future improve management of patients undergoing treatments with assisted reproductive technology.

Key words: embryo morphology/IVF/Müllerian inhibiting substance/ovarian reserve/pregnancy rate

Introduction

Numerous studies demonstrate follicular depletion and decreasing pregnancy rates as women age. Poor ovarian reserve generally portends diminished ovarian response to controlled ovarian stimulation during IVF cycles, leading to reduced numbers of oocytes and embryos, and, in severe cases, to cycle cancellation. Poor ovarian reserve also predicts decreased fertility rates, though how declining ovarian reserve relates to decreased fertility rates remains incompletely understood (Bancsi *et al.*, 2002). Clinicians employ a number of parameters to estimate ovarian reserve (Ng *et al.*, 2003), including follicular phase inhibin B levels (Seifer *et al.*, 1997), ovarian volume (Syrop *et al.*, 1995, 1999; Lass *et al.*, 1997), total antral follicle count (Tomas *et al.*, 1997), ovarian stromal blood flow (Zaidi *et al.*, 1996; Engmann *et al.*, 1999; Kupesic *et al.*, 2003), and basal or clomiphene citrate-stimulated FSH levels. Basal FSH level remains the most widely used assay for ovarian reserve, but provides only very limited prediction of embryo quality.

Pre-antral and early antral follicles secrete Müllerian inhibiting substance (MIS), also named anti-Müllerian hormone (AMH), a glycoprotein hormone in the transforming growth factor β superfamily (Cate *et al.*, 1986; Baarends *et al.*, 1995), suggesting MIS may more directly reflect ovarian reserve. Barely detectable at birth, MIS production peaks after puberty (Vigier *et al.*, 1984; Hirobe *et al.*, 1992; Rajpert-De Meyts *et al.*, 1999). Post-puberty, serum MIS levels during the early follicular phase decrease progressively with age, until becoming undetectable at menopause (De Vet *et al.*, 2002). MIS levels correlate better than inhibin B, E_2, FSH and LH measured on cycle day 3 with number of antral follicles seen on ultrasound (De Vet *et al.*, 2002; Fanchin *et al.*, 2003b). Circulating levels of MIS correlate with primordial

follicle recruitment, making it a potential marker for ovarian ageing. MIS helps to regulate the sensitivity of ovarian follicles to FSH, explaining its role in the prediction of ovarian reserve. MIS, however, also plays important roles throughout folliculogenesis, after primordial follicle recruitment (Durlinger *et al.*, 2002) and through FSH-sensitive (Durlinger *et al.*, 2001; McGee *et al.*, 2001) follicular growth, so that we postulate that MIS might also influence oocyte and embryo quality, as well as oocyte quantity.

We measured MIS levels at the time of HCG administration during IVF, and then assessed the impact of the MIS level on ongoing pregnancy probability and embryo quality, as estimated by embryo morphology, implantation rate and the ability to generate at least five embryos of ≥6 cells 3 days after fertilization.

Materials and methods

Our data were extracted from a computerized data base of 257 patients who underwent IVF between January and May of 2002 at the WIH Division of Reproductive Medicine and Infertility. We studied only a single fresh cycle in this interval from each subject. The study was approved by the Institutional Review Board (IRB) of Women and Infants' Hospital. All patients gave written, informed consent, allowing their data to be used for scientific purposes.

Ovulation induction using either the GnRH agonist leuprolide acetate (Lupron; TAP Pharmaceuticals, North Chicago, IL, USA) or GnRH antagonist ganirelix acetate (Antagon; Organon, West Orange, NJ, USA) was carried out using standard protocols, including recombinant FSH, Follitropin-α (Gonal-F; Serono, Randolph, MA, USA), recombinant FSH Follitropin-β (Follistim; Organon), highly purified urinary FSH (Bravelle; Ferring Pharmaceuticals, Tarreytown, NJ, USA), and/or HMG (Repronex; Ferring Pharmaceuticals). Transvaginal ultrasound follicle monitoring during ovarian stimulation used a Toshiba SAL 77B (Toshiba, Tokyo, Japan) or Sonosite 180 Plus (Sonosite, Bothell, WA, USA) ultrasound. A solid-phase, ligand-labelled, competitive chemiluminescent immunoassay (DPC Immulite Estradiol, Diagnostic Products Corp., Los Angeles, CA, USA) measured estradiol. FSH was measured by a competitive two-site chemiluminescent assay from the same manufacturer. Clinicians administered 10000 IU of hCG (Profasi, Serono; Pregnyl, Organon; Novarel, Ferring) s.c. when at least one follicle reached a mean diameter of 18 mm. MIS levels were analysed from routine blood samples obtained on the day of hCG administration. Oocytes were collected by transvaginal ultrasound-guided aspiration ~36 h after hCG injection, and insemination was performed by standard IVF or ICSI. Light microscopic evaluation established fertilization 14–18 h later. We performed either cleavage stage or blastocyst embryo transfers, depending on availability of the number of high quality embryos on day 3. At transfer we used ultrasound to guide embryo placement to the mid-uterine cavity. A serum β-HCG pregnancy test was performed 14 days after retrieval and repeated 2 days later if positive. Ongoing pregnancy was established by at least one ultrasonographically confirmed viable fetus within the uterus 6 weeks after embryo transfer.

Morphological assessment of embryos consisted of evaluation of embryos transferred on day 2 or 3 for cell number, percentage of fragmentation and blastomere symmetry. Ongoing clinical pregnancy rate (OPR) was calculated as the probability of one or more viable fetuses detected at 6 week post-retrieval ultrasound, divided by the number of embryo transfers performed. Implantation rate (IR) was calculated as the number of sacs on 4 week post-retrieval ultrasound divided by the number of embryos transferred. Data were analysed for statistical significance using Student's *t*-test, χ^2, and Pearson or Spearman coeffient as appropriate.

Serum samples obtained on the day of HCG administration were stored at –20°C during the study period, until assayed for MIS. The enzyme-linked immunosorbent assay (ELISA) used to measure human MIS has been described previously (Hudson *et al.*, 1990; Hirobe *et al.*, 1992; Lee *et al.*, 1996). Samples were analysed in duplicate at six serial dilutions in the laboratory of one of the authors (DTMc) and results reported as the mean of three dilutions falling within the linear portion of the standard curve, constructed using a four-parameter logistical curve fitting DeltaSoft II (BioMetallics, Inc., Princeton, NJ, USA). The sensitivity of this assay was 0.5 ng/ml and the intra-assay and inter-assay coefficients of variation were 9 and 15% respectively. The MIS ELISA does not cross-react with LH, FSH, activin, inhibin or TGF-β, nor with bovine or rodent MIS.

Statistical analysis

We characterized selected clinical characteristics by mean, median and range. The range of MIS levels was considerable (0–28.5 ng/ml) (Figure 1). The cause of this variability is not apparent since these patients were unselected IVF subjects, but in view of this feature and the skewed distribution, we resorted to log transformation of the MIS values before analysis. Non-parametric testing (Spearman correlation) also was used for both FSH and the log of MIS values. We assessed the ability of both hormones (MIS and FSH) to predict the probability of patients having five or more 6-cell embryos or a lesser number of 6-cell embryos using the area under a receiver operating characteristic (ROC) curve (Hanley and McNeil, 1982). Sensitivity of the hormones defined the association of higher levels of MIS or lower levels of FSH

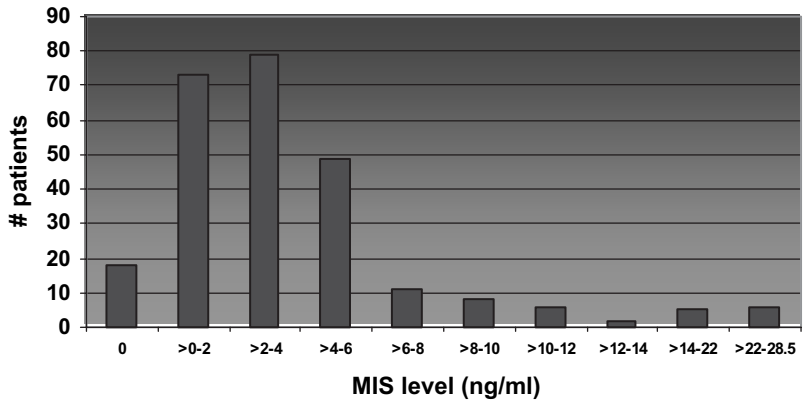

Figure 1. The distribution of Müllerian inhibiting substance (MIS) levels.

with a greater number of 6-cell embryos. Specificity was defined by an association of lower levels of MIS or higher levels of FSH with a lower number of 6-cell embryos. The sum of sensitivity and specificity defined the validity of the biomarker (area under the curve), as compared to the number of 6-cell embryos. We carried out statistical analysis with the SPSS version 11 software package.

Results

Of 257 patients studied, 234 had embryos available for transfer. Of these, 216 (92.3%) underwent transfer of cleavage stage embryos and the remaining 18 had blastocyst transfer. Mean MIS level was 4.1 ± 4.8 ng/ml (range 0–28.5), and median MIS level was 2.7 ng/ml, with distribution of MIS as shown in Figure 1. Table I summarizes these results and other parameters of the entire patient cohort.

MIS levels inversely correlated with day 3 FSH ($r = -0.194$, $P = 0.002$). Table II displays the correlation of both MIS and day 3 FSH with the following: age, body mass index (BMI), number of mature follicles, estradiol at time of HCG administration, embryo morphology score, number of oocytes retrieved and number of 6-cell embryos. Predicting the number of oocytes retrieved was common to both day 3 FSH ($P < 0.0001$) and MIS ($P = 0.011$). MIS alone, however, correlated significantly with embryo score and number of 6-cell embryos ($P = 0.038$, $P = 0.001$ respectively), whereas day 3 FSH did not. MIS and day 3 FSH both correlated significantly with patient age ($P = 0.008$, $P = 0.028$ respectively), number of mature follicles ($P < 0.001$ for both), and estradiol levels on day of HCG administration ($P < 0.001$, $P = 0.001$ respectively). Neither MIS nor FSH correlated with BMI.

Figure 2 presents an ROC curve to demonstrate that a larger number of ≥6-cell embryos is associated with both a lower day 3 FSH and a higher day of HCG MIS level. MIS significantly exceeds day 3 FSH ($P < 0.001$) in this ability [area under the curve (AUC) for MIS was 0.647 whereas the AUC for FSH was 0.406]. Nonetheless, these values also demonstrate that neither parameter is a particularly good predictor of better embryo quality.

The number of 6-cell embryos and its relationship to day 3 FSH and MIS values is expressed in Figure 3 as bubble size. The bubble is located according to the combination of MIS and day 3 FSH levels and larger bubble size indicates a greater frequency of individuals at that value. A vertical line indicates the median MIS level.

IR was greater in patients with MIS >2.7 ng/ml (95/339, 28%) than in those with values of <2.7 ng/ml (51/303, 16.8%) ($P < 0.001$). Similarly OPR (58/125, 46.4%) was superior when MIS was >2.7 ng/ml when compared to those with levels <2.7 mg/ml (38/109, 34.9%) although this level did not reach statistical significance ($P = 0.084$) (Figure 4).

Discussion

The ability to predict ovarian reserve and response to ovarian stimulation continues to be an important component of infertility treatment. Patients wish to know their probability of conception to help in making difficult decisions about pursuing additional treatment with their own oocytes, or to desist, and pursue alternatives such as oocyte donation or adoption. Clinicians

Table I. Summary of patient data

	Mean (SD)	Median (range)
Müllerian inhibiting substance (ng/ml)	4.1 (4.8)	2.7 (0–28.5)
Early follicular FSH level (mIU/ml)	6.6 (2.9)	5.9 (1.2–24.6)
Age (years)	34.9 (4.2)	35 (23–44)
Cycle number	1.8 (range 1–4)	1 (1–8)
Body mass index[a] (kg/m^2)	25.8 (6.9)	23.6 (17–56.1)
Peak estradiol level (pg/ml)	1645 (1004)	1433 (167–4524)
Retrieved oocytes (*n*)	11.8 (7.6)	10 (0–45)
6-Cell embryo number[b]	4.5 (4.5)	4 (0–33)

Calculated for: [a]203 patients, [b]171 patients.

Table II. Spearman correlations (ρ) of Müllerian inhibiting substance (MIS) on day of HCG administration and day 3 FSH levels with age, body mass index (BMI) and other outcome measures

	MIS		FSH	
	ρ	*P*	ρ	*P*
Age (years)	–0.164	0.008	0.137	0.028
Body mass index[a]	–0.033	NS	–0.036	NS
No. of mature follicles	0.331	0.001	–0.276	0.001
Estradiol at time of HCG	0.346	0.001	–0.204	0.001
Oocytes retrieved	0.330	0.001	–0.280	0.001
Embryo score[b]	0.142	0.038	0.016	NS
No. of 6-cell embryos[c]	0.245	0.001	0.041	NS

Calculated for: [a]203 patients, [b]214 patients, [c]171 patients.
ρ = Spearman correlation; NS = not significant.

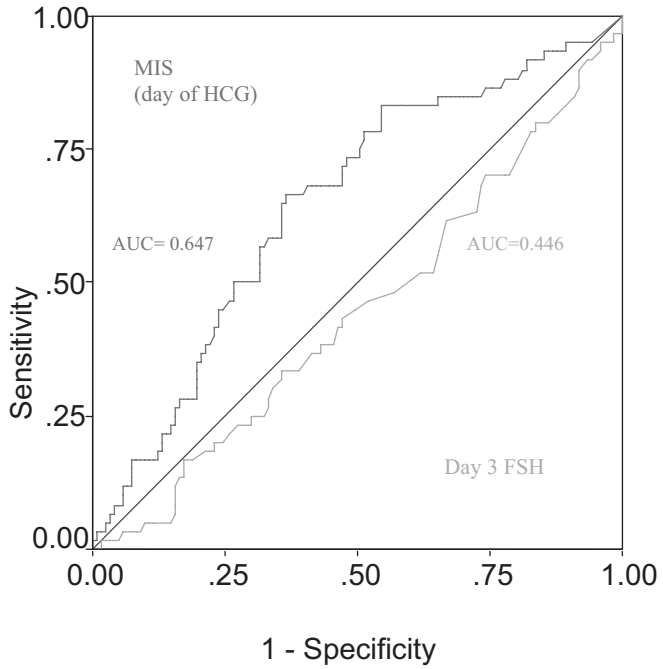

Figure 2. Receiver operating characteristic (ROC) curve, comparing the predictive value of Müllerian inhibiting substance (MIS) on day of HCG administration and day 3 FSH in attainment of morphologically favourable embryos. AUC = area under the curve.

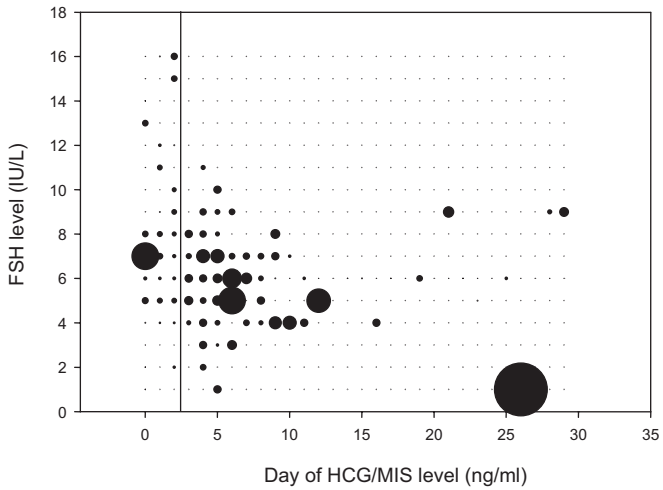

Figure 3. Bubble graph showing the relationship between Müllerian inhibiting substance (MIS) and FSH with the number of ≥6 embryos. Bubble size indicates the number of ≥6-cell embryos. The smallest bubbles indicate 'no data' at that coordinate. The vertical line indicates median MIS level.

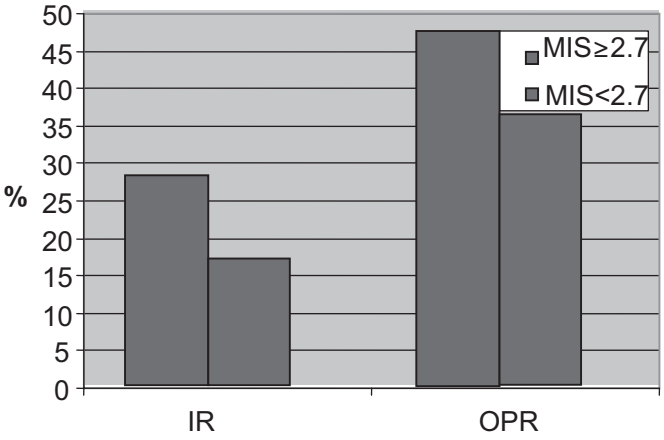

Figure 4. Implantation rate (IR) and ongoing pregnancy rate (OPR) for patients whose Müllerian inhibiting substance (MIS) values were ≥2.7 or <2.7 ng/ml.

also need to estimate the probability of embryo implantation in order to counsel patients on the number of embryos to transfer, to optimize pregnancy rates and reduce multiple gestation rates. MIS, a member of the transforming growth factor-β family produced by granulosa cells of the early developing follicle, provides an excellent marker for ovarian reserve and response to drugs used to evoke ovarian stimulation. (De Vet *et al.*, 2002; Fanchin *et al.*, 2003a,b; Seifer *et al.*, 2002; Van Rooij *et al.*, 2002a,b). We report here a relationship between MIS levels and embryo morphology, as measured by ability to generate a greater number of 6-cell embryos, better embryo morphology and possibly pregnancy outcome. We found the expected inverse correlation between day of HCG/MIS and basal FSH levels. Basal FSH and day of HCG/MIS both significantly correlated with numbers of mature follicles and oocytes retrieved. Only HCG/MIS levels, however, predicted a greater number of ≥6-cell embryos, a higher embryo score and a better IR.

This study does not itself explain the physiological basis of these findings. We postulate, however, that continued recruitment of additional antral follicles during the stimulatory phase of IVF results in higher MIS levels in individuals destined to produce better quality embryos and to have a better reproductive outcome.

Ovarian reserve typically refers to the size of the cohort of primordial follicles. Quality of oocytes is, however, an even more important characteristic, though more difficult to measure. Our study shows that the MIS level after stimulation seems to reflect not only ovarian reserve, but also better embryo morphology. The addition of the MIS level at the time of HCG administration to existing parameters of ovarian reserve, and perhaps in combination with other criteria, may in the future help to identify patients who have failed an initial IVF attempt but who are likely to respond favourably to subsequent IVF treatment. More research will be required to confirm this finding and to explore its aetiology.

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