J Assist Reprod Genet (2013) 30:745–751
DOI 10.1007/s10815-013-9996-y

ASSISTED REPRODUCTION TECHNOLOGIES

What is the optimal threshold of serum Anti-Müllerian hormone (AMH) necessary for IVM treatments?

Alon Kedem · Gil M. Yerushalmi · Ettie Maman · Rina Hemi · Mirit Hanochi · Ariel Hourvitz

Received: 31 January 2013 / Accepted: 9 April 2013 / Published online: 23 April 2013

Abstract
Purpose To assesse circulating levels of Anti-Müllerian hormone (AMH) as a predictor of oocyte number and their potential to mature in vitro in both normo-ovulatory (NO) women and in women with Polycystic Ovary Syndrome (PCOS) undergoing in vitro maturation (IVM) treatments.
Methods We prospectively studied NO women and women diagnosed with PCOS, (age range 21–39 years) underwent IVM treatments at our center. Serum AMH levels were quantified before each cycle and correlated to oocytes number, maturation and fertilization during in vitro maturation.
Results 104 NO and 30 PCOS IVM cycles were followed with retrieval of a total of 672 and 491 oocytes, respectively. In NO women, the serum AMH level positively correlated with the number of oocytes retrieved, (R=0.6; P <0.0001) the number of M2 oocytes at 24 and 48 h (R=0.4; P <0.01; R=0.26 p<0.007, respectively) and with the total number of M2 oocytes (R=0.47; P<0.0001). In the PCOS group, the serum AMH level positively correlated only with the number of oocytes retrieved (R=0.43; P <0.03). Receiver operating characteristic (ROC) analyses showed that a cutoff AMH level of 1.56 (ng/ml) could identify patients with 5 or more oocytes at OPU with a sensitivity of 83 % and a specificity of 75 %. An AMH level of 1.63 (ng/ml) was the threshold for 5 or more matured oocytes (sensitivity=81 %, specificity=53 %).
Conclusions Serum AMH may be used as a marker to identify candidates for IVM treatment in both NO and PCOS women.

Keywords Anti-Müllerian hormone · In vitro maturation · Oocyte maturation · PCOS

Capsule The identification of cycles able to produce numerous oocytes will considerably improve the efficiency of IVM treatments. We would suggest that an AMH cut-off concentration could guide the appropriateness of IVM therapy in selected cases, reducing the number of recruited cycles where suboptimal oocyte numbers are likely to be retrieved.

A. Kedem · G. M. Yerushalmi · E. Maman · A. Hourvitz
IVF Unit, Department of Obstetrics and Gynecology, Sheba Medical Center, Tel Hashomer, Ramat-Gan, affiliated with the Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

R. Hemi · M. Hanochi
Institute of Endocrinology, Sheba Medical Center, and Tel Hashomer, affiliated with the Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

A. Kedem (✉)
IVF Unit, Department of Obstetrics and Gynecology, Sheba Medical Center, Tel Hashomer 52621, Israel
e-mail: kedem2001@gmail.com

Introduction

In vitro maturation (IVM) of immature oocytes is an emerging technology providing a hormone-free or mild hormonal stimulation environment for fertility treatment. The aspiration of immature oocytes from small- and medium-sized antral follicles followed by their maturation in vitro, presents an attractive alternative to the hormonal stimulation of patients undergoing in vitro fertilization (IVF). Currently, the IVM technique remains controversial in humans because of its suboptimal pregnancy rates when compared with regular IVF treatments and as such its use is restricted to specific indications. These include women at risk of hyperstimulation syndrome [1–4, 16, 18, 24], those women with cancer requiring immediate fertility preservation and those patients who cannot receive hormonal stimulation [6, 17]. In view of the low pregnancy rate with IVM, a reliable and available tool needs to be discovered which is capable of predicting a subgroup of patients most likely to benefit from this novel treatment. Recent studies have shown that the number of retrieved oocytes is the most significant predictive factor of successful IVM treatments [9].

Anti- Müllerian hormone (AMH) is a dimeric glycoprotein and a member of the transforming growth factor-beta superfamily, [6] being exclusively produced by granulosa cells from the primary to the large antral follicle stage [25] and secreted by the ovarian follicles into the circulation. It has been suggested that AMH levels may effectively represent the ovarian follicular pool where recently, several large prospective studies have reported that its measurement may have potential clinical application in the prediction of the quantitative and qualitative ovarian response during assisted reproductive technologies [7, 11, 14, 15, 21, 23, 25]. As a result, AMH is currently being used as a novel clinical marker of ovarian reserve as well as a guide to the response to gonadotropin [8]. Our recent data would suggest that AMH expression by granulosa cells and its secretion level within the follicular fluid of small- and medium-sized follicles is strongly correlated with the serum AMH (*unpublished results*). Moreover, our recent studies have suggested that there is also a correlation between follicular fluid (FF) AMH levels and oocyte development during IVF cycles [13].

From this data it is proposed that serum AMH might not only be a useful tool for the prediction of the number of retrieved oocytes, but also that it may be a potential marker of their capacity to mature in-vitro. In spite of the large number of studies investigating the role of AMH as a marker of ovarian reserve and response during IVF, there are limited data concerning its role in IVM, where recently, Fadini et al. have suggested that it may be used to identify suitable normo-ovulatory (NO) IVM candidates with sufficient oocyte retrieval [8]. As far as we are aware, there are no available data concerning the measurement of serum AMH levels in those women with PCOS undergoing IVM treatment. The aim of this study was to investigate the role of serum AMH as a predictor of oocyte number and their potential to mature in vitro in both NO and PCOS patients undergoing IVM treatments.

Methods

Patients~

We prospectively studied women who underwent IVM protocols in our fertility center (the IVF Unit, Sheba Medical Center Israel) between March 2008 and October 2010. Approval for the conduct and analysis of the study was provided by the local hospital Ethics Committee with written informed consent being obtained from all study participants. The study included NO and PCOS patients with patient demographics represented in Table 1. Inclusion criteria for IVM patients were: Age≤38 years, body mass index<31. Patients included in the NO group underwent IVM to avoid hormonal therapy and patients treated for fertility preservation. The inclusion criteria for NO patients were: Regular menstrual cycles (25–34 days) and morphologically and endocrinologically normal ovaries. Patients in the PCOS group were diagnosed according to the Rotterdam criteria (Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS)) [19]. All PCOS women must had two of the following three manifestations: Irregular or absent ovulation, elevated levels of androgenic hormones, and/or enlarged ovaries containing at least 12 follicles each.

Table 1 Characteristics of 100 IVM patients included in the study

	Norm-ovulatory	PCOS
Number of patients	80	20
Number of cycles	104	30
Age (years)	31.5±5	29±4
BMI	22±2	23±2
FSH (IU/l)	8.2±3	6.7±3
Serum AMH (ng/ml)	2.7±2.5	8.4±4

Values given as mean±St. Dev.

IVM protocol

Patients underwent an IVM cycle according to a previously described protocol [9]. Briefly, a baseline evaluation including an hormonal profile (AMH, FSH, estradiol and progesterone levels) and an ultrasound scan were performed on day 2–3 of the menstrual cycle. On day three, 150 IU/day of recombinant follicle-stimulating hormone (FSH) was commenced for 3 days. A second evaluation was then performed on day 6 of the menstrual cycle. Following this, an injection of 10,000 IU of chorionic gonadotropin (hCG) (Pregnyl; Organon, Oss, Holland) was administered when the endometrial thickness was at least 6 mm and when the dominant follicle was 12 mm in diameter. Transvaginal oocyte retrieval was scheduled 36–38 h after hCG injection. Metaphase II oocytes underwent intracytoplasmic sperm injection (ICSI) as previously described [22]. Immature oocytes were incubated in IVM medium (Sage; Trumbull, CT) supplemented with 75 IU FSH and 75 IU Luteinizing Hormone (LH) (Ferring, Keil, Germany). Oocyte maturation was assessed after 24 and 48 h in culture and mature oocytes were fertilized by ICSI. Eighteen hours after the ICSI procedure, fertilization was assessed using the established pronuclei (PN) scoring system [20]. Laboratory outcome measurements included the number of retrieved oocytes, the number of mature oocytes on the day of the aspiration, the number of newly mature oocytes after 24 and 48 h, the fertilization

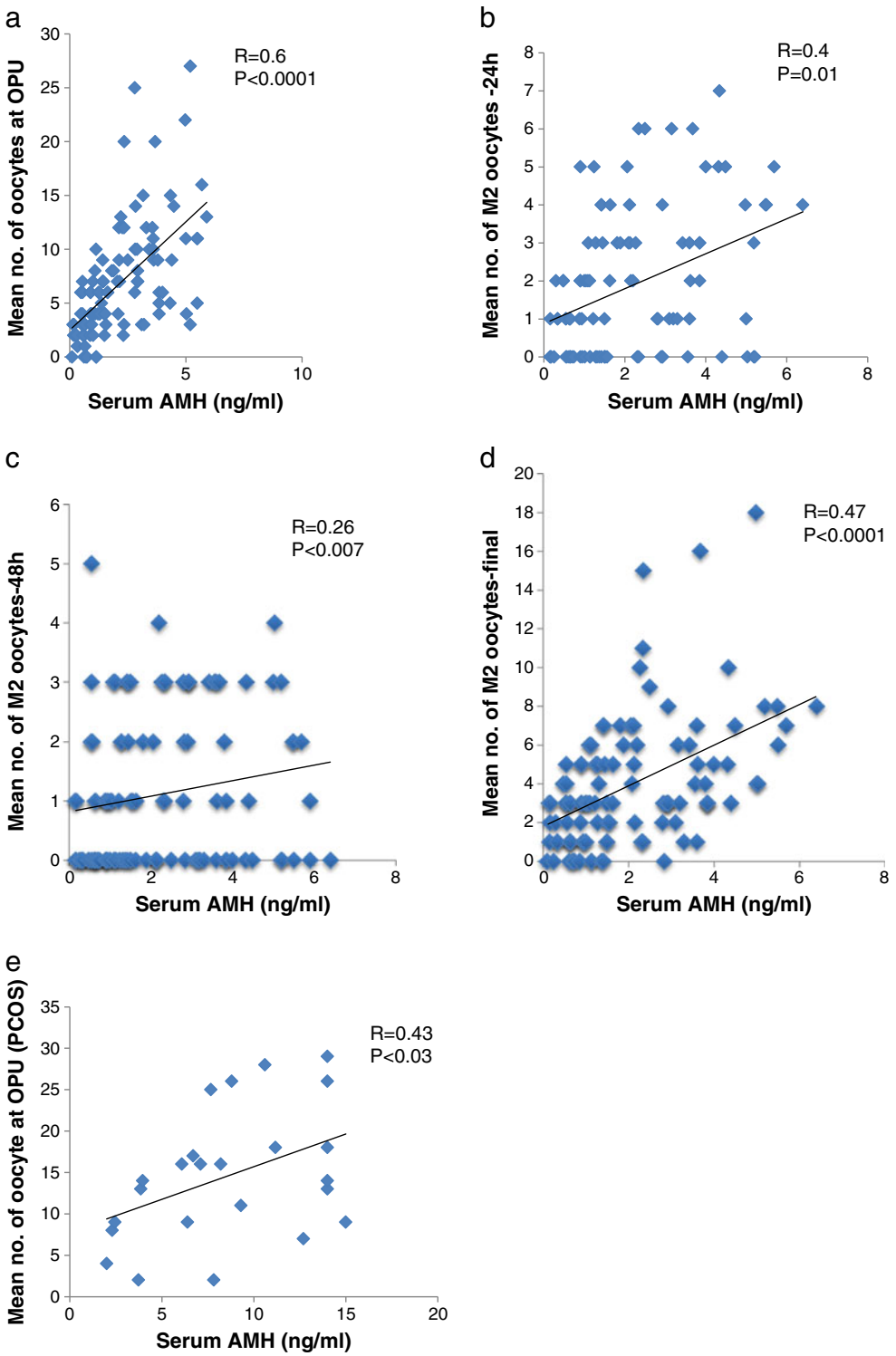

Fig. 1 Correlations of serum AMH levels and laboratory outcomes in NO and PCOS patients. **a** Mean number of retrieved oocytes on OPU day of NO patients. **b** Mean number of oocytes that completed their maturation in the first 24 h following OPU (NO patients). **c** Mean number of oocytes that completed their maturation between 24 and 48 h following OPU (NO patients). **d** Mean total number of matured oocytes ready for fertilization in NO patients including mature oocytes of OPU. **e** Mean number of retrieved oocytes on OPU day of PCOS patients

rates and the number of embryos appropriate for transfer. Maturation rates were calculated as the number of newly matured oocytes divided by the number of the immature oocyte pool over a defined period of time.

AMH measurements in serum

Serum AMH levels were measured on the third day of menstruation. The serum was separated and frozen in aliquots at −80 °C for future analysis with measurement being performed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions [Diagnostic Systems Laboratory, Webster, TX]. The intra- and inter-assay coefficients of variations (CVs) were 4.6 and 8.0 %, respectively.

Statistics

All statistical procedures were conducted with PASW Statistics, Release Version 18.0.0 software (SPSS, Inc., 2009, Chicago, IL). Paired comparisons were made using the paired two sided Student's *t*-test assuming unequal variances. The relationship between any two continuous variables was assessed by correlation when they were independent from each other and by simple regression when there was dependency. ROC curves were calculated in an attempt to determine a cutoff value for the serum AMH level with the first criterion of analysis being the retrieval of at least 5 immature oocytes. This oocyte number was considered as critical based on previous recent studies which have suggested that recovery of at least 5 immature oocytes are necessary to ensure the chances of achieving a pregnancy [9]. *P*-values<0.05 were considered significant.

Results

Patient demographics~

One hundred IVM patients met our inclusion criteria, including 80 in the NO group undergoing 104 IVM cycles with an average AMH serum level of 2.7±2.5 ng/ml. Twenty PCOS patients underwent 30 IVM cycles with an average AMH serum level of 8.4±4 ng/ml where the stimulation dose and duration of the IVM protocols were both fixed in accordance with the treatment protocol. All stimulation cycles were successfully reaching the egg retrieval criteria.

Normo-ovulatory (NO) group~

There was a positive correlation between the serum AMH level and the number of oocytes retrieved (R=0.6; P <0.0001, Fig. 1a) although there was no correlation between the number of mature oocytes on the day of OPU and the serum AMH level. Analysis of the number of newly matured oocytes 24 h after OPU showed a clear correlation with AMH levels (R=0.40; P <0.01, Fig. 1b) with a weaker but still significant correlation at 48 h (R=0.26; P <0.007, Fig. 1c). The total number of matured oocytes significantly correlated with the serum AMH level (R=0.47; P <0.0001, Fig. 1d). In addition there were negative correlations between patient's age, AMH levels and number of oocytes retrieved (R=0.23; P <0.01 and R=0.34; P <0.002 respectively). In an attempt to identify the optimal serum AMH cut-off levels for optimal immature and mature oocyte retrieval, ROC curves were constructed. These showed an AMH threshold level of 1.56 (ng/ml) that identified patients with 5 or more oocytes at OPU (sensitivity= 83 %, specificity=75 %; Fig. 2) and an AMH level of 1.63 (ng/ml) which defined the threshold for 5 or more matured oocytes ready for fertilization (sensitivity=81 %, specificity=53 %; Fig. 3).

On the basis of these findings, an AMH level of 1.6 ng/ml was chosen as the threshold concentration to assess laboratory outcomes in the two patient groups (Table 2). The mean number of retrieved oocytes in the NO group with serum AMH levels>1.6 (ng/ml) was almost 2.5 times more than that of patients with serum AMH levels<1.6 (ng/ml) (9±5.5 vs. 3.7±2.3, respectively; P<0.001). The maturation rate on the day of OPU was significantly higher in patients with an AMH level >1.6 (ng/ml) when compared with patients whose AMH levels<1.6 (ng/ml) (0.2±0.3 vs. 0.1±0.1 respectively, P<0.01). There was a significantly higher number of in vitro newly matured oocytes obtained at 24 h when the AMH levels>1.6 (ng/ml) compared with AMH levels<1.6 (ng/ml) (2.8±2.7 vs. 1.1±1.3, respectively; P<0.001) and a higher final number of matured oocytes when the serum AMH levels>1.6 (ng/ml) (1.6±2.1 vs. 0.8±1.1, respectively, P<0.001). Finally, NO patients with AMH levels exceeding 1.6 ng/ml when compared with those where the serum AMH level was<1.6

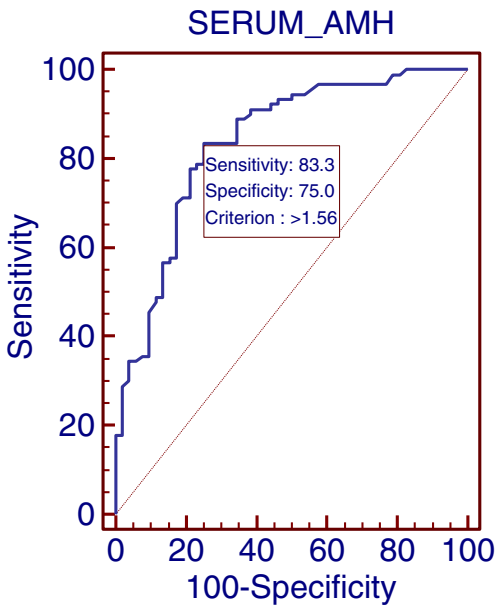

Fig. 2 ROC curve for the relationship between serum AMH levels and the retrieval of≥5 immature oocytes

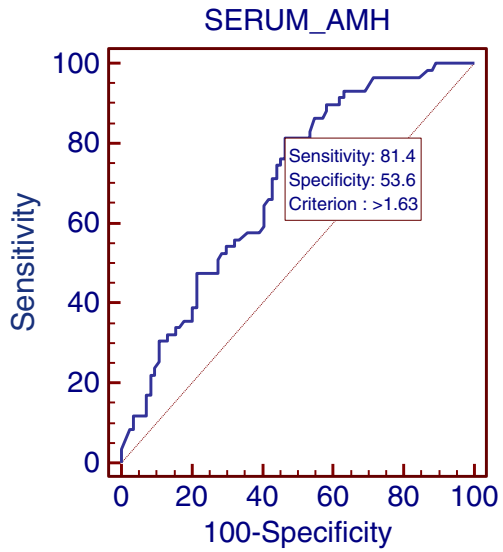

Fig. 3 ROC curve for the relationship between serum AMH levels and the retrieval of≥5 matured oocytes ready for fertilization

(ng/ml), had a significantly higher number of fertilized oocytes (2PN) (3.5±2.6, vs. 2.2±1.5; $P<0.005$) and a greater total number of embryos appropriate for transfer (2.3±2.8 vs. 1.4±1.4, respectively, $P<0.0001$).

Polycystic ovary syndrome (PCOS) group~

In this group there was a significant positive correlation only between serum AMH levels and the number of oocytes retrieved ($R=0.43$, $P<0.03$; Fig. 1E). In addition there was a negative correlation between patient's age and number of oocytes retrieved ($R=0.52$; $P<0.002$) and non significant negative correlation between patient's age and AMH levels ($R=0..32$; $P<0.08$). The laboratory outcomes of oocytes obtained from PCOS patients are presented in Table 2 with a total of 491 oocytes being retrieved. The mean number of oocytes on the day of OPU was 4 times higher than those obtained from the NO patients (15±9) and there were consistently higher numbers of mature oocytes during the different culture periods in the PCOS cases when compared with the NO patients. Despite these findings, the maturational rates between the patient groups were comparable.

Discussion

The part of the study which assessed normo-ovulatory (NO) patients undergoing IVM treatment, showed a direct correlation between measurable serum AMH levels and the

Table 2 Laboratory outcomes of 134 IVM cycles in NO and PCOS patients. An AMH threshold of 1.6 ng/ml was identified for obtaining at least 5 matured oocytes

	Normo-ovulatory			PCOS	
	AMH<1.6 ng/ml (n=37/55)	AMH>1.6 ng/ml (n=43/49)	P	(n=20/30)	P
Mean serum AMH level (ng/ml)	0.8±0.4	3.2±1	0.001	8.5±4	0.001
Total no. of oocytes retrieved	200	472		491	
Mean no. of oocyte at OPU	3.7±2.3	9±5.5	0.001	15±9	0.001
Mean no. of matured oocytes at OPU:	0.66±1	0.76±1.2	NS	1.4±1.7	0.01
Maturational rate (OPU)	0.2±0.3	0.1±0.1	0.01	0.1±0.1	NS
Mean no. Of matured oocytes oocytes (24 h)[a]	1.1±1.3	2.8±2.7	0.001	4.2±3	0.001
Maturational rate (24 h)	0.5±0.3	0.37±0.3	NS	0.36±0.2	NS
Mean no. Of matured oocytes (48 h)[b]	0.8±1.1	1.6±2.15	0.001	2.3±3.2	0.01
Maturational rate (48 h)	0.7±0.2	0.5±0.2	NS	0.5±0.2	NS
Mean final no. of matured oocytes:	2.5±1.9	5.3±4.2	0.001	7.7±5	0.01
Final maturational rate	0.7±0.3	0.55±0.28	NS	0.5±0.2	NS
Mean no. of 2PN	2.2±1.5	3.5±2.6	0.005	5.5±2.8	0.01
Fertilization rate	0.6±0.3	0.7±0.2	NS	0.74±0.2	NS
No. of cryo-preserved embryos	1.4±0.7	4.1±3.1	0.001	3.6±2.2	0.01
ET	2.3±1.1	2±0.8	NS	2.6±0.7	NS
Total Embryo no.	1.4±1.4	2.3±2.8	0.0001	3.6±2.2	0.01

Values given as mean±St. Dev.

ET embryos transfer

[a] Mean no. of oocytes that completed their maturation in the first 24 following OPU (M2 oocytes on OPU day are excluded)

[b] Mean no. of oocytes that completed their maturation between 24 and 48 h following OPU (M2 oocytes of the first 24 h are excluded)

number of oocytes retrieved as well as the number of newly matured oocytes. For PCOS patients, AMH levels correlated with the total number of oocytes retrieved with this group producing consistently greater numbers of mature oocytes when compared with the NO group. ROC curves described an AMH cut-off level with high sensitivity and specificity, of 1.6 ng/ml for the critical production of 5 or more matured oocytes. This cut-off provided a higher mean number of oocytes, a higher maturation rate, a higher number of newly matured oocytes, a higher number of fertilized oocytes and a greater total number of embryos appropriate for transfer.

Other studies have also shown that in IVM cycles, the number of available oocytes is the single most important factor correlating with a successful procedural outcome in NO patients [5, 8, 9]. Our findings are in agreement with Fadini et al. [8] who also showed a correlation between AMH levels and the number of oocytes harvested, The final number of matured oocytes suitable for fertilization is dependent not only on their quantity but also upon their quality and maturation potential in in vitro culture systems. In this respect, our study reports for the first time to the best of our knowledge the association between AMH levels and the maturational status of oocytes, where on the day of the OPU, we did not detect any correlation. By 24 h and 48 h, however, a significantly positive correlation was clearly evident. This was unassociated with any correlation between maturational rates and AMH levels with the exception of the OPU day, suggesting that patients with higher AMH levels result in a higher number of retrieved oocytes without any compromise in their final maturational rate and hence, with a significantly higher number of embryos for transfer. This would imply that despite the fact that the serum AMH level predicts ovarian response and may act as a surrogate marker of ovarian reserve, that it is only a limited quality indicator during IVM treatment.

In an attempt to identify a cut-off AMH level, ROC curves were calculated with the principal criterion of analysis being the retrieval of at least 5 immature oocytes. This oocyte number has previously been positively associated with the chances of a pregnancy during IVM therapy [5, 9, 10]. In a further attempt to increase the sensitivity and specificity of AMH, a threshold cut-off level was identified for the retrieval of 5 matured oocytes, where a level of 1.63 ng/ml yielded a sensitivity and a specificity of 81 % and 53 % respectively. This slightly higher AMH level than previously reported is recommended to obtain the critical number of mature oocytes that are ready for fertilization. One of the difficulties with IVM treatments especially when applied to regularly cycling women, is the worse clinical outcome when compared with conventional IVF. In this regard, it is proposed that this stricter criterion for production of mature oocytes primed for fertilization could be crucial for identifying in advance those truly non-responsive cases and hence, could avoid inefficient IVM treatments.

To the best of our knowledge, this the first study that aims to investigate the possible correlation of serum AMH levels and laboratory outcomes in PCOS patients undergoing IVM treatments. In these cases, the average AMH level was 8.5 ng/ml, which was twice as much as that observed in the high-level AMH NO group, with a consistently higher number of retrieved oocytes and mature oocytes at all stages of culture in the IVM-treated PCOS patients. This was unaccompanied by any effect on the maturational potential of oocytes. The finding of a strong correlation between AMH levels and the number of oocytes retrieved in PCOS patients suggests that even at the higher AMH range, the AMH level remains a relatively sensitive tool in predicting the number of oocytes retrieved. Other oocyte parameters did not correlate with the AMH level in this group and it may be that the narrow concentration range and the small number of study participants contributed to these negative results. We did not consider pregnancy rates as one of our outcome end points, as there may be many confounding variables which affect these rates such as endometrial receptivity which is an extremely variable factor in IVM cycles. Equally, it is accepted that a limitation of our study is that the majority of embryos remained frozen and were not transferred, largely as a result of our recruitment criteria for IVM treatment.

In summary, the identification of cycles able to produce numerous oocytes will considerably improve the efficiency of IVM treatments, where we would propose that women who do not reach the AMH threshold might benefit where possible from standard ovarian stimulation. In the same way that cut-off AMH levels have been proposed for the modulation of ovarian stimulation in IVF cycles, [12] we would suggest that a similar cut-off concentration can guide the appropriateness of IVM therapy in selected cases, reducing the number of recruited cycles where suboptimal oocyte numbers are likely to be retrieved.

Acknowledgments The authors are greatly indebted to Prof. Andrew Zbar of MEDEDIT, Sheba Medical Center for the English editing.

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