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Follicular fluid anti-Müllerian hormone: a predictive marker of fertilization capacity of MII oocytes

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Abstract *Purpose* The present study aimed to correlate anti-Müllerian hormone (AMH) levels in follicular fluid (FF) with oocyte maturity stages, morphological quality of metaphase II (MII) oocyte and fertilization capacity of MII oocytes.
Methods A total of 92 infertile women undergoing controlled ovarian stimulation and intracytoplasmic sperm injection were analyzed. Patients were divided into two groups according to age: <35 years ($n = 43$) and ≥35 years ($n = 49$). An FF sample was obtained from a single dominant follicle in each patient for a total of 92 follicular fluid samples analyzed. AMH levels in serum and follicular fluid were measured by enzyme-linked immunosorbent assay. Mature MII oocytes, zygotes, and embryos were assessed for morphological quality.
Results Serum AMH levels were significantly higher in patients aged <35 years. No correlation was observed between FF AMH level and oocyte maturation stages or morphological quality of MII oocyte. Significantly lower FF AMH levels were observed in fertilized MII oocytes than in non-fertilized MII oocytes in patients aged <35 years (2.56 ± 2.0 ng/ml vs. 4.81 ± 4.14 ng/ml; $p = 0.032$).
Conclusions The present study revealed no correlation between FF AMH and oocyte maturity stage or morphological quality of MII oocyte. However, FF AMH might be a predictive marker for fertilization capacity of MII oocytes.

Keywords Anti-Müllerian hormone · Embryo quality · Fertilization capacity · Follicular fluid · Intracytoplasmic sperm injection · Oocyte quality

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Introduction

Oocyte quality is a key factor of success in artificial reproductive technology (ART). The intrinsic developmental potential of an oocyte has a crucial role in fertilization and embryo development [1]. Oocyte quality is affected by the age of the woman, ovarian reserve, controlled ovarian stimulation protocol, and the composition of follicular fluid [2, 3]. Follicular fluid (FF) is a product of secretory activity of granulosa and thecal cells and provides a very important microenvironment for the development of oocytes [4]. Some biochemical substances of the FF surrounding the oocyte may play a critical role in determining oocyte quality and consequently the capacity of fertilization and embryo development [5].

From July 2009 to August 2012 in Croatia, a restrictive law regulating assisted reproduction limited the number of oocytes that could be inseminated to a maximum of three

oocytes per cycle. Any remaining mature oocytes were cryopreserved, and embryo cryopreservation was forbidden. The selection of three female gametes for IVF/ICSI treatment was made on the basis of morphological aspects of the oocyte. In the case of conventional IVF, the evaluation of oocyte maturity was based on the expansion and radiance of the cumulus-corona complex surrounding the collected oocyte [6]. In the case of ICSI, metaphase II (MII) oocyte of good morphology implies a clear, moderately granular cytoplasm, a small perivitelline space, an intact first polar body, and a colorless zona pellucida [7, 8]. Due to the small number of oocytes in which insemination was permitted and the difficulties regarding morphological assessment in the cases of conventional IVF, law restrictions had negative effects on fertilization and pregnancy rates.

An assessment of morphology often fails to predict the oocytes fertilization and developmental capacity [9] and until today, many studies have been made to find a biochemical predictor of the functional viability of oocyte and embryo in follicular fluid [5]. It was showed that follicular fluid anti-Müllerian hormone (AMH), may play a critical role in determining the highest quality oocyte [3, 5]. Serum AMH has been evaluated as a novel clinical marker of ovarian reserve [10–13] with a poor ability to predict pregnancy after IVF [14]. Several studies have found a positive correlation between serum AMH levels and oocyte quality [15, 16], fertilization rate [17, 18], embryo morphology [19, 20] and blastocyst development [21], but results are inconsistent [22–25]. Very few studies have been published so far on the relationship of follicular fluid AMH levels and the quality of oocytes and embryos, and results are still controversial [26–32].

With the intention to minimize the negative effect of law restrictions on fertilization and pregnancy rate, we focused our research on oocyte selection and clarifying the relationship between follicular fluid AMH and oocyte quality. The aim of the present study was to assess whether FF AMH level might be a predictive marker for the selection of mature MII oocytes and whether FF AMH levels correlate with the morphological quality of MII oocytes and the fertilization capacity of MII oocytes.

Materials and methods

This study was performed at the Reproductive Unit of the Department of Obstetrics and Gynecology, Clinical Hospital Center Rijeka, University of Rijeka. Ninety-two patients, aged 26–43 (mean age 34 ± 4 years), undergoing controlled ovarian stimulation and ICSI treatment at our Center between August 2010 and July 2011 were included in this study, regardless of the previous treatment history. The inclusion criteria for women were normal ovulatory cycles and a body mass index (BMI) ranging from 18 to 28 kg/m^2. Couples with a male diagnosis of severe oligoasthenozoospermia (fresh sperm concentration $<1 \times 10^6$/ml, motility <30 %) were excluded from the study. Two groups of patients were formed according to age: <35 ($n = 43$) and ≥35 years ($n = 49$). Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and anti-Müllerian hormone (AMH) were determined. Women with endocrine abnormalities, such as polycystic ovarian syndrome or hyperprolactinemia, were excluded from the study.

All patients received the luteal down-regulation protocol with the gonadotropin-releasing hormone (GnRH) agonist triptorelin (Decapeptyl, Ferring Pharmaceuticals, Düsseldorf, Germany). After pituitary suppression was achieved, ovarian stimulation was initiated with recombinant FSH (rFSH) (follitropin alfa, Gonal-F, Serono, Geneva, Switzerland) or human menopausal gonadotropin (HP-hMG) (Menopur, Ferring Pharmaceuticals A/S, Copenhagen, Denmark). Ovulation was induced with hCG (Choragon, Ferring Pharmaceuticals, Düsseldorf, Germany) when more than two follicles with a diameter of ≥17 mm were present. Oocyte retrieval was performed 34–36 h after hCG injection under transvaginal ultrasound guidance. Embryo transfer was performed 3 days later. Luteal support (Utrogestane, Laboratoires Besins International, Montrouge, France) was provided from the day of embryo transfer until 12 weeks of gestational age or negative serum hCG test (3 weeks after embryo transfer). Each pregnancy was confirmed as a normal intrauterine pregnancy with a live embryo by ultrasonography.

Follicular fluids (FF) were aspirated under transvaginal ultrasound guidance. The FF from the single dominant follicle of each patient (which is aspirated first) was separated and maintained at steady temperature conditions (37 °C) until the oocyte was identified and isolated. The oocyte from the dominant follicle was cultivated separately (Fertilization Medium, Cook, Limerick, Ireland). FF samples were centrifuged at 500 *g* for 15 min to remove cellular elements, aliquoted and frozen at −20 °C.

Blood samples were collected on day 3 of the menstrual cycle and the serum was stored at −20 °C. FSH, LH, and PRL concentrations in serum were determined by electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany). AMH levels in serum and follicular fluid were measured by enzyme-linked immunosorbent assay (Immunotech-Beckman-Coulter, Marseilles, France). The assay range was 0–20.4 ng/ml with a functional sensitivity of 0.08 ng/ml. Intra-assay and inter-assay coefficients of variation were <8 %.

Two to four hours after retrieval, oocytes were denuded using the enzyme hyaluronidase (Hyadase, Cook, Limerick, Ireland). Denuded oocytes were assessed for the

maturity stage. MII oocytes were identified by the presence of the first polar body. Metaphase I oocytes (MI stage) were identified by the absence of the germinal vesicle (GV) and the first polar body. Prophase I oocytes (PI stage) were identified by the presence of the germinal vesicle. Atretic and aged oocytes were recognized by breakdown of the zona pellucida during the denuding procedure. The ICSI procedure was performed 3–6 h after oocyte retrieval as published previously [33]. MII oocytes were assessed for morphological quality before and during the ICSI procedure on 200× magnification (Olympus IX51, Japan). Three morphologic and one functional parameter were assessed: the presence and morphology of the first polar body, the presence of central granularity, the presence of inclusions and vacuoles, and the resistance of the plasma membrane at ICSI [34–37]. MII oocytes were graded using a three-point scoring system: oocytes without any irregularities were graded as excellent-quality, oocytes with one irregularity were graded as good-quality, and oocytes with two or more irregularities were graded as poor-quality oocytes. Graded and injected oocytes were cultured separately.

The fertilization rate was assessed 16–18 h after the ICSI procedure (Day 1). Fertilized oocytes (zygotes) were graded with respect to the size of the two pronuclei, the arrangement of nucleolar precursor bodies, and the presence of the halo effect, as described previously [38–40]. Zygotes were graded using a three-point scoring system: zygotes without any irregularities were graded as excellent-quality, zygotes with one irregularity were graded as good-quality, and zygotes with two or more irregularities were graded as poor-quality zygotes.

Embryo cleavage rate and morphology were evaluated 64–76 h after ICSI (Day 3). Embryo morphology was graded according to the number of blastomeres, the evenness of blastomeres, and the relative proportion of fragmentation [6, 41, 42]. A three-point scoring system was used for embryo grading: embryos with seven or more cells, equally sized blastomeres, and <20 % fragmentation, were graded as excellent-quality; embryos with four to six cells, equally or unequally sized blastomeres, and <20 % fragmentation were graded as good-quality; embryos with less than four cells or with >50 % fragmentation were graded as poor-quality.

Statistical analysis

Depending on the data characteristics (e.g., group size, measurement scale of a parameter, group variance, and data distribution), parametric or non-parametric tests for group comparison were applied. Student's *t* tests were used to examine differences between patients aged <35 and ≥35 in patients characteristics and ovarian response (Table 1).

Table 1 Patients' characteristics, ovarian response, maturity stages of oocytes, morphological quality of MII oocytes, zygotes and embryos, and fertilized oocytes in patients aged <35 and ≥35 years

	<35 years ($n = 43$) M ± SD	≥35 years ($n = 49$) M ± SD	*p*
BMI (kg/m^2)	23.30 ± 3.26	23.26 ± 3.09	0.956
FSH (IU/L)	7.13 ± 1.87	7.77 ± 2.32	0.148
LH (IU/L)	5.10 ± 1.65	5.71 ± 1.36	0.056
PRL (mIU/L)	299.88 ± 92.95	287.97 ± 96.02	0.548
AMH serum (ng/mL)	1.48 ± 1.76	0.85 ± 0.81	0.034*
Oocytes retrieved	7.09 ± 3.69	6.88 ± 3.46	0.773
	% (*N*/total)	% (*N*/total)	*p*
Maturity stages of oocytes			
MII stage	86.0 (37/43)	89.9 (44/49)	0.580
MI stage	4.7 (2/43)	6.1 (3/49)	0.757
PI stage	0 (0/43)	2.0 (1/49)	–
Atretic/aged	9.3 (4/43)	2.0 (1/49)	0.125
Morphological quality of MII oocytes			
Excellent-quality	29.7 (11/37)	29.5 (13/44)	0.859
Good-quality	35.1 (13/37)	18.2 (8/44)	0.083
Poor-quality	35.1 (13/37)	52.3 (23/44)	0.122
Fertilized oocytes (%)	73.0 (27/37)	72.7 (32/44)	0.975
Morphological quality of zygotes			
Excellent-quality	40.7 (11/27)	28.1 (9/32)	0.308
Good-quality	51.9 (14/27)	50.0 (16/32)	0.888
Poor-quality	7.4 (2/27)	21.9 (7/32)	0.124
Morphological quality of embryos			
Excellent-quality	16.7 (5/30)	23.8 (5/21)	0.527
Good-quality	43.3 (13/30)	14.3 (3/21)	0.028*
Poor-quality	40.0 (12/30)	61.9 (13/21)	0.124

Values in parenthesis are number/total number of analyzed cells. *M ± SD* mean values ± standard deviation; * $p \leq 0.05$

The χ^2 tests were used to examined differences between patients aged <35 and ≥35 in maturity stages of oocytes, morphological quality of MII oocytes, zygotes and embryos, and fertilized oocytes (Table 1). Student's *t* tests were used to examine differences between MII oocytes and immature/atretic oocytes in follicular fluid AMH levels (Tables 2, 3). To examine differences in follicular fluid AMH levels between fertilized oocytes and non-fertilized oocytes Student's *t* tests were used (Tables 2, 3). When comparisons were made between morphological excellent-quality, good-quality, and poor-quality MII ooyctes and embryos in follicular fluid AMH levels analyses of variance (ANOVAs) were applied (Table 2, 3). In some cases (e.g., uniquely group sizes in Tables 2, 3), the results of parametric tests (*t* test and ANOVA) were re-examined using non-parametric tests (the Mann–Whitney *U* test and

Table 2 Follicular fluid AMH levels compared between mature MII oocytes and immature/atretic oocytes, between three morphological stages of MII oocyte, fertilized and non-fertilized oocytes, and between three morphological stages of embryos in patients aged <35 years

	FF AMH (ng/ml) (M ± SD)	*p*
MII oocytes ($n = 37$)	3.18 ± 2.86	0.284
Immature/atretic oocytes ($n = 6$)	1.88 ± 1.34	
Excellent-quality MII oocytes ($n = 11$)	2.73 ± 2.36	0.828
Good-quality MII oocytes ($n = 13$)	3.34 ± 3.99	
Poor-quality MII oocytes ($n = 13$)	3.42 ± 1.94	
Fertilized oocytes ($n = 27$)	2.56 ± 2.00	0.032*
Non-fertilized oocytes ($n = 10$)	4.81 ± 4.14	
Excellent-quality embryos ($n = 5$)	2.13 ± 0.97	0.644
Good-quality embryos ($n = 13$)	3.14 ± 2.34	
Poor-quality embryos ($n = 12$)	2.69 ± 2.07	

M ± *SD* mean values ± standard deviation; * $p \leq 0.05$

Table 3 Follicular fluid AMH levels compared between mature MII oocytes and immature/atretic oocytes, between three morphological stages of MII oocytes, fertilized and non-fertilized oocytes, and between three morphological stages of embryos in patients aged ≥35 years

	FF AMH (ng/ml) (M ± SD)	*p*
MII oocytes ($n = 44$)	2.12 ± 1.30	0.652
Immature/atretic oocytes ($n = 5$)	1.85 ± 0.89	
Excellent-quality MII oocytes ($n = 13$)	2.47 ± 1.31	0.491
Good-quality MII oocytes ($n = 8$)	1.82 ± 0.80	
Poor-quality MII oocytes ($n = 23$)	2.03 ± 1.44	
Fertilized oocytes ($n = 32$)	1.99 ± 1.19	0.431
Non-fertilized oocytes ($n = 12$)	2.32 ± 1.50	
Excellent-quality embryos ($n = 8$)	1.50 ± 0.91	0.117
Good-quality embryos ($n = 13$)	1.99 ± 1.46	
Poor-quality embryos ($n = 14$)	2.57 ± 0.92	

M ± SD = mean values ± standard deviation; * $p \leq 0.05$

the Kruskal–Wallis test). The results of the non-parametric tests supported our conclusions based on the results of the parametric tests. Results are presented in tables as mean ± standard deviation (SD) and number/total number (%). Statistical significance was set at $p < 0.05$ for all analyses. Data were analyzed using the software Statistical Package for Social Sciences version 17 (SPSS, USA).

Results

No significant differences were observed in BMI, FSH, LH, and PRL levels among groups. Serum AMH levels were significantly lower in patients aged ≥35 years compared to patients aged <35 years (Table 1). There was no significant difference in the number of oocytes retrieved after controlled ovarian stimulation between groups according to age (Table 1).

No significant differences were observed with respect to two different gonadotropins used in controlled ovarian stimulation (rFSH and HP-hMG) in terms of mean age, BMI, FSH, LH, PRL, serum AMH level, number of oocytes retrieved, embryo quality rate, and FF AMH level (data not shown).

There was no significant difference in percentages of maturity stages of oocytes, morphological quality of MII oocytes, fertilized oocytes, and morphological quality of zygotes between groups (Table 1). Patients aged <35 years had significantly higher percentages of morphological good-quality embryos (Table 1).

No significant difference of FF AMH levels between MII oocytes and immature/atretic oocytes were observed in patients <35 years (Table 2) and in patients ≥35 years (Table 3). There was no significant difference in the FF AMH levels in relation to the morphological quality of MII oocytes and embryos in patients <35 years (Table 2) and in patients ≥35 years (Table 3).

FF AMH levels were significantly lower in fertilized MII oocytes than in non-fertilized MII oocytes in patients <35 years (Table 2); however, there was no significant difference of the FF AMH levels between fertilized and non-fertilized MII oocytes in patients aged ≥35 years (Table 3).

Discussion

Oocyte quality is one of the most important parameter of success in IVF/ICSI procedures. However, morphological quality of oocytes noted during in vitro culture cannot always predict successful fertilization and developmental capacity of oocytes. The role of follicular fluid AMH as indicator of oocyte quality is not yet clarified. By correlating oocyte maturity and morphological quality of MII oocyte noted during ICSI procedure with follicular fluid AMH levels, the present investigation aimed to clarify this question.

Taking into account that female fertility declines with the advancing age of woman and that age-related reproductive failure results from diminished oocyte quality [43], it was decided to subdivide patients into two groups according to age (<35 years and ≥35 years). A negative correlation of serum AMH to female age was found, as described previously [44–46]. Despite data from literature reporting that serum AMH is good predictor of oocyte yield [45, 46], the present study failed to show a correlation

between the oocyte yield and serum AMH levels (Table 1). Similar results were also described by Takahashi et al. [31].

This study analyzed percentages of maturity stages of oocytes and percentages of morphological quality of MII oocytes, zygotes, and embryos in patients aged <35 and ≥35 years. Each group consisted of single dominant oocyte of each patient. Percentages of maturity stages of analyzed oocytes and morphological quality of MII oocytes were similar regardless of the patient age and serum AMH level. Percentage of morphological good-quality embryos was significantly higher in patients aged <35 years which support previously published results that the success of IVF mainly depend on maternal age [14, 47].

Some studies demonstrated a clear relationship between serum AMH levels and the quality of oocytes and embryos [15, 16, 19, 21], while others showed a lack of a consistent correlation [22–24, 48]. One recent study suggested an alteration of the follicular metabolism in conventional gonadotrophin stimulated IVF and excluded any relevant impact of AMH serum concentration on the follicular fluid AMH concentration [50]. However, a limited number of studies have analyzed quality of oocyte and embryo with regard to FF AMH levels. The present study revealed no correlation between FF AMH levels and maturity stages of oocytes. Similarly, previous studies described FF AMH concentrations having no effect on embryo quality [28, 29]. In contrast to our results, others showed a negative correlation between FF AMH and oocyte quality [27, 30] and a recent study [28] showed AMH is highly expressed and secreted in preovulatory follicles containing immature and atretic oocytes than in follicles containing MII oocytes. Inconsistency in literature can be result in difference of procedure. The present investigation measured AMH levels in single dominant follicle of each patient while others [27, 30] estimated AMH concentration in pooled follicular fluids. Also, Mehta et al. [30] estimated the maturity of oocyte prior conventional IVF according to cumulus-oophorus radiance which is often difficult to assess. Morphological assessment of MII oocyte prepared for ICSI (without cumulus-oophorus cells), can provide more accurate information about the quality of the MII oocyte. In contrast to previous observations, that different serum AMH levels can predict the morphological quality of an oocyte [15, 16], this study found no relationship between FF AMH levels and morphological quality of MII oocytes.

The present study showed a significant negative correlation between FF AMH levels and fertilization potential of MII oocytes in patients aged <35 years. Fertilized oocytes in this group had significantly lower levels of FF AMH in comparison to non-fertilized oocytes. Higher levels of FF AMH in non-fertilized oocytes suggest that, regardless the same morphological quality of MII oocytes, oocytes from follicles with higher levels of FF AMH had a poor fertilization potential. However, we found no correlation in patients aged ≥35 years. A disadvantage of the present study is its small sample size, which could be a cause of incompatible results in the two patients' groups. Present results are consistent with the recent study [30] which demonstrated higher percentages of fertilization in low FF AMH group. In contrast to our results, some studies [26, 31] found a positive relationship between FF AMH and fertilization rate. Others found no significant correlation between fertilization rate and serum AMH or FF AMH levels [13, 28, 32]. The inconsistency in literature may in part be explained by sperm quality that can affect fertilization and the application of different fertilization procedures (conventional IVF vs. ICSI). In our study, the ICSI method was used because the IVF method is not optimal for the analysis of oocyte quality. We are aware that ICSI itself can also affect zygote and embryo quality and development, but in the hands of one person, interference of methodology is somehow standardized. Previous study in mice suggests that oocyte can regulate AMH expression and may play a role in intra-follicular regulation of the rate of follicle growth [49]; therefore, we assume that oocytes with good fertilization potential had a good utilization of AMH from follicular fluid.

In conclusion, this study demonstrated that FF AMH levels were significantly lower in fertilized oocytes than in non-fertilized oocytes in patients aged <35 years. It appears that FF AMH levels have no correlation to oocyte maturity stage and morphological quality of MII oocyte but might be a predictive marker of fertilization capacity of MII oocytes in younger patients. Further studies are needed to clarify this hypothesis.

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Conflict of interest The authors declare that they have no conflict of interest. The authors have full control of all primary data and agree to alow the journal to review data if requested.

Ethical standards The study design was approved by the Ethics Committee of the Clinical Hospital Center Rijeka, and written consent was obtained from all participants prior to their inclusion in the study. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The authors have full control of all primary data and agree to alow the journal to review data if requested.

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