Research Article

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Midkine can not be accepted as a new biomarker for unexplained female infertility

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Abstract

Objectives: This study aimed to investigate whether a growth factor and a cytokine midkine (MK) can be a new biomarker for the diagnosis and treatment of unexplained female infertility (UFI) cases.

Methods: Serum (S), follicle fluid (FF), and cumulus cells (CCs) of the patients aged 20–42 years, diagnosed with male factor (MF) and UFI were used. Patients underwent Intracytoplasmic Sperm Injection (ICSI). The anti-Müllerian hormone (AMH) and MK levels with other hormone levels (FSH, LH, E2, PRL, INHB, TSH), the oocyte and embryo qualities, the fertilization and pregnancy rates, and cumulus cells (Cell number and ultrastructure, apoptosis rate) were evaluated. Student-T-test was performed and $p<0.05$ was considered statistically significant.

Results: The lowest numbers of CCs were found at UFI ($p<0.05$). The lowest apoptosis rate with the highest CC viability rate was evaluated at MF ($p<0.05$). The lowest AMH and MK levels (S, FF) were detected at UFI in comparison to MF ($p<0.05$). MK and AMH levels of non-pregnant subjects were much lower than pregnant subjects ($p<0.05$). In addition, these levels were lower in the subjects above 35 age ($p<0.05$). Structural analysis of CCs showed that the number of lytic cells with cell remnants and apoptotic bodies was higher in non-pregnant subjects. It seems that MK did not show any resistance to both AMH and apoptosis.

Conclusions: MK can not be accepted as a new biomarker for the diagnosis and treatment monitoring of UFI cases.

Keywords: unexplained female infertility; midkine; cumulus cell; follicular fluid; anti-Müllerian hormone

Introduction

Anti-Müllerian hormone (AMH) is a gonadal hormone that is a member of the growth and differentiation superfamily of transforming growth factor beta with a weight of 140 kDa, in a disulfide-linked homodimeric glycoprotein structure [1].

Antral follicles in size up to 8 mm in the ovaries (pre-antral and small antral follicles) are released by granulosa cells, preventing the development of primordial follicles, thus it is involved in maintaining the ovarian reserve. Since it is not affected by gonadotropins even though it is released from the ovaries, and the levels are minimally changed or unchanged during the menstrual cycles, it is accepted as the most reliable and commonly used marker in the clinic via the elimination of other biomarkers originated from ovaries as estradiol [1, 2]. It was also shown to contribute to the definitive diagnosis of common female diseases with poor ovarian response, polycystic ovarian syndrome (PCOS) [2, 3].

However, in recent studies, the changes in AMH levels interindividual and intraindividual have led to questioning the reliability of AMH in the determination of ovarian reserve [4–19]. In these studies, AMH levels were changed due to (1) the biological variation and age (Antral follicle number, especially in young women in the menstrual cycle) [4, 5], (2) the ethnic group [6], (3) the overweight [7], (4) vitamin D levels [8], (5) the AMH receptor and AMH polymorphisms [9], (6) the genetic variants [10], (7) the smoking [11], (8) the contraceptive use [12], (9) the pregnancy [13], (10) different AMH detection tests [14–19]. AMH is a good

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indicator of oocyte quantity, but in no case does it reflect oocyte health or the chance of pregnancy [13]. It seems that age is still the strongest parameter in the determination of success rates with fertility treatments [13].

Midkine (MK), a 13 kDa-weighted growth factor with cytokine actions, is a member of the pleiotrophin/heparin-bound growth-related molecule family. MK is involved in the growth, survival, repair, migration, and reproduction of the organism [20–30,50]. MK can be detected in the mid-gestation phase of embryogenesis at high levels and then decreases in adults [20]. This protein is expressed by a variety of cells under physiological and pathological conditions [21]. The inhibition of the *MK* gene in the treatment of several diseases such as immunity and blood pressure-related diseases and cancer resulted in a successful prognosis [20–22]. In addition, MK can be used to treat several cases of burns, tissue damage, or tissue loss to make recovery and/or regeneration [23–25].

There is a limited number of studies on MK in reproductive health [26–29,50]. MK is secreted from the theca and granulosa cells of large follicles. MK leads to the estradiol release and the proliferation of granulosa cells in growing follicles [7–10]. It is also shown that granulosa cells can secrete MK without any effect of FSH [26–28,50]. MK leads to the proliferation of primordial germ cells via the increase of mitotic pathways. The deficiency at *MK* and pleiotrophin (*PTH*) genes lead to a decrease in the interval of the oestrus stage via decreasing the number of follicles and this gene deficiency can also cause vaginal disorders [26–28,50]. It was shown that MK levels can be affected by vitamin D levels as same as AMH. The deficiency in immunomodulator vitamin D levels increases MK levels markedly [29]. The study done by Sakcak et al. (2023) showed that serum MK level was significantly higher in pregnant women with pre-eclampsia because of the contribution of MK in complicated inflammatory processes leading to pre-eclampsia [30].

Unexplained female infertility (UFI) is defined as the pregnancy failure of a woman having healthy ovaries with a normal or low ovarian capacity and regular menstrual cycles with no apparent defect that can be diagnosed despite unprotected intercourse for one year [31, 32]. Exclusion criteria for UFI were determined and summarized as (1) PCOS, (2) congenital adrenal hyperplasia, (3) androgen-secreting tumors, (4) Cushing syndrome, (5) male infertility, (6) tubal pathologies, (7) anovulation, (8) hyperprolactinemia, (9) hypothalamic menstruation (amenorrhea), (10) previous ovarian surgery, (11) ovarian tumors, (12) uterine anatomical disorders, (13) intraperitoneal adhesions, (14) endometriosis and other pelvic pathologies, (15) thyroid dysfunction and diabetes, (16) repeated pregnancy losses and (17) autoimmune diseases [31, 32]. AMH and AMH receptor II (*AMHRII*) gene variants have also been shown to be effective in normal estrogenic and normal ovulation stages in unexplained cases of infertility [33].

Differences in AMH levels seen in healthy individuals according to variables such as the biological variation described above may create ambiguities in identifying and monitoring pathological conditions such as UFI. For these reasons, besides healthy individuals, additional and/or new biomarkers are needed to determine the correct ovarian reserve markers with their cut-off values and test techniques that can be used safely in clinics nationally and internationally for the identification and monitoring of UFI. This study aimed to investigate whether serum MK protein levels can be used as a new biomarker in the evaluation of ovarian capacity and oocyte quality in patients diagnosed with UFI undergoing Intracytoplasmic Sperm Injection (ICSI) and whether there was a connection with AMH.

Materials and methods

Patient profile

This was a single-centered, prospective study. The study was carried out following the provisions of the Helsinki Declaration and started after the approval of the Biruni University Non-Interventional Ethics Committee (No: 2017/5-1).

The study proceeded at Memorial Şişli Hospital In Vitro Fertilization (IVF) Center with 270 women aged 20–45 years grouped as a male factor (n=90, control group) and UFI (n=90). These women were diagnosed according to the criteria of the Rotterdam Consensus Conference in 2004 determined by the Council of Europe Human Reproduction and Embryology Association and the American Reproductive Medicine Association.

Inclusion criterias in UFI

Women between the age of 20–42 years old who fails to achieve pregnancy despite unprotected intercourse for one year, although they have a normal or low ovary capacity, healthy ovaries, and regular menstrual cycles, were included in this study.

Exclusion criterias in UFI

Significant, identifiable diseases and cases (PCOS, congenital adrenal hyperplasia, androgen-secreting tumors, Cushing syndrome, male infertility, tubal pathologies, anovulation, hyperprolactinemia, hypothalamic menstruation (amenorrhea), previous ovarian surgery, ovarian tumors, uterine anatomical disorders, intraperitoneal adhesions, endometriosis, other pelvic pathologies, thyroid dysfunction and diabetes, other hormonal disorders, recurrent pregnancy loss, and autoimmune diseases), the usage of vitamin D and any other medications, hypovitaminosis D and hypervitaminosis D cases were excluded from the study because it's known that they can change AMH levels [29].

Patient samples and main outcome criteria

Demographic data, fertilization rate, embryo number, embryo quality, MI-MII, and GV oocyte ratios, and antral follicle count (AFC) were evaluated in patients undergoing ICSI [34–37]. Ultrasonographic antral follicle count was done on the third day of the menstrual cycle. 12 days after the transfer, human chorionic gonadotropin beta (hCGβ) was examined by Electrochemiluminescence immunotest (ECLIA) and those who had exponentially increased (hCGβ) levels after two days were considered pregnant. The blood taken on the third day of the menstrual cycle from healthy individuals and patients is separated into a serum. Follicule-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), prolactin (PRL), thyroid stimulating hormone (TSH), and AMH levels were investigated by using ECLIA, and inhibin B (INHB) with MK levels were detected by using enzyme-linked immunosorbent test (ELISA). MK and AMH levels were also evaluated in follicle fluid (FF) taken on the day of oocyte pick-up (OPU). In addition, the number of CCs was counted by hemocytometry and viable, dead and apoptotic CC indexes were evaluated by flow cytometry, and the ultrastructure of CCs was evaluated by transmission electron microscopy (TEM) on the day of OPU. All the remaining products were destroyed after the experiments.

Serum isolation from blood

The blood of healthy individuals and patients taken on the third day of the menstrual cycle was put into yellow-capped vacuum tubes with clot activator and acrylic-based gel. The tube, which was gently reversed 5–6 times and mixed gently, was centrifuged at 1,500 *g* for 10 min.

Cumulus cell and follicular fluid isolation

Follicle fluids containing follicles reaching 15 mm in diameter were taken by using sterile needles from each patient individually. Then, the follicles were removed from the follicle fluid. According to CCs, oocytes were evaluated as firs-degree immature, second-degree intermediary, third-degree mature, fourth-degree postmature, and fifth-degree atretic [34]. After the follicles were removed from the follicle fluid, 40 IU/mL hyase was applied for 10 min to remove CCs around the oocyte. Three CC layers were left. CCs removed from the oocyte were centrifuged for 10 min at 2,000 rpm with the hyase-containing medium and the supernatant was discarded. Thus, erythrocyte, leukocyte, medium with hyase, and residual follicular fluid were removed. Cells were counted by using a hemocytometer. After FFs were centrifuged at 300 *g* for 15 min, the upper liquid was taken into 1 mL cryo tubes for use in ECLIA and ELISA processes. The vitrification was applied and stored in the liquid nitrogen tank [35, 36].

Transmission electron microscopy

The first step of the ultrastructural analysis with TEM was performed with the first fixation by keeping the cells collected on the OPU at 4 °C for 30 min in 2.5 glutaraldehyde [35, 37]. After washing three times for 5 min with PBS, samples were kept at 4 °C for 30 min with 1 % osmium tetraoxide (OsO_4) for the second fixation. Then, the third fixation with 1 % uranyl acetate was done at 4 °C for 15 min. CCs were embedded into the white part of an egg and incubated in 70 % ethanol for two days. Following hardening, the ascending degrees of ethanol (70 %, 90 %, 96 %, and two times 100 % ethanol) were applied at room temperature for 10 min. Then, they were kept in 1/1 and 1/3 ratios of propylene oxide/embedding medium (EMBed 812 kit, EMS, Hatfield, PA, USA) for 50 min, and in pure embedding medium for 70 min, respectively. They were embedded in capsules filled with pure embedding medium and stored in an oven at 60 °C for 18 h. The capsules were cut into 65–70 nm thickness by ultramicrotome and these thin sections were transferred onto the nickel grids. Finally, 5 % uranyl acetate for 15 min and Reynold's solution for 5 min was applied. Samples were examined with the transmission electron microscope (JEOL JEM-1011 Peabody, MA, USA) and photographs were taken at different magnifications.

Flow cytometry

Viable, dead, and apoptotic CC ratios were determined by using a commercial kit based on cytometric Annexin-V-Fluorescent isothiocyanate/propidium iodide (Anneksin-V-FITC/PI) double staining. Some minor changes were made to the method proposed by the kit (FITC Annexin V Apoptosis Detection Kit II; BD 556570, BD Sciences, San Jose, CA, USA) [38]. Cumulus cells were washed, and a binding buffer consisting of 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer (HEPES), sodium chloride, and calcium chloride was added. CCs were incubated with 5 µL Anneksin V-FITC and 5 µL PI at 22 °C and in the dark for 15 min. Then, measurements were taken on the flow cytometry device (BD Biosciences, FACS Calibur, USA). As a result of the device analysis, histograms were evaluated. At histograms, the left lower part (Annexin V–, PI–) and the left upper part were shown as viable cells and dead cells, respectively. In addition, the right lower part (Anneksin V+, PI–)and right upper part (Anneksin V+, PI+) indicated early apoptotic cells and late apoptotic cells, respectively. Total apoptotic cell ratios were evaluated by summing up early and late apoptotic cell ratios.

MK levels by ELISA

The evaluation of total protein levels in serum and FFs was performed using the commercial protein kit (Quick Start™ Bradford Protein Assay 1; Bio-Rad Laboratories, Inc. Munich, Germany) based on the Bradford method ELISA process. MK protein levels in serum and FFs were determined using the commercial ELISA kit (CDYELISA; Cellmid Limited, Sydney, Australia) by making some changes following the manufacturer's instructions [38]. One hundred µL of test samples, positive controls (One of them is lysate from T98 glioblastoma cells which is highly expressed MK, the other one is included in the ELISA kit) and the MK standards (0, 25, 50, 100, 250, 500, 750 and 1.000 pg/mL) were added into MK-coated 96-well microplates. Standards, controls, and samples were placed in wells in triplicate. They were kept at room temperature for 2 h with continuous shaking in the orbital shaker. After each step, the except for stopper solution application, four washes were performed with the wash buffer provided by the kit. Then detector antibody (100 µL/well) was added to the wells and they were incubated for 1 h at room temperature with continuous shaking in the orbital shaker. 100 µL of streptavidin-peroxidase solution was added per well and they were kept at room temperature for 30 min. After, they were incubated with 100 µL of substrate solution at room temperature in the dark with continuous shaking on the orbital shaker for 10 min. After detecting the blue color, 100 µL of stopping solution was added without pouring

the substrate solution out. When the yellow color formation was observed indicating that the reaction was stopped, they were measured at a wavelength of 450 nm using an ELISA microplate reader (Multiskan GO UV/Vis; Thermo Scientific Corporation, Germany) within 3 min.

AMH levels by ECLIA

AMH levels were done using the ECLIA-based commercial kit (Roche Diagnostics GmbH, Germany, catalog number: 06331076 190). After 50 µL of serum and FFs were placed into the well coated with monoclonal antibody specific for mammalian AMH, and labeled with ruthenium complex. Then, biotinylated monoclonal antibody was added and they were incubated for 9 min. Streptavidin-coated microparticle was added and allowed to bind to a biotinylated antibody for 9 min. All operations were carried out using the Roche Cobas e601 device.

The detection of other hormone levels

The ECLIA-based commercial kits provided by Roche Diagnostics GmbH (Germany) used for the detection of FSH (Catalog number: 11775863 122), LH (Catalog number: 11732234 122), PRL (Catalog number: 03203093 190), E2 (Catalog number: 06656021 190), TSH (Catalog number: 11731459 122) and hCG (Catalog number: 03271749 190) and The ELISA based commercial kit provided from Novus Biologicals (USA) used for the detection of INH B (Catalog number: NBP2-66432).

Statistical analysis

The results were evaluated for statistical significance using the IBM SPSS Statistics (Version 25) program. The Student-t-test was performed. Results are shown as means ± standard deviation (SD). $p<0.05$, $p<0.01$, $p<0.001$ values were considered statistically significant.

Results

Patient profile

The demographic data is shown in Table 1. In comparison to the control group (male factor), it's seen that fertilized oocyte number, fertilization percentage, embryo number, and good quality embryo number with the pregnancy rate were lower in the UFI group.

Flow cytometry analysis

In comparison to the control group, the UFI group had lower total cumulus cell number and viable cumulus cell rate, however, they had higher early and late apoptotic cell rates meaning that higher total apoptotic cell rates and higher dead cell rates (Table 2).

Table 1: The demographic data.

Variables	The control group (n=90)	The UFI group (n=90)
Age	31.31 ± 4.84	32.26 ± 4.90[a]
BMI	25.30 ± 4.55	24.95 ± 4.35[1]
Endometrium thickness, mm	10.68 ± 2.06	10.01 ± 2.05[1]
AFC	7.89 ± 4.00	6.71 ± 3.41[a]
Oocyte number	8.37 ± 4.70	7.97 ± 4.41[1]
GV	0.67 ± 0.39	0.92 ± 0.23[a]
MI	0.14 ± 0.45	0.08 ± 0.11[a]
MII	6.13 ± 3.55	6.32 ± 2.89[a]
Fertilized oocyte number	4.82 ± 2.76	4.39 ± 2.90[1]
Fertilisation rate, %	45 ± 7.6	43 ± 6.7[a]
Embryo number	4.71 ± 2.80	4.47 ± 2.80[1]
Good quality embryo number (day 3)	2.79 ± 1.65	2.1 ± 1.76[a]
Pregnancy rate (Sac+), %	26.7 ± 6.23	23.4 ± 3.12[a]

Results are shown as means ± SD. [a]$p<0.05$, [b]$p<0.01$, and [c]$p<0.001$ statistically significant compared to the control group, [1]$p>0.05$ not statistically significant compared to the control group. AFC, antral follicle count, BMI, body mass index, GV, germinal vesicle (blocked oocyte maturation at the prophase stage of meiosis), MI, (1) meiotic metaphase, MII, (2) meiotic metaphase.

Table 2: The apoptotic index of cumulus cells.

Variables	The control group (n=90)	The UFI group (n=90)
Number of cumulus cells	158.652 ± 56.507	148.762 ± 57.300[a]
Viable cell rate, %	65.38 ± 20.71	56.70 ± 9.56[b]
Early apoptotic cell rate, %	9.81 ± 8.22	11.54 ± 8.24[a]
Late apoptotic cell rate, %	19.80 ± 7.54[z]	24.19 ± 10.84[a,z]
Total apoptotic cell rate, %[d]	29.61 ± 15.76	35.73 ± 19.08[c]
Dead cell rate, %	6.11 ± 4.14	7.57 ± 6.27[a]

Results are shown as means ± SD. [a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$ statistically significant compared to the control group. [d]Percentage of total apoptotic cells is indicated in the table as the sum of early and late apoptotic cell percentages.

Hormone levels with midkine levels

It's clearly shown in Table 3 that the UFI group has higher sE2, sPRL, sINHB, sTSH levels in comparison to the control group. However, sMK and ffMK levels with sAMH and ffAMH levels were lower in the UFI group.

The analysis of AMH and MK levels by pregnancy outcome

Table 4 showed that the UFI group has lower sAMH and ffAMH with sMK and ffMK levels in comparison to the control group both in the pregnancy-positive and pregnancy-negative groups. However, these levels detected in the

pregnancy-positive group were much higher than in the pregnancy-negative group.

The analysis of AMH and MK levels by age in pregnancy-negative individuals

The UFI group has lower sAMH and ffAMH with sMK and ffMK levels in comparison to the control group both in the pregnancy-positive and pregnancy-negative groups. However, these levels detected in the group aged over 35 were much lower than in the group aged equal and under 35 (Table 5).

Table 3: The hormone and MK protein analysis of serum and follicle fluid.

Variables	The control group (n=90)	The UFI group (n=90)
sFSH, mIU/mL	6.41 ± 2.14	6.61 ± 2.34[d]
sLH, mIU/mL	4.23 ± 3.52	4.25 ± 2.09[d]
sLH/sFSH	0.66	0.65[d]
sE2, pg/mL	41.63 ± 21.47	44.82 ± 22.49[a]
sPRL, ng/mL	14.48 ± 7.10	19.16 ± 9.39[a]
sINH B	67.70±32.45	69.90 ± 31.78[a]
sTSH, mIU/L	1.68 ± 0.38	2.00 ± 0.42[b,c]
sAMH, ng/mL	2.89 ± 2.52	2.64 ± 2.43[a]
sMK, pg/mL	252.2 ± 54.1	250.3 ± 32.2[a]
ffAMH, ng/mL	5.31 ± 0.52	4.9 ± 1.22[d]
ffMK, pg/mL	355.2 ± 57.1	349.05 ± 48.02[a]

Results are shown as means ± SD. [a]p<0.05, [b]p<0.01, and [c]p<0.001 statistically significant compared to the control group, [d]p>0.05 is not statistically significant compared to the control group. s, serum, ff, follicle fluid.

Ultrastructural evaluation of cumulus cells of non-pregnant subjects in terms of age

The control group of non-pregnant subjects

According to the results obtained from non-pregnant subjects (n=40) in the control group at age 35 and under age 35, some of the CCs lost cell integrity due to disrupted cell membranes. Lytic cells were frequently seen in this group. Vacuolization in the cytoplasm was also detected in some cells. Mild mitochondria damage was detected in the same group. Few lipid droplets were also seen (Figure 1A). Subjects aged over 35 diagnosed as a male factor has also lipid droplets as well as the control group at age 35 and under age 35. Lytic cells with cell remnants were much higher than the control group at age 35 and under age 35. Apoptotic bodies were also detected in the same group (Figure 1C). The number of vacuoles detected in this group was much higher than in the control group at age 35 and under age 35 (Data not shown). In addition, severe mitochondria damage characterized by cristae damage, and the loss of mitochondria shape was also detected in the same group (Figure 1C).

Table 4: The analysis of AMH and MK levels by pregnancy-positivity and pregnancy-negativity.

	Pregnancy (+) (n=90)		Pregnancy (−) (n=90)	
Variables	The control group (n=45)	The UFI group (n=45)	The control group (n=45)	The UFI group (n=45)
sAMH, ng/mL	2.94 ± 2.38	2.77 ± 2.67[a]	2.88 ± 2.47	2.50 ± 2.38[a,C]
sMK, pg/mL	258 ± 59	257±35[d]	246 ± 49	242 ± 29[a,B]
ffAMH, ng/mL	6.13 ± 0.64	5.60 ± 1.25[a]	4.49 ± 0.40	4.20 ± 1.19[a,B]
ffMK, pg/mL	370 ± 64	365 ± 49[a]	340 ± 50	332 ± 47[a,C]

Results are shown as means ± SD. [a]p<0.05, [b]p<0.01, and [c]p<0.001 statistically significant compared to the control group; [A]p<0.05, [B]p<0.01 and [C]p<0.001 statistically significant compared to the pregnancy negative group at the UFI group, [d]p>0.05 is not statistically significant compared to the control group. s, serum, ff, follicle fluid.

Table 5: The analysis of AMH and MK levels by age in pregnancy-negative individuals.

Variables	Pregnancy (−) (n=90)			
	Age≤35 (n=45)		Age>35 (n=45)	
	The control group (n:24)	The UFI group (n:21)	The control group (n:22)	The UFI group (n:23)
sAMH, ng/mL	3.23 ± 2.61	2.93 ± 2.81[a]	2.65 ± 2.15	2.60±2.52[d,A]
sMK, pg/mL	264 ± 63	252 ± 36[c]	240 ± 52	231 ± 34[a,C]
ffAMH, ng/mL	6.17 ± 0.67	5.79 ± 1.29[a]	4.45 ± 0.61	5.40 ± 1.21[b,A]
ffMK, pg/mL	383 ± 70	380±51[d]	357 ± 57	349 ± 46[d,C]

Results are shown as means ± SD. [a]p<0.05, [b]p<0.01, and [c]p<0.001 were statistically significant compared to the control group, [A]p<0.05, [B]p<0.01 and [C]p<0.001 were statistically significant compared to the group aged above 35 at the UFI group; [d]p>0.05 is not statistically significant compared to the control group. s, serum, ff, follicle fluid.

The UFI group of non-pregnant subjects

Due to the results obtained from non-pregnant subjects (n=40) in the UFI group at age 35 and under age 35, the same ultrastructural changes were observed as well in the control group at the same age, however, the number of lytic cells with cell remnants and mitochondria with mild damage was much higher in this group (Figure 1B). Although the alterations of ultrastructure in the UFI group consisting of patients aged above 35 were very similar to the UFI group aged 35 and under age 35, the higher number of lytic cells and cell remnants with the presence of apoptotic bodies and severe mitochondria damage have briefly described the differences between these two groups (Figure 2D).

Ultrastructural evaluation of cumulus cells of pregnant subjects in terms of age

The control group of pregnant subjects

Due to the results obtained from pregnant subjects in the control group at age 35 and under age 35, some of the CCs have cell integrity, intact nuclei and continuous cell membranes, and healthy, intact mitochondria (Intact outer and inner membranes, oval shape). Some CCs of the control group were found to have lipid droplets in different sizes. In addition, vacuoles were rarely seen (Figure 2A). Patients aged over 35 diagnosed as a male factor have the almost same structure except for frequently seen vacuolization in

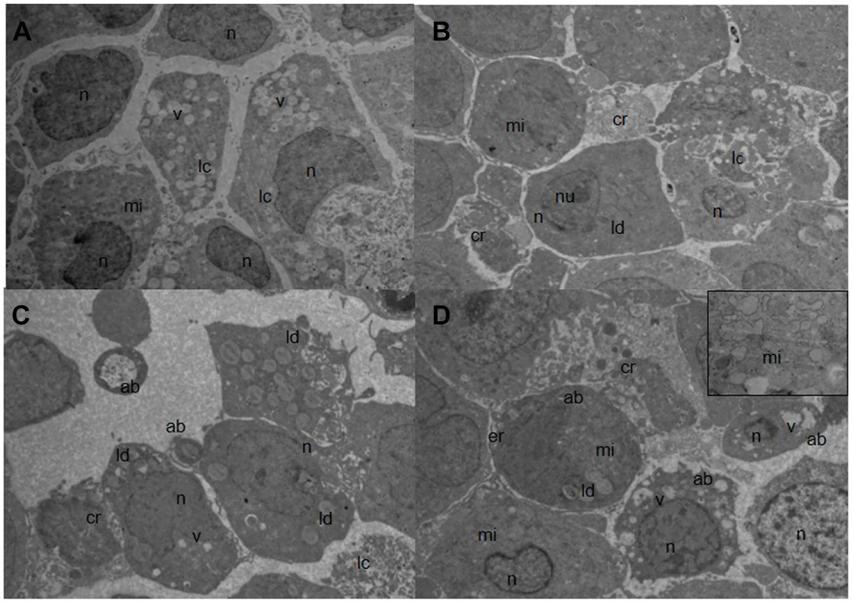

Figure 1: The evaluation of transmission electron micrographs of cumulus cells of non-pregnant patients: (A) male factor, age≤35, (B) UFI, age≤35, (C) male factor, age>35, (D) UFI, aged 35. The original magnification of all micrographs is ×7,500, except for a small micrograph at d (×10k). ab, apoptotic body, cr, cell remnant, er, endoplasmic reticulum, lc, lytic cell, ld, lipid droplet, mi, mitochondria, n, nucleus, nu, nucleolus, v, vacuole.

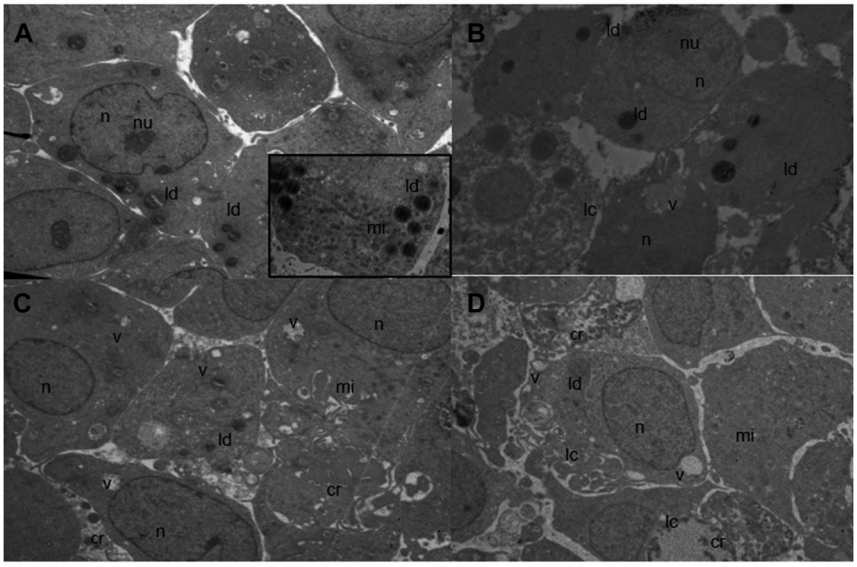

Figure 2: The evaluation of transmission electron micrographs of cumulus cells of pregnant patients: (A) male factor, age≤35, (B) UFI, age≤35, (C) male factor, age>35, (D) UFI, age>35. The original magnification of all micrographs is ×7,500, except for a small micrograph at c (×10k). ab, apoptotic body, cr, cell remnant, lc, lytic cell, ld, lipid droplet, mi, mitochondria, n, nucleus, nu, nucleolus, v, vacuole.

the cytoplasm, and the presence of mild mitochondria damage. In addition, lytic cells and cell remnants were also rarely observed (Figure 2C).

The UFI group of pregnant subjects

According to the results obtained from pregnant subjects in the UFI group at age 35 and under age 35, The integrity of the cell and nucleus membranes was also preserved in the UFI groups as the control group (Figure 2B). Although there were not many cells, in some cells, vacuoles of various sizes were also observed. These vacuoles were much higher than the control group. In addition, some of the cells were found to have lipid droplets of varying sizes. In addition, a greater number of lytic cells were also seen in this group than in the control group (Figure 2C). In comparison to the patients aged 35 and under, vacuoles in different sizes in the cytoplasm and lytic cells with cell remnants, and mild mitochondria damage were observed slightly higher in many cells of the patients aged over 35 (Figure 2D).

Discussion

In our study, we investigated whether MK levels can be evaluated as a biomarker for ovarian reserve in female unexplained infertility cases with its relationship of cumulus cell and Anti-Müllerian Hormone or not. An oocyte interacts with the surrounding somatic cells during folliculogenesis resulting in the release of the oocyte from a quiescence state to ovulation, fertilization, and zygote formation. At the later step of folliculogenesis, when the follicle cavity is formed granulosa cells differentiate into cumulus and mural granulosa cells and finally, they formed the cumulus-oocyte complex (COC) [39]. Bidirectional communication between the oocyte and cumulus cell is very important for the oocyte at the gain of competence. Cumulus cells involve in meiotic arrest and cytoplasmic maturation. The cumulus cells export cyclic AMP, calcium, other metabolites, and some signals for the control of transcription in the enclosed oocyte [39]. Growth factors were secreted by oocytes for the promotion of cumulus cell differentiation and proliferation and the maintenance of their differentiated state with the prevention of their transition to mural granulosa cells. The exchange of these compounds via gap junctions which connect their membranes is vital for this process [39].

A programmed cell death named apoptosis is started by physiological stimulation. Apoptosis is essential for the maintenance of tissue homeostasis and the elimination of defective/damaged cells without the presence of an inflammatory response. But pathological processes i.e. neoplasms, the treatment resistance with anti-neoplastic agents (The lack of apoptosis), organ-tissue atrophy, and hypoplasia (The excess of apoptosis) can also be triggered by apoptosis [40–42]. Apoptosis occurs during follicular development (Ovarian follicular atresia, luteal regression) [43]. Follicles that will not ovulate undergo atresia via apoptosis and this is important for fertility. Besides, granulosa cells go also apoptosis in follicular growth. Atresia can be seen at any phase of folliculogenesis, however, the antral stage is when most follicles undergo apoptosis. GCs, the first apoptotic cell population in atretic follicles, start follicular atresia, however, growing healthy follicles don't show apoptotic GCs. The number of apoptotic GCs gradually increases in atretic follicles [44].

Although apoptosis is a normal, healthy physiological process during dominant follicle selection, dominant follicles can prevent ovulation and decrease oocyte quality. Consequently, apoptosis occurs in GCs and affects pregnancy outcomes negatively after IVF [40–43]. When the demographic data in our study were examined, we found that although the number of oocytes was similar in the groups, the M2 oocyte ratio and fertilization rate were low in the UFI group. Likewise, PR was found to be low. This situation reflects the characteristics of the UFI group. In our study, we found the apoptotic index to be high in the UFI group. Bosco et al. showed that the DNA Fragmentation Index (DFI), a marker of oocyte competence, is higher in arrested embryos than in the transferred blastocysts [40, 41]. İdil et al. investigated granulosa cell apoptosis in patients with unexplained infertility (Mean age: 32.20 ± 4.24) and tubal factor (Mean age: 32.40 ± 1.40). They found that the apoptosis rate was significantly higher in the unexplained infertility group [45]. Fan et al. performed a study with 164 women aged 21–46 years undergoing IVF/ICSI cycles and they found that worse ovarian response was strictly correlated with a high apoptosis rate of mural granulosa cells, with fewer egg and embryo numbers in IVF/ICSI, as well as with age. In addition, they also concluded that the early apoptosis rate of cumulus cells might also influence clinical pregnancy [46]. Lee et al. performed a study with 34 patients aged ranging from 26 to 44 years who suffered from unexplained infertility and tubal factor. They found that apoptosis is higher in unfertilized oocytes than in fertilized oocytes [47]. However, in contrast to previous studies by Bosco et al. (2017), İdil et al. (2004), and Fan et al. (2019), no difference was found in the incidence of cumulus cell apoptosis according to embryo quality in their study. In our current study, the total apoptotic rate of cumulus cells with the death rates in the UFI group was higher than in the control group. Ultrastructure evaluation of these CCs also showed that the number of cumulus cells with apoptotic appearance, and lytic cumulus

cells with cell remnants was higher in the UFI group than the control group. Regardless of the group type, it's observed that the number of lytic cumulus cells with cell remnants and apoptotic bodies increased. In addition, although severe mitochondria damage was observed in all non-pregnant subjects, the number of damaged mitochondria was higher in the UFI group aged over 35. In addition, all of this was correlated with lower fertilized oocyte number, lower fertilization percentage, lower embryo number, lower good quality embryo number, and lower pregnancy rate in this current study. In addition, MK levels were higher in pregnant subjects than in non-pregnant subjects. MK levels of non-pregnant and pregnant subjects aged above 35 were much lower than other non-pregnant and pregnant subjects aged equally and under 35. This structural scheme also explains the reason damaged CC cells had lower MK levels and that CCs could not show resistance to apoptotic progress triggered by age and other reasons via increasing MK levels.

In the evaluation of findings of the hormones that were analyzed in the present study, the FSH and LH values were similar in both groups. However basal PRL, INHB, TSH, and E2 values were found to be statistically higher in the UFI group compared to the control group. We found that AMH and MK values were lower in both FF and serum for all groups. In addition, AMH and MK values were found to decrease in the pregnancy-negative group and the group aged above 35 for all groups.

There are many factors as hormones, growth factors, and cytokines [FSH, E2, inhibins and Insulin-like Growth Factor (IGF)-I, Epidermal Growth Factor (EGF), basic Fibroblast Growth Factor (bFGF), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6)] in folliculogenesis [40, 41]. These factors can interact with each other and they are produced by GCs. Therefore, they can direct the follicles to apoptosis or survival [40, 41]. Bosco et al. found that the cumulus cells of oocytes that can produce blastocysts have a higher protein kinase B/DFI ratio. They concluded that the relationship between apoptosis and survival molecules can be used as a marker to select the best oocytes [40]. Ikeda et al. previously reported that the application of MK to the *in vitro* maturation (IVM) culture of bovine cumulus-enclosed oocytes (CEOs) led to the enhancement of the developmental competence to the blastocyst stage after IVF and they concluded that the effect of MK might be mediated by its action upon mural granulosa cells and cumulus cells that closely surround the oocyte [48]. Ikeda et al. matured denuded oocytes (DOs) in IVM medium with or without MK (200 ng/mL) in the presence or absence of isolated cumulus cells, after that they were fertilized *in vitro*. They searched the effects of MK on the developmental competence of DOs to the blastocyst stage. In addition, they also prepared the conditioned media of granulosa cells cultured with or without 200 ng MK and searched their effects on the IVM of DOs during their acquisition of developmental competence to the blastocyst stage after IVF. They found that MK increases the developmental competence of bovine oocytes via cumulus and granulosa cells. In their study, they showed MK-induced suppression of the apoptosis of cumulus cells at the IVM process of CEOs. MK may promote the production of some putative soluble factor(s) in part by its anti-apoptotic effects on cumulus cells [48]. In our current study, the levels of a survival factor of MK both in serum and follicular fluid were significantly lower than in the control group. When we tried to correlate these levels with apoptosis, we didn't get what we expected. We presumed that MK levels in UFI which significantly higher apoptosis was detected would be higher than in the control group.

Hirota et al. searched the alterations of MK levels at the FF and luteinized granulosa cells (LGC) taken from women aged between 33 and 41 years and diagnosed for tubal factor and/or male factor undergoing IVF and embryo transfer [49]. They detected MK protein in FF and they found that both granulosa cells and theca cells of large follicles expressed MK mRNA. In the current study, we also detected MK protein in FF and these levels were much higher than the levels detected in serum. In the same study by Hirota et al., the promotion of BrdU uptake in LGC by MK was also detected meaning that the proliferation index of CCs was positively correlated with MK levels. In our current study, we also detected a positive correlation between the proliferation of CCs and MK levels that lower MK levels found in the UFI group had also lower CCs' proliferation index in comparison to the control group.

Bersinger et al. investigated 13 cytokine levels in serum and follicle fluid of patients with an average age of 35.3 going under stimulated IVF cycle with conventional gonadotropin and patients with an average age of 34.2 going natural IVF cycle diagnosed as a male factor, tubal factor, and unexplained infertility [50]. They found that IL-4, TNF-α, and Interferon gamma-induced protein 10 levels were lower in the FF than serum, but IL-6,-8,-10,-18, monocyte chemoattractant protein-1, VEGF ve leukemia inhibitory factor levels were higher in the FF than serum [50]. Up to now, no study investigated MK levels in serum and follicular fluid in unexplained infertility cases was found. In the current study, MK levels with AMH levels were found lower in UFI cases in comparison to the control group both in serum and FF. However, MK levels found in FF were significantly much more higher than MK levels in serum.

In the same study, it was found that the concentration of most of these markers in serum increased more in the stimulated IVF cycles than in the natural cycle groups, but this increase was not found in follicular fluid [50]. Taken

together, they concluded that the follicular defense system is not affected by gonadotropin stimulation, and follicular hormone concentrations are affected by exogenous gonadotropins, but follicular cytokines are not affected. In parallel with this study, we also found that MK levels examined in the current study were not affected by gonadotropin stimulation in terms of the comparison of the UFI group with the control group.

The study done by Somigliana et al. emphasized that fecundity decreases and ends with age, discerning between unexplained infertility and age-related infertility becomes more and more difficult day by day with the increase of the woman's age [51]. They showed by using a mathematical model that the rate of false positive diagnoses of unexplained infertility increases rapidly after 35 years of age. In addition, they also concluded that older women erroneously diagnosed with unexplained infertility may receive inappropriate therapies during Assisted Reproductive Technologies processes. In our current study, the AMH levels of the UFI group in serum and follicle fluids both in the pregnancy-positive group and the pregnancy-negative group were lower than in the control group. The same results were also observed for the MK levels. When we evaluated pregnancy negativity in terms of age, we detected that the AMH and MK levels of the UFI group in serum and follicle fluids in women aged over 35 were significantly lower than those aged equal and under 35. Our results may be interpreted that we got these results due to the decrease of cumulus cell number with the oocyte number and/or the increase in apoptosis of CCs which secrete MK and AMH. Consequently, MK does not help to overcome the problem of discriminate age-related infertility and unexplained infertility.

Nikiforov et al. used two different IVM mediums that were supplemented with and without recombinant human midkine to assess the improvement of *in vitro* maturation of oocytes [52]. They found that the addition of midkine improved the maturation rate (34 vs. 27 %). Although the detailed mechanism of action and signaling pathways activated by MK are currently unknown, MK acts indirectly on cumulus cells and triggers the secretion of factors acting on the oocyte which are important for the healthy maturation of oocyte [52].

The limitations of the study could be listed as follows: (1) other female-related infertility diseases such as a tubal factor, endometriosis, and PCOS could be evaluated and compared to UFI, (2) a multicentered study could be done to increase heterogeneity, (3) the number of patients could be increased, (4) immunohistochemistry study could be applied to evaluate the levels of MK in cumulus cells, (5) genetic evaluation could be added to understand the way of MK activity in DNA and RNA levels.

We concluded that MK does not contribute to the progression of UFI and it can not be accepted as a new biomarker for unexplained female infertility.

Research ethics: The study was carried out in accordance with the provisions of the Helsinki Declaration and started after the approval of Biruni University Non-Interventional Ethics Committee (No:2017 / 5-1).

Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission. Mine Ergüven: Conceived and designed analysis, collected data, contributed analysis tools, performed analysis, wrote the paper; Semra Kahraman: collected data, contributed analysis tools, performed analysis; Caroline Pirkevi: collected data, contributed analysis tools, performed analysis; Tülay İrez: collected data, contributed analysis tools, performed analysis.

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Data availability: The raw data can be obtained on request from the corresponding author.

References

1. Dewailly D, Andersen CY, Balen A, Broekmans F, Dilaver N, Fanchin R, et al. The physiology and clinical utility of anti-Mullerian hormone in women. Hum Reprod Update 2014;20:370–85.
2. Garg D, Tal R. The role of AMH in the pathophysiology of polycystic ovarian syndrome. Reprod Biomed Online 2016;33:15–28.
3. Cohen J, Chabbert-Buffet N, Darai E. Diminished ovarian reserve, premature ovarian failure, poor ovarian responder–a plea for universal definitions. J Assist Reprod Genet 2015;32:1709–12.
4. Broer SL, Broekmans FJ, Laven JS, Fauser BC. Anti-Müllerian hormone: ovarian reserve testing and its potential clinical implications. Hum Reprod Update 2014;20:688–701.
5. Overbeek A, Broekmans FJ, Hehenkamp WJ, Wijdeveld ME, van Disseldorp J, van Dulmen-den Broeder E, et al. Intra-cycle fluctuations of anti-Müllerian hormone in normal women with a regular cycle: a re-analysis. Reprod Biomed Online 2012;24: 664–9.
6. Seifer DB, Golub ET, Lambert-Messerlian G, Benning L, Anastos K, Watts DH, et al. Variations in serum müllerian inhibiting substance between white, black, and Hispanic women. Fertil Steril 2009;92: 1674–8.
7. Buyuk E, Seifer DB, Illions E, Grazi RV, Lieman H. Elevated body mass index is associated with lower serum anti-mullerian hormone levels in infertile women with diminished ovarian reserve but not with normal ovarian reserve. Fertil Steril 2011;95:2364–8.

8. Dennis NA, Houghton LA, Jones GT, van Rij AM, Morgan K, McLennan IS. The level of serum anti-Müllerian hormone correlates with vitamin D status in men and women but not in boys. J Clin Endocrinol Metab 2012; 97:2450–5.
9. Kevenaar ME, Themmen AP, Rivadeneira F, Uitterlinden AG, Laven JS, van Schoor NM, et al. A polymorphism in the AMH type II receptor gene is associated with age at menopause in interaction with parity. Oxford, England: Human Reproduction; 2007:2382–8 pp.
10. Schuh-Huerta SM, Johnson NA, Rosen MP, Sternfeld B, Cedars MI, Reijo Pera A. Genetic variants and environmental factors associated with hormonal markers of ovarian reserve in Caucasian and African American women. Hum Reprod 2012;27:594–608.
11. Dólleman M, Verschuren WM, Eijkemans MJ, Dollé ME, Jansen EH, Broekmans FJ, et al. Reproductive and lifestyle determinants of anti-Müllerian hormone in a large population-based study. J Clin Endocrinol Metab 2013;98:2106–15.
12. Dólleman M, Faddy MJ, van Disseldorp J, van der Schouw YT, Messow CM, Leader B, et al. The relationship between anti-Müllerian hormone in women receiving fertility assessments and age at menopause in subfertile women: evidence from large population studies. J Clin Endocrinol Metab 2013;98:1946–53.
13. Cedars MI. Evaluation of female fertility-AMH and ovarian reserve testing. J Clin Endocrinol Metab 2022;107:1510–19.
14. Nelson SM, La Marca A. The journey from the old to the new AMH assay: how to avoid getting lost in the values. Reprod Biomed Online 2011;23:411–20.
15. Fleming R, Nelson SM. Reproducibility of AMH. Hum Reprod 2012;27: 3639–42.
16. Rustamov O, Smith A, Roberts SA, Yates AP, Fitzgerald C, Krishnan M, et al. Anti-Mullerian hormone: poor assay reproducibility in a large cohort of subjects suggests sample instability. Hum Reprod 2012;27: 3085–91.
17. Kumar A, Kalra B, Patel A, McDavid L, Roudebush WE. Development of a second generation anti-Müllerian hormone (AMH) ELISA. J Immunol Methods 2010;362:51–9.
18. Schipper I, Visser JA, Themmen A, Laven JS. Limitations and pitfalls of antimüllerian hormone measurements. Fertil Steril 2012;98:823–4.
19. Welsh P, Smith K, Nelson SM. A single-centre evaluation of two new anti-Mullerian hormone assays and comparison with the current clinical standard assay. Hum Reprod 2014;29:1035–41.
20. Jones DR. Measuring midkine: the utility of midkine as a biomarker in cancer and other diseases. Br J Pharmacol 2014;171:2925–39.
21. Filippou PS, Karagiannis GS, Constantinidou A. Midkine (MDK) growth factor: a key player in cancer progression and a promising therapeutic target. Oncogene 2020;39:2040–54.
22. Muramatsu T, Kadomatsu K. Midkine: an emerging target of drug development for treatment of multiple diseases. Br J Pharmacol 2014; 171:811–3.
23. Iwashita N, Muramatsu H, Toriyama K, Torii S, Muramatsu T. Expression of midkine in normal and burn sites of rat skin. Burns 1999; 25:119–24.
24. Crouch EE, Bhaduri A, Andrews MG, Cebrian-Silla A, Diafos LN, Birrueta JO, et al. Ensembles of endothelial and mural cells promote angiogenesis in prenatal human brain. Cell 2022;185: 3753–69.e18.
25. Tsai SL, Baselga-Garriga C, Melton DA. Midkine is a dual regulator of wound epidermis development and inflammation during the initiation of limb regeneration. Elife 2020;9:e50765.
26. Shen W, Park BW, Toms D, Li J. Midkine promotes proliferation of primordial germ cells by inhibiting the expression of the deleted in azoospermia-like gene. Endocrinology 2012;153:3482–92.
27. Minegishi T, Karino S, Tano M, Ibuki Y, Miyamoto K. Regulation of midkine messenger ribonucleic acid levels in cultured rat granulosa cells. Biochem Biophys Res Commun 1996;229:799–805.
28. Muramatsu H, Zou P, Kurosawa N, Ichihara-Tanaka K, Maruyama K, Inoh K, et al. Female infertility in mice deficient in midkine and pleiotrophin, which form a distinct family of growth factors. Gene Cell 2006;11:1405–17.
29. Serinkan Cinemre FB, Cinemre H, Karacaer C, Aydemir B, Nalbant A, Kaya T, et al. Midkine in vitamin D deficiency and its association with anti-Saccharomyces cerevisiae antibodies. Inflamm Res 2016;65: 143–50.
30. Sakcak B, Turgut E, Denizli R, Farisogullari, Tanacan A, Yazihan N, et al. Comparison of serum midkine levels between pregnant women with and without pre-eclampsia: a case-control study from a tertiary hospital. Int J Gynaecol Obstet 2023;162:287–291.
31. Tafvizi F, Masomi M. Comparison of serum leptin level in women with unexplained infertility and fertile women in Iran. J Obstet Gynecol India 2016;66:466–70.
32. Casadei L, Manicuti C, Puca F, Madrigale A, Emidi E, Piccione E. Can anti-Müllerian hormone be predictive of spontaneous onset of pregnancy in women with unexplained infertility? J Obstet Gynaecol 2013;33:857–61.
33. Rigon C, Andrisani A, Forzan M, D'Antona D, Bruson A, Cosmi E, et al. Association study of AMH and AMHRII polymorphisms with unexplained infertility. Fertil Steril 2010;94:1244–8.
34. Effio E, Bermúdez A, Gutiérrez Nájar A, Orbea M. Tasa de embarazo en embriones criopreservados según la calificación morfológica clásica y la integridad de las blastómeras a la descongelación [Pregnancy rate in cryopreserved embryos based on the classical morphologic scoring and the integrity of blastomeres at thawing]. Ginecol Obstet México 2001;69:431–8.
35. Tanriverdi G, Denir S, Ayla S, Bilir A, Oktar H, Cepni I, et al. Notch signaling pathway in cumulus cells can be a novel marker to identify poor and normal responder IVF patients. J Assist Reprod Genet 2013;30: 1319–26.
36. Kahraman S, Çetinkaya CP, Çetinkaya M, Tüfekçi MA, Ekmekçi CG, Montag M. Is there a correlation between follicle size and gene expression in cumulus cells and is gene expression an indicator of embryo development? Reprod Biol Endocrinol2018;16:69.
37. Taghizabet N, Khalili MA, Anbari F, Agha-Rahimi A, Nottola SA, Macchiarelli G, et al. Human cumulus cell sensitivity to vitrification, an ultrastructural study. Zygote 2018;26:224–31.
38. Üner M, Yener G, Ergüven M. Design of colloidal drug carriers of celecoxib for use in treatment of breast cancer and leukemia. Materials for Biological Applications. Mater Sci Eng C 2019;103:109874.
39. Biase FH, Kimble KM. Functional signaling and gene regulatory networks between the oocyte and the surrounding cumulus cells. BMC Genom 2018;19:351.
40. Bosco L, Chiarelli R, Roccheri MC, Matranga D, Ruvolo G. Relationship between apoptosis and survival molecules in human cumulus cells as markers of oocyte competence. Zygote 2017;25:583–91.
41. Almeida CP, Ferreira M, Silveira CO, Campos JR, Borges IT, Baeta PG, et al. Clinical correlation of apoptosis in human granulosa cells-A review. Cell Biol Int 2018;42:1276–81.
42. Ferrero H, Delgado-Rosas F, Garcia-Pascual CM, Monterde M, Zimmermann RC, Simon C, et al. Efficiency and purity provided by the

existing methods for the isolation of luteinized granulosa cells: a comparative study. Hum Reprod 2012;27:1781–9.
43. Jančar N, Kopitar AN, Ihan A, Klun IV, Bokal EV. Effect of apoptosis and reactive oxygen species production in human granulosa cells on oocyte fertilization and blastocyst development. J Assist Reprod Genet 2007;24:91–7.
44. Matsuda F, Inoue N, Manabe N, Ohkura S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. J Reprod Dev 2012;58:44–50.
45. Idil M, Cepni I, Demirsoy G, Ocal P, Salihoğlu F, Senol H, et al. Does granulosa cell apoptosis have a role in the etiology of unexplained infertility? Eur J Obstet Gynecol Reprod Biol 2004;112:182–4.
46. Fan Y, Chang Y, Wei L, Chen J, Li J, Goldsmit S, et al. Apoptosis of mural granulosa cells is increased in women with diminished ovarian reserve. J Assist Reprod Genet 2019;36:1225–35.
47. Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW. Cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. J Assist Reprod Genet 2001;18:490–8.
48. Ikeda S, Saeki K, Imai H, Yamada M. Abilities of cumulus and granulosa cells to enhance the developmental competence of bovine oocytes during in vitro maturation period are promoted by midkine; a possible implication of its apoptosis suppressing effects. Reproduction 2006; 132:549–57.
49. Hirota Y, Osuga Y, Nose E, Koga K, Yoshino O, Hirata T, et al. The presence of midkine and its possible implication in human ovarian follicles. Am J Reprod Immunol 2007;58:367–73.
50. Bersinger NA, Kollmann Z, Von Wolff M. Serum but not follicular fluid cytokine levels are increased in stimulated versus natural cycle IVF: a multiplexed assay study. J Reprod Immunol 2014;106:27–33.
51. Somigliana E, Paffoni A, Busnelli A, Filippi F, Pagliardini L, Vigano P, et al. Age-related infertility and unexplained infertility: an intricate clinical dilemma. Hum. Reprod. 2016;31:1390–6.
52. Nikiforov D, Junping C, Cadenas J, Shukla V, Blanshard R, Pors SE, et al. Improving the maturation rate of human oocytes collected ex vivo during the cryopreservation of ovarian tissue. J Assist Reprod Genet 2020;37:891–904.