# Inhibin A may be the Black Horse for Determination of the Optimal Triggering Time and Decision-making for Oocyte Retrieval

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## Abstract

**Objectives:** Assessment of the applicability of estimated serum inhibin A (INHA) and/or INHB versus estradiol (E2) levels to determine the triggering time during controlled ovarian stimulation (COS) that might allow the retrieval of the optimal number of oocytes.

**Patients:** 196 infertile women assigned to receive COS using the flexible antagonist protocol gave blood samples for ELISA estimation of serum levels of E2 and INHs on day-2 of the cycle and on the day of retrieval depending on having serum E2 levels >2000 pg/ml and the optimal number of follicles; 11-15 follicles of ≥16 mm in diameter. The study outcome is the blinded distinguishing ability of estimated serum levels of E2 and INHs between cases that might have <11, 11-13, and 14-15 mature follicles on transvaginal ultrasonography (TUV) imaging on the day of triggering.

**Results:** Serum E2 levels at the time of triggering (>2000 pg/ml) showed sensitivity and specificity rates of 89.8% and 40.6% to distinguish women who had <11 mature follicles after COS. Serum levels of INHA (≥723 ng/ml) showed significantly higher diagnostic performance for differentiation between patients according to the number of mature follicles compared to serum E2 and INHB levels with significant area under the curve (AUC) for differentiating patients had <11 or >14 mature follicles, while E2 and INHB failed for this respect. Statistical analyses defined serum INHA as the significant predictor for canceling depending on the maturation of a low number of follicles after COS and for the presence of 14-15 mature follicles and deciding triggering and oocyte retrieval.

**Conclusion:** Estimation of serum E2 is an unreliable marker for differentiating women according to several mature follicles. Estimated serum levels of INHA are the best biomarker for the identification of women who might have <11 follicles and women who might have ≥14 mature follicles with high specificity.

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## Introduction

According to the WHO, infertility can be defined as failure to achieve a clinical pregnancy through 12 or more months of unprotected regular sexual intercourse in apparently healthy couples (1). Infertility is a worldwide problem with a high prevalence that was estimated to affect about 15% of couples [(2)].

Assisted reproductive technology (ART) as defined by the American Center for Disease Control is any fertility-related treatment in which eggs or embryos are manipulated. In-vitro fertilization (IVF), cryopreservation, and intracytoplasmic sperm injection (ICSI) are by far the most common ART procedure performed [(3)].

Inhibins (INH) belong to a large family of glycoprotein hormones and growth factors and are members of the transforming growth factor-β family [(4)]. INHB is composed of a common α-subunit that is linked to 1-2 β-subunits by disulfide linkage; βA in inhibin A (INHA) or βB in inhibin B (INHB) [(5)]. Gonadal-derived INHs act in an endocrine manner to suppress the synthesis of follicle-stimulating hormone (FSH) by pituitary gonadotrope cells [(6)] by blocking the signaling by activins, which are homodimers of β-subunits, in gonadotrope cells of the anterior pituitary [(5)].

Timing for the human chorionic gonadotrophin (hCG) triggering during ART is still unsettled because too early triggering will result in immature oocytes and endometrium with insufficient luteal phase, while with too late triggering the spontaneous LH surge will be already occurred with subsequent wrong timing of oocyte retrieval; thus the perfect timing for hCG triggering, which might be defined as the time probably allows achievement of the highest possible success rates [(7)], is crucial for ART outcome but unfortunately is still under debate.

## Objectives

This study aimed to evaluate the success rate to determine the triggering timing that allows the retrieval of the optimal number of oocytes depending on the estimation of serum levels of estradiol (E2), INHA, and INHB alone or in combination in conjunction with transvaginal ultrasonography (TVU) imaging.

## Design

Prospective comparative non-randomized study.

## Setting

IVF centers at Benha University Hospital and multiple private hospitals.

## Patients

All infertile women attending these centers were evaluated for exclusion and inclusion criteria.

### Exclusion criteria

The presence of endometriosis, systemic diseases that may affect the outcomes, very poor ovarian reserve and refusal to participate in the study, manifest hypertension, diabetes mellitus, chronic kidney disease, obesity grades II or III, uterine anomalies, immunological infertility, autoimmune diseases, maintenance on immunosuppressant therapy, or refusal to participate in the study.

### Inclusion criteria

Infertile women younger than 40 years, free of exclusion criteria, and who signed the written consents were included in the study.

### Ethical considerations

The study protocol was approved in November 2021 and after the end of the study trial and case collection the final approval by No.: RC50.10.22 was obtained.

**Blindness**

Blood samples were obtained by an assistant who was blinded about the study protocol and sent as numbered innominate tubes to the lab physician who was also blinded about the indications for the requested investigations

**Study procedure**

All the enrolled women were clinically evaluated for the collection of demographic and clinical data. Then, patients underwent TVU to determine the number of antral follicles in each ovary and the collective number was calculated to determine baseline antral follicle count (AFC) as previously documented by the Practice Committee of the American Society for Reproductive Medicine for measuring the ovarian reserve [8]. During ovarian stimulation (OS), patients were frequently monitored for the follicular response by TVU for follicle number and size which was determined as the mean value of two of the orthogonal diameters of the follicle. The optimal number of follicles for deciding for oocyte retrieval was 11-15 follicles of ≥16 mm in diameter as previously documented [9]. Regarding serum E2 as an indicator for retrieval, as previously documented serum E2 on a day assigned for oocyte retrieval at <2000 pg/ml will yield few total numbers of oocytes and low-quality embryos [10].

**Controlled Ovarian Stimulation (COS) protocol**

The COS according to the flexible antagonist protocol was performed as a daily subcutaneous injection of 300-450 IU of Gonapure (150 IU/ml amp, Minapharm, Al-Amyrea, Cairo, Egypt) starting on the 2nd day of the cycle. When the dominant follicle reached 14 mm, cetrorelix (250 µg amp, Cetrotide®, Merck, Germany) therapy in a dose of 250 µg/day was started till the day of Human chorionic gonadotrophin (hCG) injection. The hCG injections were given in the form of Epifasi (5000 U amp, Epico, Al-Amyrea, Cairo, Egypt) injection; 10000 units as triggering agent, and oocyte retrieval was performed 36-hr later.

**Blood Sampling**

Two blood samples were obtained from each enrolled woman under complete aseptic conditions. Blood samples were allowed to clot and centrifuged at 3000rpm for 10 minutes and the supernatant was aspirated and collected in Eppendorf tubes that were numbered by an assistant who was blinded about the required investigations and their indications. Blood samples were collected on the 2nd day of the cycle before the start of ovarian stimulation and at the end of ovarian stimulation on the day of final oocyte maturation.

**Investigations**

1. Human estradiol (E2) using Abcam ELISA kit (Cat. No. ab285329).
2. Human Inhibin-A and Inhibin-B using Abcam ELISA kit (Cat. No. ab285329 and ab119449, respectively).

**Study outcome**

The study outcome is the ability of estimated serum levels of E2, INHA, and INHB to distinguish between cases that showed <11, 11-13, and 14-15 oocytes on TUV imaging on the day of hCG triggering in comparison to the retrieved number of the oocyte as the gold standard for comparison.

**Statistical analysis**

Results were analyzed using IBM® SPSS® Statistics (Version 22, 2015; Armonk, USA) by applying the paired t-test for analysis of intra-group variance and Chi-square test (X2 test) for analysis of non-numeric data. Kaplan-Meier Regression analysis was used to suggest the most probable cutoff point of serum levels of studied variables for prediction of the number of mature follicles on time of TVU. The receiver characteristic curve (ROC) analysis was used to determine the best predictor for the decision-making regarding to cancel or continue as judged by the significane of the area under curve (AUC) in relation to the area under the reference line (AUC=0.5) for Windows statistical package. Significance was considered if P value was <0.05.

## Results

The study included 196 women fulfilling the inclusion criteria after the exclusion of 4 women of obese grade II or III, 3 women who had immunological infertility, 3 women who had uterine anomalies, 5 women who had endometriosis, and 3 women who were older than 40 years. Also, during the follow-up for the response to COS, 12 women were missed and were excluded from the study as shown in figure 1; patients' enrolment data are shown in table 1.

**Table (1): Enrolment data of studied infertile women**

| Variables | | Findings |
|---|---|---|
| **Age (years)** | **<25** | 40 (20.4%) |
| | **25-29** | 85 (49.4%) |
| | **30-35** | 58 (29.6%) |
| | **>35** | 13 (6.6%) |
| | **Mean (±SD)** | 28.5±4.2 |
| **Body mass index (kg/m²)** | **Average weight (<25)** | 6 (3.1%) |
| | **Overweight (25-30)** | 69 (35.2%) |
| | **Obese I (>30-34.99)** | 121 (61.7%) |
| | **Mean (±SD)** | 30.3±2.5 |
| **Duration of infertility (years)** | **1-2** | 116 (59.2%) |
| | **3-5** | 74 (37.7%) |
| | **>5** | 6 (3.1%) |
| | **Mean (±SD)** | 2.4±1.3 |

At the triggering time as decided according to TVU-determined AFC, the detected AFC, and serum levels of E2, INHA, and INHB were significantly higher in comparison to the corresponding data determined on day 2 of the cycle. TVU detected 69 women (35.2%) had <11 oocytes, 83 women (42.4%) had 11-13 oocytes and 44 women (22.4%) had 14-15 oocytes (Table 2).

**Table (2): Mean AFC and serum levels of E2, INHA, and INHB of the studied women**

| Variables | | | Findings |
|---|---|---|---|
| **Bilateral antral follicular count** | **Day 2 of the cycle** | | 4.5±0.9 |
| | **At Triggering time** | **<11** | 69 (35.2%) |
| | | **11-13** | 83 (42.4%) |
| | | **14-15** | 44 (22.4%) |
| | | **Mean (±SD)** | 11.7±2.2 |
| **Serum E2 (pg/ml)** | **Day 2 of the cycle** | | 66.2±45.8 |
| | **At Triggering time** | | 2856.7±967.8 |
| **Serum Inhibin-A (ng/ml)** | **Day 2 of the cycle** | | 8.9±3.5 |
| | **At Triggering time** | | 639.6±178.5 |
| **Serum Inhibin-B (ng/ml)** | **Day 2 of the cycle** | | 146.1±123.3 |
| | **At Triggering time** | | 4405±3117.9 |

Using Kaplan-Meier regression analysis to define the cutoff points for serum levels of E2, INHA, and INHB at which the possibility of having oocyte number of 11-15 was increased, showed that serum levels of E2, INHA, and INHB at cutoff points of 3300pg/ml, 723 ng/ml and 5835 ng/ml, respectively might predict the increased possibility of getting 11-15 oocyte on retrieval by about 65%, 50% and 70%, respectively (Fig. 2a-c).

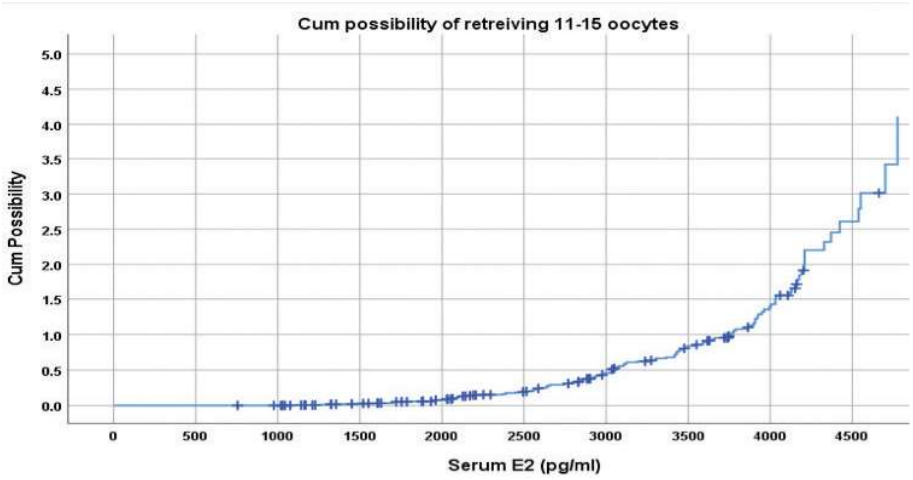


**Fig. (2a): Kaplan-Meier regression of serum E2 levels for defining a cutoff point for prediction of 11-15 oocyte retrieval**

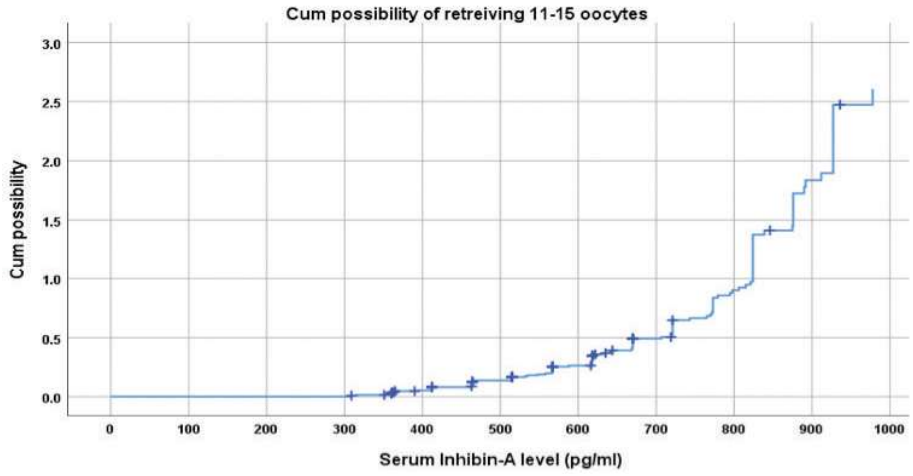


**Fig. (2b): Kaplan-Meier regression of serum INHA levels for defining a cutoff point for prediction of 11-15 oocyte retrieval**

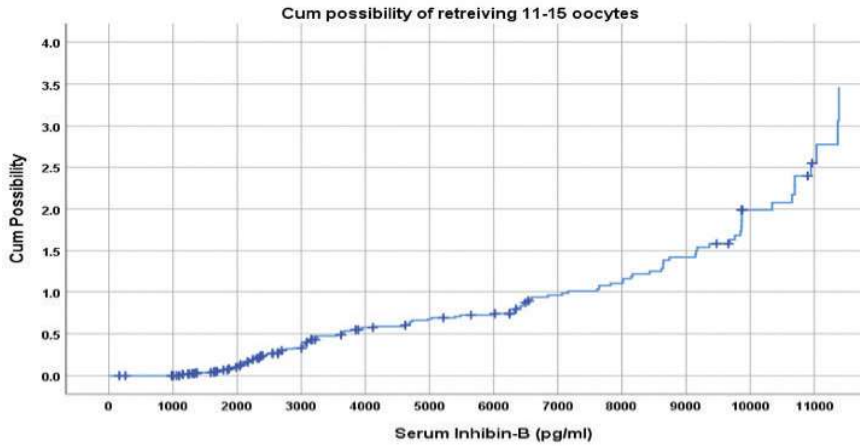


**Fig. (2c): Kaplan-Meier regression of serum INHB levels for defining a cutoff point for prediction of 11-15 oocyte retrieval**

Evaluation of the diagnostic performance of the suggested cutoff points for the lab variables to discriminate women had 11-15 mature follicles as shown in table 3, defined significantly higher diagnostic performance of serum INHA in comparison to that of E2 (P=0.0005) and INHB (P=0.0028) with the non-significant difference between the diagnostic performance of E2 and INHB (P=0.711).

**Table (3): The diagnostic performance of the suggested cutoff point for serum levels of E2, INHA, and INHB to discriminate women had 11-15 mature follicles compared versus the TVU findings**

| Variables \ Markers | | Serum Estradiol (pg/ml) | Serum Inhibin-A (ng/ml) | Serum Inhibin-B (ng/ml) |
|---|---|---|---|---|
| Cutoff point | Value (±SE) | 3300±69.3 | 723±13.9 | 5835±283 |
| | % of the increased possibility | 65% | 50% | 70% |
| | 95% CI of the value | 3170-3440 | 696-750 | 5280-6390 |
| Sensitivity rate (%) | | 54.33 (45.3-63.2) | 64.1 (55.28-72.3) | 56.3 (46.9-65.4) |
| Specificity rate (%) | | 72.5 (60.4-82.5) | 96.9 (89.3-99.6) | 77.9 (67-86.6) |
| Positive predictive value (%) | | 78.4 (70.6-84.6) | 97.7 (91.4-99.4) | 79.8 (71.6-86.1) |
| Negative predictive value (%) | | 46.3 (40.4-52.3) | 57.3 (51.5-62.9) | 53.6 (47.7-59.4) |
| Accuracy (%) | | 60.7 (53.5-67.6) | 75 (68.3-80.9) | 64.8 (57.7-71.5) |

Evaluation of the discriminative ability of the estimated lab variables between the studied women according to the number of mature follicles that might be detected on TVU defined serum INHA as the significant predictor for canceling depending on the maturation of a low number of follicles (n<11 follicles) after COS (Fig. 3a), while serum E2 and Inhibin-B showed non-significant AUC for prediction of such low number of mature follicles, so cancellation decision could not depend on both markers. However, serum E2 and INHB could define women who may have 11-13 mature follicles with significant AUC, while INHA showed non-significant AUC for this targeted number of follicles as shown in figure 3b. For prediction of the possibility of the presence of 14-15 mature follicles, serum INHA may be a significant indicator for triggering and oocyte retrieval for this high number of follicles, while serum E2 and INHB could not discriminate these women (Table 2, Fig. 3c).

**Table (3): Receiver characteristic curve analysis of serum biomarkers for discrimination between the studied women according to the number of mature follicles that might be detected on TVU**

| No. of oocytes | <11 | | | 11-13 | | | | 14-15 | |
|---|---|---|---|---|---|---|---|---|---|
| **Variables** | **AUC (SE)** | **P-value** | **95% CI** | **AUC (SE)** | **P-value** | **95% CI** | **AUC (SE)** | **P-value** | **95% CI** |
| **Serum estradiol** | 0.498 (0.064) | 0.971 | 0.372-0.623 | 0.712 (0.069) | 0.014 | 0.576-0.847 | 0.598 (0.051) | 0.079 | 0.496-0.701 |
| **Serum Inhib-in-A** | 0.366 (0.051) | 0.045 | 0.266-0.466 | 0.545 (0.120) | 0.605 | 0.309-0.780 | 0.663 (0.056) | 0.004 | 0.554-0.772 |
| **Serum Inhib-in-B** | 0.402 (0.064) | 0.141 | 0.277-0.623 | 0.690 (0.084) | 0.028 | 0.526-0.854 | 0.592 (0.051) | 0.098 | 0.492-0.693 |

AUC: Area under the curve; SE: Standard error; CI: Confidence interval

Regression analysis of the three lab variables assured the finding of ROC curve analysis as regards the prediction of the presence of 14-15 mature follicles and defined serum INHA as the significant predictor for the timing of oocyte triggering and retrieval (β=0.378, P<0.001), while excluded E2 and INHB.

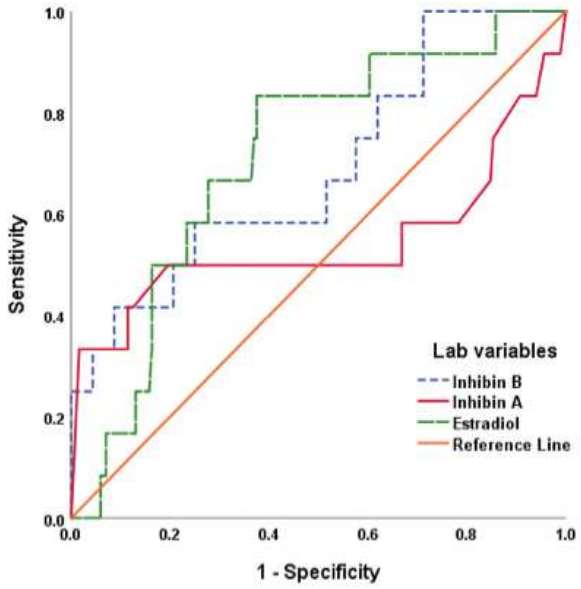


**Fig. (3a): ROC curve analysis for identification of women who had <11 mature ovarian follicles on COS and cancellation of triggering**

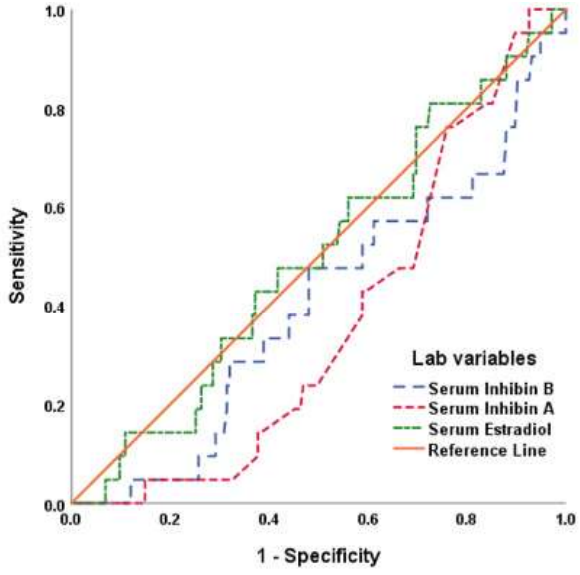


**Fig. (3b): ROC curve analysis for identification of women who had 11-13 mature ovarian follicles on COS**

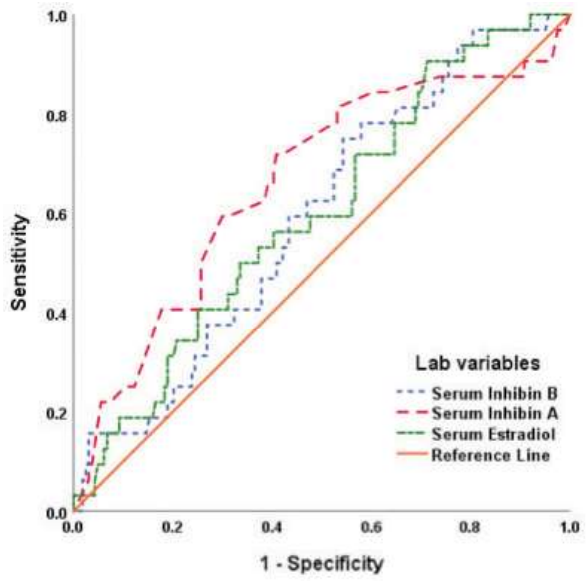


**Fig. (3c): ROC curve analysis for identification of women who had 14-15 mature ovarian follicles on COS and triggering indication**

## Discussion

This study tried to resolve the dilemma of the relationship between the timing of triggering after COS and the number of retrieved oocytes, evaluation of the diagnostic performance of the previously documented cutoff point of serum E2 at the time of triggering (>2000 pg/ml) to distinguish women had >11 mature follicles after COS [9] showed sensitivity rate of 89.8%, while the specificity rate was 40.6% due to the detected high number of false positive cases (59.4%) among women had <11 mature follicles. This finding allows for suggesting the unreliability of the dependence on the estimation of serum E2 as a marker for initiation of triggering and oocyte retrieval and points to the need for another differentiating marker. In line with this data, a previous study documented that the maturity rate of ovarian follicles did not significantly differ among E2 levels [11]. Also, a recent study documented that oocyte maturity was associated with E2 concentration and follicle size as well as the interaction of both parameters; however, the live birth rate per follicle showed a non-significant difference according to follicles sizes at the time of oocyte retrieval [12]. Moreover, the observed data and the provided suggestion goes in hand with the recently documented that E2 measurement is unreliable as a determinant of oocyte maturity and to determine the optimal time point for triggering and attributed this to the multi-follicular growth of follicles of varying size on OS that yields supra-physiological serum E2 levels [13].

On contrary, the estimation of serum levels of INHs could differentiate patients according to the number of mature follicles that might be detected on TVU and help the decision-making for canceling or proceeding, wherein serum levels of INHA at the cutoff point of 723 ng/ml showed significantly higher diagnostic performance for differentiation between patients according to the number of mature follicles in comparison to serum E2 and INHB levels and showed significant AUC for differentiating patients had <11 or >14 mature follicles, while E2 and INHB failed for this respect, but their high levels showed high AUC for defining women had 11-13 mature follicles, while AUC for INHA was non-significant.

These findings supported the previously reported that on the day of final oocyte maturation serum INHA is strong, while serum E2 is moderately correlated to the number of follicles ≥15 mm and to the number of retrieved and mature oocytes with AUC of 0.91 and 0.84, respectively and concluded that serum INHA may be a more powerful tool in conjunction with TVU in defining the triggering time and in the decision making for oocyte retrieval than E2 [14].

The reported relation between high serum levels of INHA and a high number of mature follicles which was not evident for INHB could be attributed to the previously detected in an animal study that found INHA, not INHB can affect follicular maturity through impairing the synthesis of FSH via the competitive binding to activin type II receptors, which stimulates FSH production,

especially in the presence of the TGFβ type III receptor (15). Another in-Vitro study attributed the prolonged effect of INHA on ovarian follicles during OS to the finding that cumulin, which is a heterodimer of the oocyte-secreted factors bone morphogenetic protein 15 and growth differentiation factor 9 that regulate folliculogenesis and ovulation rate, did not significantly alter the ovarian INHA secretion or action, irrespective of the presence of FSH, while cumulin exerts paracrine control of FSH-induced regulation of INHB [16].

Also, experimental treatment of the primary granulosa cells that were isolated from ovarian follicles with different concentrations of INHA for 24 h resulted in increased cell viability in a dose-dependent manner of INHA with significant enhancement of the mitochondrial membrane potential, improvement of the progression of the G1 phase of the cell cycle and increased cell number in the S phase and decrease of the apoptotic rate in granulosa cells with higher INHA concentrations [17]. Another study attributed the relationship between INHs and the number of mature follicles to the discordant pattern of secretion of ovarian INHs where smaller follicles produce INHB, while the dominant follicle produces INHA [5]. Recently, an In-Vivo animal study, reported that the functional molecule of the INHA gene can induce follicular development via regulation of proliferation and apoptosis of granulosa cells with increased secretion of folliculogenesis-related hormones [18].

## Conclusion

Estimation of serum biomarkers in samples that were obtained on the date assumed for initiation of oocyte triggering might predict the number of mature follicles before TVU examination. Estimation of serum E2 is an unreliable marker for differentiating women according to several mature follicles. Estimated serum levels of INHA are the best biomarker for the identification of women who might have <11 follicles and women who might have ≥14 mature follicles with high specificity. The diagnostic performance of INHB is in the gray zone and its reliability is uncertain.

## Limitation

Estimation of the studied cytokines in follicular fluid was missed and so its relation to serum levels was not performed. Also, the relation between these markers and serum FSH was not considered.

## Recommendations

Further studies to evaluate the relation between serum INHs and successful pregnancy outcomes of ICSI were mandatory.

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