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Correlation between Resistin and Follistatin hormones with pregnancy outcome in samples of Iraqi women undergoing ICSI cycle.

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ABSTRACT

Resistin and follistatin levels have been found to be high in polycystic ovarian syndrome (PCOS) patients, that play role in pathophysiology of PCOS and pregnancy status of women having ICSI cycle. This study aimed to determine the relationship between the hormone levels and pregnancy outcome in PCOS and non-PCOS women undergoing intracytoplasmic sperm injection (ICSI) program. The study was done at Higher Institute for Infertility Diagnosis and Assisted Reproductive Technologies from 2022 to 2023. Eighty infertile women participating in the IVF/ICSI treatment. They were split to control (40 non-polycystic ovarian syndrome- PCOS women) and 40 women for PCOS group. They were subdividing to subgroups' according to body max index (BMI; normal and overweight / obese). ELISA method was used to measure hormone level at day 2 and follicular fluid and serum at day of ova pick up. About 38.8% of study participants were got pregnant. Means of serum resistin and follistatin in day 2 and OPU were significantly higher in patients with PCOS and normal BMI level while follistatin at day 2 and in follicular fluid (FF) were significantly higher in participants who got pregnant. Follistatin level at day 2 > 9.89 ng/ml and in FF > 15.06 ng/ml and might be as a predictor for occurrence of pregnancy after ICSI. The cutoff point of follistatin level in the serum at day two and in FF at the day OPU was found to be a predictor of pregnancy following ICSI, while no correlation was found between resistin and pregnancy outcome.

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KEYWORD

Resistin, Follistatin, Pregnancy Rate, ICSI Cycle, Ova Pick

1. Introduction

Polycystic ovary syndrome (PCOS) is regarded to be as the most prevalent endocrinopathy. In women of reproductive stage of life with a range of occurrence from 8–13% (Jabeen et al., 2022 [1]). The World Health Organization (WHO) data leads to the belief that about 116 million women (3.4%) are PCOS globally (Bulsara et al., 2021 [2]) .

Its pathogenesis consists of insulin resistance (IR) and hyperandrogenism (HA), which motivate reproduction (menstrual dysfunction and infertility). The pathogenesis is not well known, but evidence suggests that aberrant ovarian angiogenesis, caused by a mismatch between several angiogenic markers, take a role in the etiology of PCOS (Liu et al., 2020[3]). Aside from infertility, PCOS is linked to obesity, IR, type-2 diabetes, dyslipidemia, cardiovascular diseases, hepatic steatosis, and endometrial cancer (Raeisi et al., 2021[4]). It was discovered that excess adiposity and increased adipokine production from adipocytes could be a link between IR and ovulation disturbances (Legro et al., 2013 [5]). Resistin, a 12.5 kD, a cysteine-rich protein from adipocytes, is thought to be a strong candidate for linking IR to an increase in adiposity. Circulating resistin levels in the blood are significantly higher in IR mice and genetically or diet-induced obese mice. Furthermore, dehydro-epiandrosterone has been shown to increase resistin expression, claiming that PCOS-related resistin and androgen synthesis may be correlated (Raeisi et al. 2021[4]).

Follistatin, a member of the transforming growth factor-b superfamily, is another type of adipokine that has been identified as a potential genetic marker for PCOS. Follistatin was discovered and isolated from follicular fluid because of its suppression of pituitary FSH secretion (Díaz et al., 2023[6]).

Lately, follistatin has been counted to have a role in the etiology of PCOS, as women diagnosed with this disorder presented with increased follistatin concentrations, unlike healthy women, independently of BMI (Wu et al. 2021 [7]). It had been observed that circulating levels of follistatin and resistin, whatever was the BMI of the woman, are higher in those with PCOS in comparison with non-PCOS women, showing that these adipokines may take a part in the pathology of PCO (Legro et al. 2013 [5]). The present study was designed to clarify the correlation between resistin and follistatin hormones with pregnancy outcomes in samples of Iraqi women undergoing the ICSI cycle

2. Patients and Methods

The study was performed for (80) infertile couples who had attended the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University in Baghdad-Iraq. The duration of the study extended from 2022 to 2023.

The sample size was 80 infertile women undergoing the ICSI cycle. All females were assessed in the Females' Infertility Clinic. The women ages included were ranged from 18 to 40 years old, which complain of primary and secondary infertility for a period more than < or equal to 25 Kg/m 2) b-Over weight / obese (>25 Kg/m 2).

Control group: Forty women who had neither no signs or symptoms of PCOS, regular periods, or endocrine problems. They were undergoing an ICSI cycle and were divided into two subgroups (20 for each) according to their BMI into: a normal weight (< or equal to 25 Kg/m 2) b-Over weight / obese (>25 Kg/m 2).

The PCOS group (no=40) represent women who suffered from the sign and symptoms of PCOS, including hormonal endocrine disturbance, classical picture of cystic ovarian changes in the U/S, with primary or secondary infertility; that group also

subdivided into two subgroups (20 for each) in according to their BMI as mentioned above. All women undergoing ICSI cycles by antagonist protocol as it is shorter less coast, and patients with PCOS cannot have agonist protocol as it's a possible complication with ovarian hyperstimulation syndrome (OHSS). All subjects received a full clarification of the goal of the study and signed the consent form. The diagnosis of PCOS depends on attain at least two of three criteria, relayed on the Rotterdam ESHRE/ASRMS sponsored PCOS consensus workshop group (Rosenfield et al. 2016[8]).

1-Inclusion criteria

The Women's ages of (18 - 40) years, couple with normal or mild male factors (e.g., Asthenospermia and oligospermia).

2- Exclusion criteria

Endocrine pathology (Hyperprolactinemia, thyroid disease, DM, Cushing syndrome). Congenital malformation of the female genital tract. Women whose follicular fluid was bloody during the oocyte retrieval because it effects on the quality. Cycles with no oocytes (empty follicles) were retrieved on the day of aspiration.

Women age more than 40 years old. 6- Women with serum FSH levels of more than 12 mIU/ml .

3- Female investigation, blood sampling, and hormones assay

The couple underwent a complete history with physical examination in an attempt to find the factors that may be the cause of infertility

All women had been tested for basal serum FSH and LH levels in the second or third day of a spontaneous or induced menstrual cycle using the Mini VIDAS apparatus. Day two serum Resistin and Follistatin was also measurement before the program of ovulation induction. In the day of oocyte retrieval, serum and follicular fluid (FF) also were assembled to measure the two markers.

Hysterosalpingography was done to the patient or if she had it already to exclude Hydrosalpinx or congenital malformations. Vaginal ultrasound scanning is used for screening the morphological appearance of the ovaries with measurement of the ovarian volumes and the antral follicle count (AFC). The uterine assessment for the presence of abnormalities, with the estimation of the endometrial thickness (Ramakrishnan et al. 2023[9]) .

4- Male Evaluation

The male partner evaluation was performed by the urologist in charge at the Male Infertility Clinic. A part from history and physical examination, a complete semen analysis was done to measure sperm concentration, motility, and morphology as recommended by WHO, 2021 (WHO [10]).

5- Controlled ovarian hyperstimulation protocol

Using GnRH antagonist protocol, it was started on the menstruation cycle day (2-3) by Recombinant FSH 75IU in a solvent-prefilled syringe of Follitropin alpha as active ingredient injection, or human menopausal gonadotropin HMG corresponding to 75 IU LH+ 75 IU FSH (menogon®/ menopure®)with menotrophin as active ingredient presented as powder and solvent for injection. They are administrated daily with a dose of (150-300)IU /day. In PCOS patients may decrease the FSH dose to 37.5 IU in sever PCOS cases(more than 20 follicles in each ovary); other non-PCOS patients or older than 35 may start with an HMG dose of 150 – 300. The GnRH antagonist as cetrotide®, as Cetrolix Acetate 0.25mg injection to prevent premature LH surge with a short time and less cost in the stimulation protocol (Demirol & Guven, 2016[11]).

6- Oocyte retrieval

When at least three follicles are 18 mm with serum E2 measured (around 100-150 pg/ml follicle), hCG was given to trigger and induce follicle maturation.

Then, after 34-36 hours of trigger administration, aspiration of oocytes was done by trans-vaginal ultrasound guide.

7- Intra cytoplasmic sperm injection (ICSI) procedure

Aspirated FF was handled to the embryologist to recognize the number and the quality of the retrieved cumulus-oocytes complexes. ICSI was done by the clinical embryologist 3-5 hours after oocyte aspiration in the laboratory. The denudation was performed by exposure to a buffered medium consisting of (40 IU/ml) hyaluronidase for enzymatic removal of corona cells and cumulus (Bonduelle et al.,1999 12]). The oocyte maturation was evaluated by noticing the presence or absence of the germinal vesicle and the first polar body. Next to the ICSI procedure, the oocyte is examined the next day to account for the fertilization rate. The embryo quality and development were checked, according to (Patrat et al.,2012 [13]).

Embryo transfer (ET) was done - 2-9 at day two (4- cells embryo stage), and on day three (6-8 cell embryo stage), Embryo transfer was done according to (Malekkheili et al., 2022[14])

8- Luteal phase

Progesterone therapy is prescribed to all women for the luteal phase support, in the form of Cyclogest (Actavis and Barnstable, UK) ® 200- 400 mg twice/day, or (Crinone, ® 8% progesterone gel, Merk) transvaginal, and IM. Treatment started from the day of oocyte retrieval until the pregnancy test. The pregnancy test was performed after 14 days post-ET. Luteal phase support was continued up to 12 weeks of gestation. Vaginal ultrasound examination was done (6 - 7) weeks after the embryo transfer to confirm clinical pregnancy.

9- Statistical analysis :

Analyzed of data were done by SPSS version 26. The data is presented as mean, standard deviation, and ranges. ANOVA were used to compare the

continuous variables accordingly for normally distributed data. The Chi-square test was utilized to assess the relationship between study groups and ICSI outcomes. ROC curve analysis was used for the determination of follistatin level at day two serum and in FF at the day of OPU for the occurrence of pregnancy after ICSI. Pearson's correlation test (r) was used to assess the correlation between continuous variables accordingly. Pearson correlation was utilized. The significance level was set at $p \leq 0.05$.

3. Results

1- Correlation of resistin and follistatin levels with certain demographic Characteristics and BMI:

Age and BMI: Correlation between resistin and follistatin levels with age and BMI is shown in **(Table 1)**. Positive correlations were detected between age and both of resistin follicular fluid at OPU (r= 0.265, P= 0.022) and serum follistatin level at OPU (r= 0.285, P= 0.01). Whereas no statistically significant ($P \geq 0.05$) correlations were detected between all other parameters.

2- Hormonal level: Correlation between resistin and follistatin levels with FSH and LH was shown in **(Table 2)**. Positive correlation was detected between LH and serum follistatin level at OPU (r= 0.279, P= 0.03). However, there was no statistically significant correlations detected between all other parameters ($P \geq 0.05$).

3- Comparison of resistin and follistatin levels between the four study groups

(Table 3) shows the comparison of resistin and follistatin levels between the four study groups. Median of serum resistin at day two serum and at day of OPU (serum & FF) and the mean of follistatin in serum at day two and day of OPU were significantly higher in terms of median Resistin:(4145.69, 6485.92, and 5831.38), and in term of Mean follistatin (15.12 ± 8.6and 19.29

± 7.9) respectively in patients with PCOS and
normal BMI level compared to other study groups. At the same time, the statistical analysis did not find significant (P ≥ 0.05) differences regarding follistatin level in the follicular fluid between study groups.

3- Comparison of resistin and follistatin levels according to ICSI outcome

The comparison of resistin and follistatin levels according to ICSI outcome is shown in **(Table 4).** Means of follistatin at day two and in FF were significantly higher (P =0.037 and 0.044, respectively) in participants who got pregnant than that in participants who didn't get pregnant.

4- Receiver operating characteristic

Receiver operating characteristic (ROC) curve analysis was constructed for follistatin level at day two and in FF as predictors for the occurrence of pregnancy after ICSI. The cutoff point of the follistatin level at day two and in FF were 9.89 ng/ml and 15.06 ng/ml, respectively. Hence, follistatin level at day 2 > 9.89 ng/ml and in FF > 15.06 ng/ml are predictors for the occurrence of pregnancy after ICSI, as a large significant area under the curve (AUC= 69.4% and 68.1% respectively), indicating a strong correlation between the high levels of follistatin at day two and in FF with getting pregnant. Follistatin level in the serum at day 2 was 55.6% sensitive, 81.8% specific, and 72.5% accurate, while, in FF was 77.8% sensitive, 67.9% specific, and 71.3% accurate; in predicting the occurrence of pregnancy after ICSI. **(Table 5)** and **(Figures 1 & 2)**

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Table (1): Correlation between resistin and follistatin levels with age and BMI

Hormonal aspects	**Age (Year)**		**BMI (kg/m2)**	
	R	**P - Value**	**R**	**P – Value**
Resistin (ng/L)				
At Day 2-serum	0.095	0.402	0.17	0.132
At OPU- FF	0.265	0.022	- 0.134	0.237
At OPU-serum	0.009	0.937	- 0.173	0.124
Follistatin (ng/ml)				
At Day 2-serum	0.172	0.128	0.015	0.895
At OPU- FF	0.065	0.566	0.114	0.313
At OPU-serum	0.285	0.01	- 0.014	0.902

Table (2): Correlation between resistin and follistatin levels with FSH and LH

Hormonal aspects	**FSH (mIU/mL)**		**LH (mIU/mL)**	
	R	**P - Value**	**R**	**P - Value**
Resistin (ng/L)				
At Day 2-serum	- 0.044	0.74	- 0.123	0.35
At OPU- FF	- 0.159	0.224	0.09	0.492
At OPU-serum	- 0.013	0.924	- 0.029	0.827
Follistatin (ng/ml)				
At Day 2-serum	0.173	0.186	0.237	0.068
At OPU- FF	- 0.017	0.897	0.104	0.431
At OPU-serum	0.09	0.492	0.279	0.03

Table (3): Embryo characteristic of infertile women participating in the present study

Hormonal Levels in FF and Serum	**Study groups**				**P–Value**
	A PCO + >25 kg/m$_2$	**B PCO + <25 kg/m$_2$**	**C Non PCOS +>25 kg/m$_2$**	**D Non PCOS +<25kg/m$_2$**	
Resistin (ng/L)	Median	Median	Median	Median	
At Day 2-serum	1519.77	4145.69	3935.11	1375.4	**0.024 ***
FF (OPU)	2104.88	6485.92	2964.57	4044.63	**0.002**
Serum (OPU)	2141.43	5831.38	2582.32	1574.48	**0.027 ***
Follistatin (ng/ml)					
At Day 2-serum	9.73 ± 7.0	15.12 ± 8.6	7.97 ± 4.0	6.22 ± 5.9	**0.002****
FF (OPU)	18.67± 9.2	23.86 ± 17.9	16.4 ± 12.7	13.83 ± 6.4	**0.126 ****
Serum OPU)	12.57 ± 7.5	19.29 ± 7.9	11.77 ± 7.8	8.76 ± 6.6+	**0.003 ****

* Kruskal-Walli's test, ** One-way ANOVA test FF: follicular fluid

Table (4): Comparison of resistin and follistatin levels in pregnant and non-pregnant women following ICSI program

Hormonal Levels in FF and Serum	**Pregnancy status**		**P – Value**
	Pregnant	Not pregnant	
Resistin (ng/L)	Median	Median	
At Day 2-serum	2771.02	2010.14	**0.787 ***
FF (OPU)	2540.98	2964.57	**0.915 ***
Serum (OPU)	2868.15	2206.61	**0.673 ***
Follistatin (ng/ml)	Mean ± SD	Mean ± SD	
At Day 2-serum	11.63 ± 7.5	8.06 ± 6.1	**0.037 ****
FF (OPU)	21.74 ± 13.0	15.67 ± 11.0	**0.044 ****
Serum (OPU)	13.69 ± 7.2	12.02 ± 8.5	**0.36 ***

* Mann-Whitney U test * Student t-test

Table (5): Diagnostic accuracy of follistatin level at day two and in FF as a predictive marker for occurrence of pregnancy after ICSI

Follistatin level	**Cut-off value**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
At day 2 (ng/ml)	9.89	55.6%	81.1%	60%	78.2%	72.5%
FF at OPU (ng/ml)	15.06	77.8%	67.9%	55.3%	85.7%	71.3%

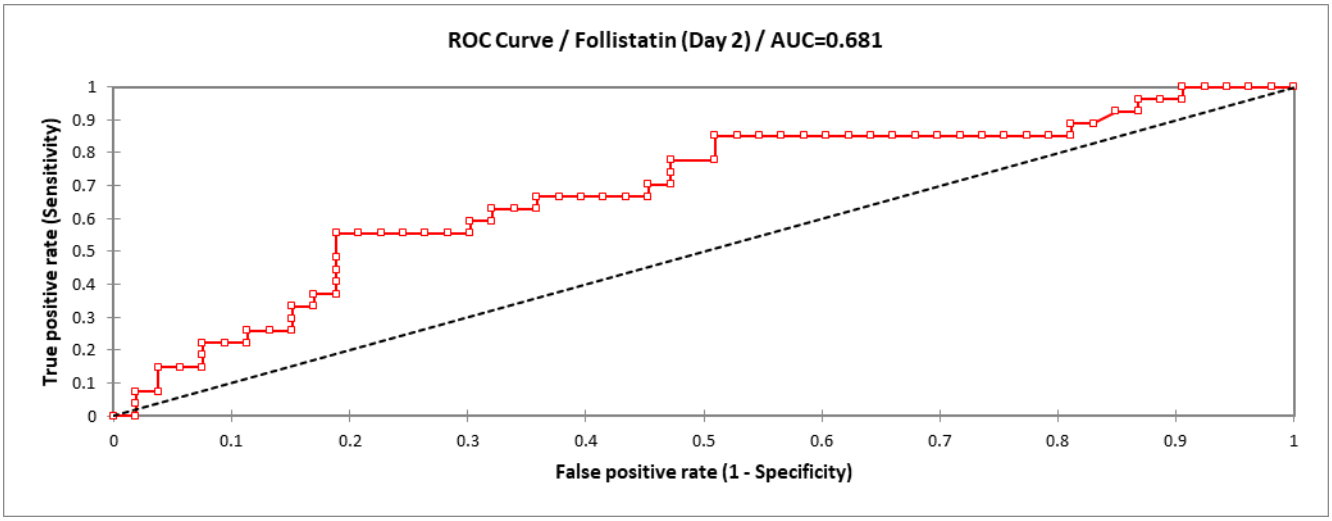

Figure (1): ROC curve for follistatin level at day two as a predictive marker for occurrence of pregnancy after ICSI

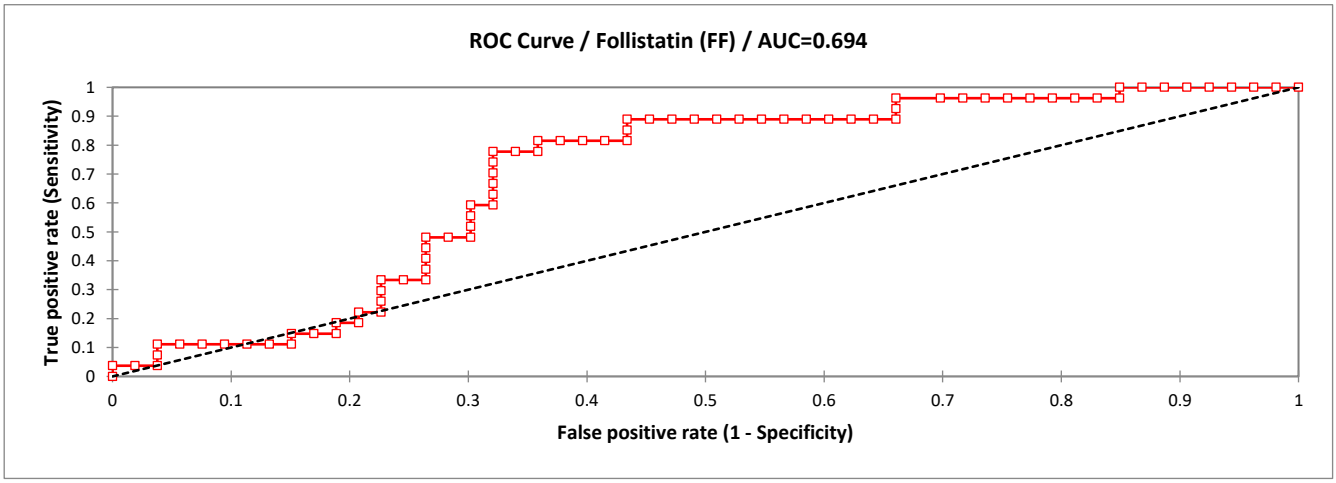

Figure (2): ROC curve for follistatin level in FF at OPU as a predictive marker for the occurrence of pregnancy after ICSI.

4. Discussion

1- Correlation of resistin and follistatin levels with different factors

Regarding to correlation with age and BMI, In the current study, positive correlations between age and both follistatine blood level and resistin follicular fluid **(Table 1),** while no

significant correlations were detected between all other parameters. One possibility for the lack of a strong association between resistin and obesity could be that in humans, macrophages rather than adipocytes are the major sources of resistin, and it is related more to other factors such as IR **(Patel et al.,2003[15])**.

Serum follistatin may act as a potential screening biomarker of PCOS regardless of age and/ or BMI. In a study done by **(Wakatsuki et al. [16])** on different age groups of women and then on those who became pregnant, they found that there was a strong correlation between age, pregnancy, and follistatin, as FSH level increased with age and has a correlation with activin/follistatin, also considering activin is present in the reproductive system .

For correlation with hormonal levels (FSH and LH) as, the current study revealed a positive correlation between LH and follistatin blood level, while no significant correlations were detected Between all other parameters **(Table 2).**

Although there was no evidence of a positive or negative impact of resistin on pregnancy outcomes, it is possible that in PCOS women, the hormone may have a detrimental effect that is the same as the effect of LH. Additionally, it is unknown whether giving a GnRH analogue for ovulation induction lowers the levels of follicular resistin and whether aspirating the follicular fluid first would be preferable **(Seow et al., 2005[17])**. In fact, the structure and function of growing follicles depend on LH. The LH peak enhances the ovum to resume meiosis and reach final maturation by blocking the ovum maturation inhibitors in the early phases of normal menstruation **(Wang et al., 2022 [18])**.

2-Comparison of resistin and follistatin levels between the four study groups

In this study **(Table 3),** patients with PCOS and a normal BMI had significantly higher median serum resistin levels on days two of serum and day of OPU serum and FF, that's to say it is more significant. In regarding to resistin, it is seen that morbidly obese people express more resistin in their adipocytes than lean people do; there may be a correlation between IR and obesity (Savage et al., 2001[19]; Zhang et al.,2011[20]). A study done by (Baldani et al., 2019[21]) clearly demonstrated a significant alteration in the secretion of resistin in lean PCOS patients compared to healthy subjects. Therefore, it is likely that other factors, independent of obesity, affect serum levels resistin in Chapter Five Discussion 83 women with PCOS, and that agrees with the current study in which PCOS with normal BMI had the highest level.

As for the next hormone, in return to our table, the Mean of serum follistatin in day two and day of OPU were higher in PCOS with a normal BMI group. An increase in follistatin may arrest follicular development and increase androgen production, and these features are seen in PCOS patients. It has been reported that circulating levels of resistin and follistatin were higher in women with PCOS compared to non-PCOS, regardless of obesity status, indicating that these adipokines might be contributing to the pathology of PCOS (Raeisi et al., 2021[4]). At the same time, the statistical analysis did not find a significant differences regarding the levels of resistin and follistatin in FF between study groups. The same finding was observed by other study (Steppan and Lazar, 2002 [22]). The rapid clearance of resistin and follistatin into the blood caused by thecal secretion of these adipokines and the abundant blood supply in this specific site of the ovary could explain this finding (Norman et al., 2001 [23]). Furthermore, it has been suggested that the high levels of resistin and follistatin in women with PCOS may have extragonadal origins were unrelated to

obesity status (Pangaribuan et al., 2011 [24]).

3-Comparison of resistin and follistatin levels according to ICSI outcome Regarding ICSI outcome **(table 4),** means of follistatin in FF and at day-2 were significantly higher in those who get pregnant, while no differences in serum follistatin, FF, and serum resistin, and at day-2 between those pregnant and those who didn't get pregnant. It has been stated that serum and FF resistin levels in women with PCOS who went through ICSI had no significant difference in women who became pregnant and those who did not (Seow et al.,2005[17]). In a study done by (Al-Dujaily et al., 2016 [25]) about the effect of Activin A, Follistatin, and fibrillin-3 Hormones on pregnancy rate in IVF programs, they found that elevation in serum follistatin (day 2-3) in pregnant women and continue to increase even more in in luteal phase and such finding in agreement to the result of the current study. The same observation was reported when the researcher found that the behavior of follistatin levels was exactly opposite that of AMH levels, and the interest finding when follistatin levels increased significantly during pregnancy and on the first day after parturition but declined afterwards, whereas AMH levels decreased significantly during pregnancy and increased after labor **(Koninger et al.,2017 [26]) .**

During pregnancy, follistatin levels tend to increase due to the high demand for growth and development of the fetus; it helps to regulate the action of with activin, which are involved in various aspects of pregnancy, such as implantation, where the fertilized egg attaches to the uterus. It helps to regulate the activity of activin, which are involved in the development of the endometrial lining for implantation, placental development, and fetal growth (Hameed et al., 2020 [27]).

4 -Receiver operating characteristic

Analysis of the ROC curve that shown in **(Table 5, figure 1&2)** constructed

follistatin level at day 2 > 9.89 ng/ml and in FF > 15.06 ng/ml are predictors for the occurrence of pregnancy after ICSI, as a large significant area under the curve (AUC= 69.4% and 68.1%, respectively) showing a significant association between the higher levels of follistatin at day two and in FF with getting pregnant. Follistatin level at day 2, it was 55.6% sensitive, 81.8% specific, and 72.5% accurate, while in FF was 77.8% sensitive, 67.9% specific, and 71.3% accurate; in predicting the occurrence of pregnancy after ICSI. It has been stated that follistatin could be showed promising diagnostic performance, and it may be considered for further studies to create a single PCOS screening method that is independent of the other diagnostic criteria with acceptable sensitivity and specificity.

6- Conclusion

Resistin and pregnancy rate did not correlate; the cutoff point of follistatin level in the serum at day two and in FF at the day OPU was found to be a predictor of pregnancy following ICSI. Resistin and follistatin may be helpful indicators in PCOS.

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Author Contribution

Alyaa S. Mohammed performed the study, and Wasan Al-Jubori Saad Al-Dujaily supervised the work .

Conflict of Interest

The authors declare no conflict of interest .

Ethical Clearance

The study was approved by the Ethical Approval Committee.

Financial Disclosure

There is no financial disclosure.

Ethical Clearance

The study was approved by the Ethical Approval Committee

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