

# Follicular Glypican-1 As A Predictive Marker Of Reproductive Outcomes In Women Undergoing IVF/ICSI

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## ABSTRACT

**Objectives:** The current study aims to determine the relationship between the follicular fluid Glypican-1 levels and the reproductive outcomes in women underwent an In Vitro Fertilization and Intracytoplasmic Sperm Injection (IVF/ICSI).

**Methods:** The current prospective study includes fifty infertile women who are undergoing IVF/ICSI treatment. Follicular fluid samples are collecting from ovarian follicles for the determination of Glypican-1 levels. Enzyme-linked immunosorbent assays (ELISA) is used to evaluate the expression of Glypican-1 levels. The relationship between follicular Glypican-1 levels and IVF/ICSI outcomes is assessing.

**Results:** There is a significant difference ( $p < 0.05$ ) between the follicular Glypican-1 levels in patients who are achieving clinical pregnancy and patients who are remaining non-pregnant ( $15.3667 \pm 5.41264$ ) and ( $22.8171 \pm 9.31019$ ), respectively.

**Conclusion:** The results of the current study suggest that the levels of follicular fluid Glypican-1 could be a useful predicting marker of IVF/ICSI treatment outcomes.

**Keywords:** Follicular fluid, oocytes, Glypican-1, IVF/ICSI.

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## INTRODUCTION

Follicular fluid (FF) produces in the follicles, contains several metabolites that play a crucial role in the follicular growth and development as well as in the maturation of oocytes. The biochemical compositions of FF reflect the functional state of the follicles and the oocyte's competence that influence the oocyte quality, fertilization, and embryo development. (1,2,3) Several studies indicated the microenvironment of FF in the human oocyte as a crucial factor for the oocyte's developmental competence; and a medium that ensures oocyte maturation. Thus, FF plays a role in the development and implantation of early embryos. (1,4)

A successful pregnancy prediction is desiring of both clinicians as well as infertile women who underwent IVF/ICSI treatment. Several sera, as well as FF markers, have been investigated concerning the prediction of pregnancy outcomes. (5) The metabolomics analysis of FF may contribute to determining predictive markers of follicle development as well as oocyte quality before fertilization, to predict the assisted reproductive technology success. (3) Glypican-1 (GPC-1) belongs to the proteoglycans family (Glypicans), attaching to three heparan sulfate (HS) chains. Proteoglycans are highly expressing proteins on the extracellular matrix, as well as the cell membrane, connecting to the cytoplasmic membrane via an exocyttoplasmic domain, anchoring by a glycosylphosphatidylinositol Glypicans via extracellular growth signals, modulating several biological processes, including morphogen gradient, organ development, and human overgrowth. (6,7) They are participating in the regulation of growth factors, adhesions, cellular proliferation, differentiation, as well as morphogenesis development. (8,9,10,11,12) Glypicans may modulate the angiogenesis branching via the enhancement of endothelial cell proliferation and controlling the expression of the angiogenic molecule. (13)

Glypican-1 expresses in most adult tissues, as well as normal ovary. (14) Acts through binding the proteins, growth factors, as well as cytokines, via the side chains of heparan sulfate. (6,15,16)

The current study describes the first study investigating the relationship between the FF Glypican-1 levels and the IVF / ICSI outcomes.

## MATERIALS AND METHODS

The current prospective study includes 50 infertile patients, who are attending the Kamal Al-Samarra'ay Specialized Hospital for IVF/ICSI treatment, in February 2019 - August 2019. All participants were informed of the study, obtaining their informed written consent. The study included infertile patients who have male and female etiology, such as male infertility, ovulatory dysfunction, fallopian tube obstruction, and idiopathic factors. The study excluded patients with liver, kidney, heart diseases, and endocrine disorders.

### Controlled ovarian hyperstimulation

All patients underwent control ovarian hyperstimulation, a long-acting GnRH agonist regimen started at the 21<sup>st</sup> day of the preceding cycle, followed by gonadotropins injections at the later 14 days to ensure pituitary down-regulation, doses adjusted according to follicle growth monitored by ultrasound; when at least 3 follicles with a diameter 18 mm are detected, an injection of human chorionic gonadotropin (hCG) performed subcutaneously 30 - 40h after the last FSH injections.

### Follicular fluid sampling and Oocyte retrieval

Approximately 34 to 36 hours after hCG administration, follicles  $\geq 17$  mm diameter retrieved under vaginal ultrasound guidance. FF collected and centrifuged to exclude blood cells and impurities, then stored at  $-70$  °C for assessment of the GPC1 and other hormone levels.

### IVF/ICSI treatment and Oocytes assessment

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The laboratory clinical embryologist has performed the procedure of IVF/ICSI. The oocyte maturity status was determined 20-30 minutes after oocyte collection. The Oocytes were assessed as a germinal vesicle (GV), prophase I, and stage II metaphase (MII). The presence of the first polar body indicated oocyte maturity (MII). (13) And the mature oocytes MII were inseminated .

### Fertilization, Embryo culture, and Embryo transfer

After IVF / ICSI performance, oocytes placing in cell culture, and evaluating results of fertilization within 16-20h. Fertilization rate and embryo formation are determining. Embryos are grading as Grade I and II embryos (good quality) with  $\leq 20\%$  cytoplasmic fragmentation and with uniform blastomeres. Grade III and IV embryos (poor quality) with  $\geq 50\%$  fragmentation of cytoplasm and unequal blastomeres. On days 2 to 3 of embryonic development, transferring fresh embryos is performing. Determining the number of transferring embryos is basing on the quality of embryo and the couple's recommendation.

### Follicular Fluid GPC1 Determination

All follicular fluid samples are examining on the same day for measuring the follicular GPC1 levels, by using a commercially available ELISA kit, Human Glypican 1 (GPC1) ELISA kit; H CUSABIO. According to the manufacturer's instructions, the sensitivity is  $< 0.39$  ng / ml, and the sample volume is 50-100 $\mu$ l with a detection range between 1.56ng/ml-100ng/ml.

### Statistical analysis

This study is a prospective study design with an analytical aspect. SPSS version 25.0 is using for the statistical analysis. Data analysis is using an unpaired t-test of association

(bivariate analysis) for evaluating the IVF/ICSI pregnancy outcome variable with the patients' demographics and hormonal data as independent variables. Independent variables with a significance level of  $P < 0.05$  in the bivariate analysis are furtherly including in the multivariate analysis of the Binary logistics regression analysis technique for determining the significant predictors associated with IVF/ICSI pregnancy success, as well as for eliminating the effect of confounding factors on the outcome variable.

### RESULTS

Table 1 was summarized the clinical characteristics of patients who underwent controlled ovarian hyperstimulation. According to clinical pregnancy outcomes, patients were divided into pregnant and non-pregnant groups. Clinical pregnancy was positive in 16 patients, while it was negative in 34 patients. Based on the analysis of data, the current results indicating there was a highly significant difference ( $p < 0.001$ ) between the ages of both groups, with an average age of both groups, was  $(25.07 \pm 4.026)$  and  $(30.13 \pm 4.704)$ , respectively.

Based on an unpaired t-association test (bivariate analysis), the current study indicating there was a significant difference ( $p < 0.05$ ) between several explanatory variables in both groups, including the number of the retrieval oocytes, endometrial thickness, fertilization rate ratio, FSH, mature oocytes MII, Grade I and Grade II embryos, as well as the number of transferred embryos. Additionally, there was a significant difference ( $p < 0.05$ ) in the level of FF GPC1 in patients who had a clinical pregnancy compared to non-pregnant patients  $(15.3667 \pm 5.41264)$   $(22.8171 \pm 9.31019)$  respectively. While other parameters, including the duration of infertility, LH, LH/FSH ratio, and the GV stage, showed no significant difference in both groups.

**Table 1 Clinical characteristics of patients undergoing IVF/ICSI-ET**

| Characteristic<br>(Mean $\pm$ SD) | Pregnant<br>(n= 16)   | Non-pregnant<br>(n= 34) | Significance     |       |              |
|-----------------------------------|-----------------------|-------------------------|------------------|-------|--------------|
|                                   |                       |                         | Mean differences | t     | P*           |
| Age (years)                       | 25.07 $\pm$ 4.026     | 30.13 $\pm$ 4.704       | 5.076            | 3.642 | <b>0.001</b> |
| Duration of infertility (yrs.)    | 4.80 $\pm$ 1.656      | 4.94 $\pm$ 1.714        | 0.143            | 0.273 | 0.786        |
| Serum E2 (pg/ml)                  | 3.8107 $\pm$ 4.88087  | 2.6907 $\pm$ 4.14900    | 1.11095          | 0.829 | 0.411        |
| LH (pmol/l)                       | 6.6067 $\pm$ 3.19183  | 6.6829 $\pm$ 2.49098    | 0.07619          | 0.091 | 0.928        |
| FSH (mIU/ml)                      | 4.7133 $\pm$ 2.07532  | 6.4943 $\pm$ 2.42134    | 1.78095          | 2.481 | <b>0.017</b> |
| LH/FSH ratio                      | 1.5840 $\pm$ 0.97516  | 1.1584 $\pm$ 0.63678    | 0.42567          | 1.836 | 0.073        |
| GPC1 in FF (ng/ml)                | 15.3667 $\pm$ 5.41264 | 22.8171 $\pm$ 9.31019   | 7.45048          | 2.887 | <b>0.006</b> |
| MI oocytes                        | 3.8667 $\pm$ 1.68466  | 2.9429 $\pm$ 1.02736    | 0.92381          | 1.972 | 0.064        |
| MII oocytes                       | 5.4000 $\pm$ 2.13140  | 3.6286 $\pm$ 1.53557    | 1.77143          | 3.317 | <b>0.002</b> |
| GV stage                          | 1.7333 $\pm$ 1.032280 | 1.7714 $\pm$ 0.87735    | 0.03810          | 0.133 | 0.894        |

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|                            |                   |                  |         |       |              |
|----------------------------|-------------------|------------------|---------|-------|--------------|
| Endometrial thickness (mm) | 9.85 ± 0.904      | 9.28 ± 0.868     | 0.570   | 2.101 | <b>0.041</b> |
| Retrieved oocytes (n)      | 11.2667 ± 3.95450 | 8.4000 ± 2.43986 | 2.86667 | 3.135 | <b>0.003</b> |
| Fertilization rate (%)     | 59.33 ± 11.169    | 47.57 ± 10.667   | 11.762  | 3.525 | <b>0.001</b> |
| Grade I embryos (n)        | 3.67 ± 1.345      | 2.23 ± 1.352     | 1.438   | 3.451 | <b>0.001</b> |
| Grade II embryos (n)       | 2.40 ± 0.986      | 1.77 ± 0.770     | 0.629   | 2.428 | <b>0.019</b> |
| Transferred embryos (n)    | 2.07 ± 0.258      | 2.40 ± 0.604     | 0.333   | 2.049 | <b>0.046</b> |

\* *P* value of unpaired *t*-test. IVF/ICSI-ES, in vitro fertilization intracytoplasmic sperm injection- embryo transfer; FF, follicular fluid, E2; Estradiol, LH, luteinizing hormone; FSH, follicle-stimulating hormone; LH/FSH ratio; luteinizing hormone/ follicle-stimulating hormone ratio. MI, prophase I, MII, metaphase II stage GV; germinal vesicle, and GPC1; Glypican-1.

Based on a statistical significance criterion of less than 0.05 in the current study, the results indicating that only the FF GPC1 was affecting the outcomes of IVF / ICSI pregnancy. Since the nine explanatory variables by bivariate (Unpaired *t*-test) analysis were significantly associated with the IVF/ICSI pregnancy outcomes, further they evaluated by multivariate analysis. These nine explanatory (independent) variables were entered simultaneously in binary logistic regression by using Enter Method.

The output was indicating there was a significant difference between the full model as well as the null model = 23.896, *P* < 0.05. The model had a sensitivity of 60% and a specificity of 91.4%, with an overall predictive accuracy of 82%. The

**Table 2 Predictors for IVF/ICSI-ET pregnancy success by logistic regression**

| Explanatory Variable       | B      | S.E.  | Walid | df | sig          | Exp(B)   | 95% C.I.        |
|----------------------------|--------|-------|-------|----|--------------|----------|-----------------|
| Age (years)                | -0.084 | 0.139 | 0.361 | 1  | 0.548        | 0.920    | (0.700 - 1.208) |
| FSH (mIU/ml)               | -0.258 | 0.225 | 1.316 | 1  | 0.251        | 0.773    | (0.498 - 1.200) |
| MII oocytes                | -0.150 | 0.462 | 0.106 | 1  | 0.745        | 0.861    | (0.348 - 2.129) |
| Endometrial thickness (mm) | -0.641 | 0.791 | 0.656 | 1  | 0.418        | 0.527    | (0.112 - 2.485) |
| Retrieved oocytes          | 0.362  | 0.425 | 0.726 | 1  | 0.394        | 1.436    | (0.625 - 3.301) |
| Fertilization rate (%)     | 0.074  | 0.061 | 1.467 | 1  | 0.226        | 1.077    | (0.955 - 1.215) |
| Grade I embryos (n)        | 0.110  | 0.603 | 0.033 | 1  | 0.855        | 1.116    | (0.342 - 3.641) |
| Grade II embryos (n)       | -0.697 | 0.865 | 0.650 | 1  | 0.420        | 0.498    | (0.091 - 2.712) |
| Transferred embryos (n)    | -0.841 | 1.296 | 0.421 | 1  | 0.517        | 0.431    | (0.034 - 5.474) |
| GPC1 protein in FF (ng/ml) | -0.133 | 0.067 | 3.955 | 1  | <b>0.047</b> | 0.875    | (0.768 - 0.998) |
| Constant                   | 7.541  | 8.220 | 0.842 | 1  | 0.359        | 1884.448 |                 |

### DISCUSSION

Several studies recognized the FF as a significant environment of oocytes development, influenced fertilization, and embryonic development. (17,18) FF is a significant source of non-invasive biomarkers for predicting the quality of oocyte and embryo. (19)

Glypican-1 continues to be well interesting in the research field to elucidate their mechanistic roles as well as diagnostic value in the clinical settings. (6)

Few studies evaluate the role of FF GPC-1 to elucidate their effect on the results of assisted reproductive technology. And this is the first report that evaluates the expression of FF GPC-1 in infertile women who received IVF/ICSI treatment as well as their association with the pregnancy outcome.

Based on the clinical characteristics of the patients, the current study shows there is a significant difference (*p* < 0.05) between the level of FF GPC1 in patients who are achieving clinical pregnancy and patients who are remained non-

fit of the model was verified furtherly by the fittest of Hosmer- Lemeshow goodness. Multicollinearity was also checked with no violation detection.

The Logistic regression coefficient, Wald test, and the odds ratio were represented by the Exponential coefficient “Exp (B)” for each of the predictors is revealed in (Table 2).

The logistic regression output showed an inverse contribution of the follicular GPC1 level to IVF / ICSI pregnancy outcomes, as a reduction of one unit of follicular GPC1 protein was a predicted to have a substantial increase of 0.875 times of a positive IVF / ICSI outcome, after a modification of the other explanatory variables included in the model (OR= 0.875; 95% CI: 0.768 - 0.998) (*P* < 0.05).

pregnant, (15.3667 ± 5.41264) and (22.8171 ± 9.31019) respectively.

One study demonstrated during the periovulatory period, GPC-1 could improve the localized signals and might restrict the GDF9 diffusion in the cumulus. Proteoglycan was upregulated rapidly by the cumulus cells rather than the granulosa cells. Consequently, this depletion of GPC1 from in vitro matrix (IVM) is compatible with a deficiency in components of the extracellular matrix (ECM). (11)

Another study identified distinct proteoglycan expression profiles on the cell membrane and the extracellular matrix of the ovary versus ovarian tumors. The study showed that the normal ovary expressed all of the HSPGs. (14)

Moreover, few studies demonstrated the GPC-1 expression in a histochemical ovarian section. During the preovulatory process, significant alteration of proteoglycans was showed during follicular development and atresia. Proteoglycan concentration was higher in membrana granulosa and FF of the atretic follicles. The upregulation of proteoglycans in

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atretic follicles than in healthy follicles might indicate the indirect contribution of proteoglycans in apoptosis of granulosa cells. (20)

Thus, the low level of Glypican-1 in healthy follicles may explain our findings which demonstrated that follicular GPC1 levels are significantly lower in patients who had a clinical pregnancy versus non-pregnant patients who underwent controlled ovarian hyperstimulation, with 60% sensitivity, 91.4 % specificity, and with a predictive accuracy of 82%. Additional studies will be required to respond to this observation.

Our data suggested that the follicular GPC1 concentration may play a role in follicular growth as well as oocyte maturation via attaching of a core protein to the glycosaminoglycan chains. Glycosaminoglycan (GAG) chains are required for the cell differentiation and may have a possible role in ovarian physiology through their involvement in a matrix formation, cell-cell and cell-matrix adhesion, cell proliferation; migration, and co-receptor activity for growth factors. (6,20)

In conclusion, our findings suggested that individuals with low follicular GPC1 levels were associated with a positive result of IVF / ICSI treatment. Follicular GPC1 could be useful as a predictive marker of IVF / ICSI outcome.

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