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# The Effect of Letrozole and N-Acetylcysteine on the Expression Levels of Genes Involved in Glucose Metabolism in Patients with Polycystic Ovary Syndrome: A Clinical Trial Study

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## Abstract

**Background:** N-acetylcysteine (NAC) is a supplement commonly used in patients with polycystic ovary syndrome (PCOS). The expansion of oocyte-associated cumulus cells (CCs) and the quality of the oocyte are critical factors influencing fertilization rates and clinical pregnancy outcomes in assisted reproductive techniques (ARTs). Genes such as phosphofructokinase (PFKP) and pyruvate kinase isoform M2 (PKM2) are involved in glucose metabolism and are crucial in the regulation of oocyte competence and developmental potential. The purpose of the current study was to evaluate the effects of letrozole and NAC on the expression of PFKP and PKM2 in CCs of PCOS patients undergoing ART.

**Methods:** The study evaluated 20 PCOS women undergoing ART to assess the effect of letrozole and NAC on the expression levels of PKM2 and PFKP genes in cumulus cells. Women were randomly assigned using a simple randomization method into four groups: control, NAC, letrozole, and NAC plus letrozole, with five women in each group. Gene expression levels of PKM2 and PFKP were measured using real-time PCR.

**Results:** The expression level of PKM2 was significantly higher in the letrozole plus NAC group compared to the control group ($p<0.05$). In NAC group, PFKP was significantly expressed compared to the control group ($p<0.05$). There were no significant differences among the other groups compared to the control group.

**Conclusion:** NAC can improve the quality of oocytes by increasing the expression level of genes involved in the glucose metabolism (PKM2, PFKP) of CCs, thereby potentially improving ART success rate in PCOS patients. Therefore, administering NAC along with letrozole can have a synergistic effect on increasing the expression level of genes associated with blastocyst quality in PCOS patients.

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## Introduction

More than 186 million people suffer from infertility worldwide (1). PCOS is a common endocrine disorder that contributes to infertility in a large number of women (2). According to the Rotterdam criteria, 6–21% at women of reproductive age are affected by PCOS (3). Body

mass index (BMI), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin concentrations are higher in PCOS patients compared to healthy individuals. Increased LH levels lead to increased androgen levels, which contribute to the development of PCOS (4).

Among other factors, chronic inflammation and oxidative stress contribute to PCOS. Additionally, elevated levels of oxidative markers exacerbate the pro-inflammatory state in women with PCOS (5).

Infertility affects approximately one in five couples of reproductive age, making it a significant global health issue. ARTs, such as frozen embryo transfer (FET), have become essential in clinical practice, allowing the reduction of the number of embryos transferred and minimizing the risk of multiple pregnancies (6). Antioxidants play a critical role in ART by neutralizing free radicals and mitigating oxidative stress, which can compromise oocyte quality and embryo development (7).

PCOS, a common endocrine disorder, contributes significantly to female infertility. Obesity, frequently associated with PCOS, may lead to additional complications, including anemia (8). Effective treatment requires medications that can modulate oxidative stress and inflammatory markers. Letrozole and NAC are two agents commonly used to improve ovulation and oocyte quality in women with PCOS (9). NAC, a potent antioxidant, enhances total antioxidant capacity by increasing glutathione levels, scavenges free radicals such as hydrogen peroxide and superoxide, inhibits the release of pro-inflammatory cytokines including TNFα and interleukins, and regulates insulin secretion and receptor function (10–12). It can also optimize *in vitro* conditions during follicular development, oocyte maturation, and cryopreservation, thereby improving oocyte maturation and survival (13).

Letrozole, an aromatase inhibitor similar to clomiphene, reduces estrogen biosynthesis and consequently decreases hypothalamic-pituitary axis inhibition. This elevates FSH secretion, promoting follicular development. Letrozole offers advantages such as a lower risk of multiple pregnancies during IUI, improved endometrial thickness, a shorter half-life (~45 *hr*), and fewer adverse effects on estrogen-sensitive tissues compared to clomiphene. Clinical studies report ovulation induction rates of 70–84%, pregnancy rates of 20–27%, and an average endometrial thickness of 7.9 *mm* following treatment (14-16).

Ovarian follicle development and ovulation are tightly coordinated processes requiring the mutual interaction of oocytes and their surrounding CCs for proper growth and maturation (17). CCs support oocyte meiosis to metaphase II, provide metabolites such as pyruvate and lactate via glycolysis, and communicate with oocytes through gap junctions, establishing a two-way functional relationship essential for fertilization and embryo development (18–25). Damage to CCs impairs oocyte metabolism, reducing fertilization potential and subsequent embryo developmental competence (10, 18, 19). The functional status of CCs serves as a reliable, non-invasive indicator of oocyte quality (26, 27).

CC expansion is essential for proper oocyte maturation, ovulation, and fertilization, and is tightly regulated by key glycolytic genes, including PFKP and PKM2, which modulate the metabolic support required for oocyte developmental competence. The expression levels of these genes in CCs serve as biomarkers reflecting oocyte developmental potential, linking gene expression related to glycolysis and cumulus expansion with oocyte growth and maturation (20, 28).

Women with PCOS often exhibit an oxidant-antioxidant imbalance due to metabolic disorders such as obesity, hyperinsulinemia, and dyslipidemia, which increase reactive oxygen species (ROS) production (29, 30). Excessive ROS can activate TNFα and nuclear factor-kappa B (NF-κB), leading to inflammatory responses that stimulate ovarian androgen production and exacerbate hyperandrogenism (31, 32).

The present study aimed to investigate the effects of NAC and letrozole on the expression of genes involved in glucose metabolism, specifically PFKP and PKM2, in CCs of women with PCOS. If these agents, particularly NAC recognized for its cost-effectiveness and favorable safety profile, successfully modulate the expression of these genes, their optimized application could enhance oocyte developmental competence and ultimately improve ART outcomes.

## Methods

***Study design:*** A randomized, non-blinded clinical trial was conducted on 20 cumulus cell mass samples collected from patients at Al-Zahra Hospital, Tabriz, Iran, following written informed consent. The study was approved by the Ethics Committee (IR.TBZMED.REC.1401.511) and registered in the Iranian Registry of Clinical Trials (IRCT

20221019056244N1). All procedures adhered to national and institutional ethical guidelines, including the "Ethics in Laboratory Studies" workshop (Code: TBZMEDE142-4463).

***Patient selection:*** PCOS patients were referred to the IVF Department of Al-Zahra Hospital and selected based on the Rotterdam criteria, requiring at least two of the following: oligo- or anovulation, clinical or biochemical hyperandrogenism, or polycystic ovarian morphology on ultrasound. Additional inclusion criteria included normal thyroid and prolactin function, non-smoking status, normal semen analysis of the male partner, and a history of at least one year of infertility. Patients with thyroid or prolactin dysfunction, uterine abnormalities, ovarian cysts larger than 6 *cm*, asthma, use of medications affecting glucose metabolism, or smoking were excluded. Hormonal evaluations, including thyroid-stimulating hormone (TSH) and prolactin, as well as diabetes screening and lipid profile assessments, were conducted, and patients with abnormal results were referred to an endocrinologist and excluded from the study.

***Randomization and group allocation:*** Patients were randomly assigned using simple randomization via the RAND function in Microsoft Excel into four groups to evaluate the individual and combined effects of letrozole and NAC on gene expression and reproductive outcomes. The control group received standard treatment consisting of gonadotropins and metformin, while Group 1 received gonadotropins, metformin, and letrozole; Group 2 received gonadotropins, metformin, and NAC; and Group 3 received a combination of gonadotropins, metformin, letrozole, and NAC. Drug administration followed standard PCOS treatment protocols without influencing clinical management.

***Intervention protocol:*** Metformin (1500 *mg* daily) was administered in all groups starting on the second or third day of the menstrual cycle and continued until the last day of the treatment protocol. Letrozole (5 *mg* daily) was administered to Groups 1 and 3, while NAC (1200 *mg* daily) was administered to Groups 2 and 3, with administration continuing daily until one day prior to hCG injection. Recombinant FSH (Cinnal-f, 150–225 *IU/day*; CinnaGen, Iran) was prescribed based on patient age, ultrasound assessment, and predicted ovarian response. Three days after gonadotropin initiation, transvaginal ultrasound was performed to measure endometrial thickness and assess the number and diameter of mature follicles. When the dominant follicle reached 13–14 *mm*, a gonadotropin-releasing hormone (GnRH) antagonist (Sterotide®, 250 *µg/day*) was administered until hCG injection, which was given at a dose of 5,000–10,000 *IU* when at least three follicles reached 17–18 *mm* in diameter.

***Oocyte retrieval and cumulus cell isolation:*** Oocyte retrieval was performed 34–36 *hr* post-hCG via transvaginal ultrasound-guided aspiration, and cumulus-oocyte complexes (COCs) were isolated from the aspirated samples. Metaphase II oocytes were identified using a stereomicroscope, achieving a yield of over 80%. To minimize contamination by granulosa cells, the COCs were washed three times in enzyme-free culture medium (Universal Medium; Bio & SELL GmbH, Germany), and cumulus cells were mechanically separated within one *hr* of oocyte retrieval. The isolated cumulus cell mass was centrifuged at 1000 × *g* for 10 *min* at room temperature, and the resulting pellet was resuspended in RNA stabilization reagent at a 5–10:1 volume ratio relative to the pellet. The samples were incubated at 4°*C* for 24 *hr*, transferred to –20°*C* for two *hr*, and then stored at –80°*C* until RNA extraction and subsequent analysis of gene expression (Figure 1).

***RNA extraction and cDNA synthesis:*** RNA extraction was performed by thawing the cryovials at 37°*C*, followed by centrifugation for 10 *min* at 10,000 *rpm*. The supernatant was discarded, and the pellet was dissolved in 1 *ml* of PBS buffer and centrifuged at 12,000 *rpm* for 10 *min*. After discarding the supernatant, 550 *µl* of TRIzol reagent was added and incubated at room temperature for 5 *min*. Subsequently, 200 *µl* of chloroform was added, samples were shaken vigorously for 15 *s*, and centrifuged at 12,000 *rpm* at 4°*C* for 15 *min*, resulting in three distinct phases: the RNA-containing aqueous phase, the DNA-containing inter-

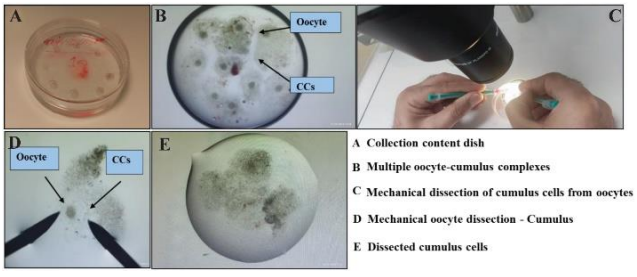


**Figure 1.** The steps of isolating the oocyte-cumulus complex from ovarian aspirate samples

phase, and the protein-containing organic phase. The upper aqueous phase was carefully aspirated and transferred to a clean, RNase-free microcentrifuge tube, to which an equal volume of cold isopropanol was added and stored overnight at –80°*C*. The samples were then centrifuged at 12,000 *rpm* for 10 *min*, the supernatant removed, and 1000 *µl* of 70% ethanol added. After vortexing, the mixture was centrifuged for 5 *min* at 10,000 *rpm* at 4°*C*, the supernatant discarded, and the microtube left open to remove residual ethanol. The RNA pellet was solubilized in 10–20 *µl* of diethyl pyrocarbonate (DEPC)-treated water. RNA concentration and purity were assessed using a NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific, USA) by measuring absorbance at 260/280 *nm* for protein contamination and 230/260 *nm* for phenol contamination. The extracted RNA was stored at –80°*C* until cDNA synthesis, which was performed using the AddScript cDNA Synthesis Kit (Addbio, Korea) according to the manufacturer's instructions.

***Real-time PCR:*** Gene expression of PFKP and PKM2 was analyzed by real-time PCR using gene-specific primers (Sinaclon, Iran) and GAPDH as a housekeeping gene (Table 1). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method:

$\Delta CT$= CT Target−CT Reference

$\Delta\Delta CT=\Delta CT$ Sample−$\Delta CT$ Calibrator

***Statistical analysis:*** Gene expression data were analyzed using GraphPad Prism 8.2.1 software. One-way ANOVA was used to compare means, followed by Tukey's post hoc test to identify statistically significant differences.

## Results

In this study, the expression level of the PFKP gene was increased in the NAC group compared to the letrozole and control groups, and the PKM2 gene was also increased in the letrozole plus NAC group compared to the letrozole, control, and NAC groups (Table 2).

***The expression level of PFKP gene:*** The results of data analysis are shown in table 2. The expression level of PFKP gene was compared between the letrozole group and the NAC group and the letrozole plus NAC group and the control group. The PFKP gene expression level in the NAC group showed a significant 2.15-fold increase compared to both the control group (p=0.0464), and the letrozole group (p=0.0197). The expression level of PFKP gene in the letrozole plus NAC group increased 1.7-fold compared to the letrozole (p= 0.1352) and control (p=0.2838) groups; however, this increase was not statistically significant. The expression level of PFKP gene was not significantly different among the other groups (Let. *vs*. control: p=0.9601/NAC+Let. *vs*. NAC: p=0.6812). These findings indicate that the administration of NAC to PCOS patients who are candidates for assisted reproductive techniques can be effective in the success of ART by increasing the expression level of PFKP genes involved in CCs glucose metabolism.

***The expression level of PKM2 gene:*** The results of data analysis in table 2 indicate that the expression level of PKM2 in the letrozole plus NAC group was significantly increased compared to the control (p=0.0001) and letrozole (p<0.0001) and NAC (p=0.0041) groups. The expression level of PKM2 in the NAC group showed a significant increase of 1.6-fold compared to the letrozole group (p<0.05). The expression level of PKM2 gene in the NAC group was increased 1.2-fold compared to the control group, but this expression was not statistically significant (p=0.2113) (Table 2). These findings suggest that the simultaneous administration of NAC and letrozole in PCOS patients who are candidates for ART may enhance its success rate by upregulating the expression of PKM2 gene, which plays a role in improving glucose metabolism in CCs.

***Comparison of baseline age and BMI among study groups:*** Comparison of age variable among the study groups showed no significant difference be-

**Table 1.** Primers used for real time- PCR

| Primer sequence | Melting temperature (Tm) | Gene | Amplicon size |
|---|---|---|---|
| F: GGCGGCTACTGTGGCTACCT<br>R: GCCTCTCTGGATGGTGGTCTT | 58ºC | PFKP | 150 |
| F: GAGTACCATGCGGAGACCATC<br>R: TTTCCATGTAGGCGTTATCCAG | 60ºC | PKM2 | 208 |
| F: AAGGTGAAGGTCGGAGTCAAC<br>R: GGGGTCATTGATGGCAACAA | 60ºC | GAPDH | 102 |

**Table 2.** Comparison of the expression level of PFKP and PKM genes in the studied groups

| PFKP | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | | | | Comparisons | Mean diff. | 95.00% CI of diff. | p-value | Summary | Adjusted p-value |
| **Control** | **Let** | **NAC** | **NAC+Let** | **Control *vs*. Let** | **0.1844** | **-0.9350 to 1.304** | **No** | **ns** | **0.9601** |
| 1.097 | 0.821994 | 2.478041 | 1.316949 | Control *vs*. NAC | -1.136 | -2.255 to -0.01650 | Yes | * | 0.0464 |
| 1.003 | 0.845104 | 1.256317 | 2.866321 | Control *vs*. NAC+Let | -0.7113 | -1.831 to 0.4081 | No | ns | 0.2838 |
| 0.993 | 0.902626 | 2.866321 | 1.027544 | Let *vs*. NAC | -1.320 | -2.440 to -0.2009 | Yes | * | 0.0197 |
| 0.987 | 0.762707 | 2.012795 | 1.704324 | Let *vs*. NAC+Let | -0.8957 | -2.015 to 0.2237 | No | ns | 0.1352 |
| | | | | NAC *vs*. NAC+Let | 0.4246 | -0.6948 to 1.544 | No | ns | 0.6812 |
| **PKM** | | | | | | | | | |
| Variable | | | | Comparisons | Mean diff. | 95.00% CI of diff. | p-value | Summary | Adjusted p-value |
| **Control** | **Let** | **NAC** | **NAC+Let** | **Control *vs*. Let** | **0.1971** | **-0.07253 to 0.4667** | **No** | **ns** | **0.1868** |
| 0.998 | 0.687009 | 1.308941 | 1.622704 | Control *vs*. NAC | -0.1897 | -0.4593 to 0.07991 | No | ns | 0.2113 |
| 1.099 | 0.991304 | 1.116049 | 1.567429 | Control *vs*. NAC+Let | -0.5901 | -0.8598 to -0.3205 | Yes | *** | 0.0001 |
| 0.987 | 0.971577 | 1.139499 | 1.452361 | Let *vs*. NAC | -0.3868 | -0.6564 to -0.1172 | Yes | ** | 0.0053 |
| 0.98 | 0.631739 | 1.264354 | 1.788066 | Let *vs*. NAC+Let | -0.7872 | -1.057 to -0.5176 | Yes | **** | <0.0001 |
| | | | | NAC *vs*. NAC+Let | -0.4004 | -0.6701 to -0.1308 | Yes | ** | 0.0041 |

Tukey's multiple comparisons test was used. Values are expressed as mean ± SEM. *p<0.05, **p<0.005, ***p=0.0001, **p<0.0001 (n=20). LET: Letrozole; NAC: N-acetylcysteine.

**Table 3.** Comparison of baseline age and BMI among study groups

| AGE | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | | | | Comparisons | Mean diff. | 95.00% CI of diff. | Significant | Summary | Adjusted p-value |
| Control | Let | NAC | NAC+Let | Control *vs*. Let | 3.400 | -5.488 to 12.29 | No | ns | 0.6978 |
| 30 | 33 | 32 | 26 | Control *vs*. NAC | -4.600 | -13.49 to 4.288 | No | ns | 0.4710 |
| 29 | 29 | 30 | 31 | Control *vs*. NAC+Let | -3.000 | -11.89 to 5.888 | No | ns | 0.7703 |
| 34 | 23 | 34 | 36 | Let *vs*. NAC | -8.000 | -16.89 to 0.8876 | No | ns | 0.0855 |
| 20 | 20 | 38 | 37 | Let *vs*. NAC+Let | -6.400 | -15.29 to 2.488 | No | ns | 0.2080 |
| 36 | 27 | 38 | 34 | NAC *vs*. NAC+Let | 1.600 | -7.288 to 10.49 | No | ns | 0.9543 |
| **BMI** | | | | | | | | | |
| **Variable** | **Comparisons** | **Mean diff.** | **95.00% CI of diff.** | **Significant** | **Summary** | **Adjusted p-value** | **Variable** | **Comparisons** | **Mean diff.** |
| Control | Let | NAC | NAC+Let | Control *vs*. Let | 1.434 | -7.875 to 10.74 | No | ns | 0.9705 |
| 43.67 | 50 | 44.3 | 59.6 | Control *vs*. NAC | -1.526 | -10.84 to 7.783 | No | ns | 0.9648 |
| 39.4 | 43.8 | 40 | 48.7 | Control *vs*. NAC+Let | -7.886 | -17.20 to 1.423 | No | ns | 0.1124 |
| 43.2 | 41 | 47.3 | 39.2 | Let *vs*. NAC | -2.960 | -12.27 to 6.349 | No | ns | 0.8000 |
| 45.3 | 36 | 45 | 50.6 | Let *vs*. NAC+Let | -9.320 | -18.63 to -0.01073 | Yes | * | 0.0497 |
| 43.2 | 36.8 | 45.8 | 56.1 | NAC *vs*. NAC+Let | -6.360 | -15.67 to 2.949 | No | ns | 0.2456 |

Tukey's multiple comparisons test was used. Values are expressed as mean±SEM. *p<0.05, **p<0.005, ***p=0.0001, **p<0.0001 (n=20). LET: Letrozole, NAC: N-acetylcysteine

tween the group receiving letrozole plus NAC and the letrozole group (p>0.05) (Table 3). However, BMI comparison revealed a significant difference between these two groups, with the letrozole plus NAC group showing a higher or lower BMI (p< 0.05) (Table 3).

## Discussion

The transcriptional profiles of CC genes have been analyzed by various groups, resulting in the identification of a set of candidate genes, of which only a few have been suggested as predictors of oocyte quality and pregnancy success (27). On the other hand, infertility in women with PCOS is a serious problem, and various approaches have been developed to improve their pregnancy rates. PCOS is a common endocrine disorder affecting women of reproductive age and requires more effective treatment options. NAC possesses two key properties (antioxidant and insulin-sensitizing effects) that influence LH and progesterone levels, as well as endometrial thickness in women with PCOS. Therefore, NAC may represent a potential therapeutic option (34).

Letrozole significantly increases the probability of pregnancy in patients with PCOS; however, the exact success rate of this treatment remains unclear and reaches, at best, approximately 20% (35). In addition, NAC as an effective drug in reducing serum insulin and testosterone can improve serum homocysteine levels and lipid profile in PCOS patients (36). Another potential therapeutic application of NAC lies in its anti-inflammatory properties. NAC may be particularly beneficial for improving BMI, fasting blood glucose (FBG), and fasting insulin (FI) levels in women with PCOS (37). The transcription factor NF-kB plays a critical role in various aspects of the inflammatory cascade and immune response by regulating the expression of related genes (38). The anti-inflammatory effect of NAC is associated with a reduction in NF-kB activity (39). Previous evidence also suggests that NAC supplementation may positively affect semen viscosity. Given that a significant proportion of infertility is due to hyperandrogenism and anovulation, the potential usefulness of NAC is notable, especially given its accessibility and minimal side effects (40). NAC may have the potential to impact specific signaling pathways in β cells and insulin target cells. Various biological properties of NAC show its potential effectiveness as a therapeutic approach in the treatment of PCOS patients (41).

PKM2 catalyzes the last step of the glycolysis and converts phosphoenolpyruvate (PEP) to pyruvate with the simultaneous production of ATP (42). In addition to its position in the glycolytic pathway, PKM2 plays a crucial role in regulating the metabolic fate of glycolytic intermediates. High activity of PKM2 leads to the rapid conversion of PEP to pyruvate, facilitating energy production (43).

The results of our study showed that the expression levels of PFKP gene in CCs of PCOS patients was higher in the NAC group than in other groups. Wathlet et al. in 2012 showed that the use of antioxidants for the treatment of infertility improves oocyte quality (44).This could be related to the significant antioxidant effect of NAC supplementation. In our study, the expression level of PKM2 in the letrozole plus NAC group significantly increased (p<0.05) compared to the letrozole and control groups (Table 3). According to the investigation by Mostajeran et al. involving 130 PCOS patients divided into two groups (one receiving letrozole plus NAC and the other receiving letrozole alone), the ovulation rate, the number of follicles larger than 18 *mm*, and the pregnancy rate were significantly higher in the letrozole plus NAC group than in the letrozole alone group (35). In a similar study conducted by Teimouri et al. in 2021 on 317 PCOS patients, it was revealed that ovulation and pregnancy rates were significantly higher in the letrozole plus NAC group compared to the letrozole alone group (40). Based on the results of the study, NAC administration increased the expression level of genes involved in glucose metabolism (PFKP, PKM2) in PCOS patients. These findings indicate that NAC administration in PCOS patients is effective in increasing the expression level of genes involved in CCs glucose metabolism and can be helpful in the treatment of PCOS patients who are candidates for ARTs.

## Conclusion

In general, based on the findings of previous studies and the present study, NAC administration increases the expression of genes involved in glucose metabolism (PFKP, PKM2) in women with PCOS and can improve the success of ARTs. The expression levels of these genes may serve as potential indicators for assessing the maturity of oocytes retrieved from human subjects. Future research directions may include investigating the expression of various genes involved in the fertili-

zation process following treatment with NAC and letrozole; conducting studies on patients with high BMI, insulin resistance, or resistance to clomiphene citrate; and exploring the effects of higher NAC doses or extended treatment durations. Additionally, examining the impact of NAC on the expression of genes and proteins related to sperm fertility in patients with sperm disorders could provide valuable insights. To further validate the findings of this study, it is recommended that larger-scale studies be conducted to clarify the roles and signaling pathways of these genes in CCs.

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## Conflict of Interest

Authors declare no conflict of interest.

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