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# Association between early embryo morphokinetics plus cumulus cell gene expression and assisted reproduction outcomes in polycystic ovary syndrome women

## BIOGRAPHY

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## KEY MESSAGE

Some embryo time-lapse kinetics parameters may be related to cumulus cell gene expression and clinical outcome. The expression of cumulus cell gene *DIAPH2* can predict intracytoplasmic sperm injection outcome following Day 3 embryo transfer. Embryo assessment and selection by time-lapse imaging and cumulus cell gene expression should be prospectively studied.

## ABSTRACT

**Research question:** Can a combination of time-lapse morphokinetic parameters and cumulus cell gene expression in polycystic ovary syndrome (PCOS) women be used to predict assisted reproductive treatment outcome?

**Design:** A total of 547 embryos from 100 intracytoplasmic sperm injection (ICSI) cycles were evaluated. Fifty women with PCOS and 50 women who were categorized as tubal factor infertility were recruited. Time-lapse records were annotated for time to pronuclear fading (tPNf), time to 2 to 8 cells (t2–t8), reverse cleavage, direct cleavage and also for the presence of multinucleation. Expression levels of three genes involved in mitotic divisions, diaphanous-related formin 2 (*DIAPH2*), nibrin (*NBN*) and NIMA-related protein kinase (*NEK4*), were measured in 100 associated cumulus cell samples using quantitative real-time polymerase chain reaction.

**Results:** Expression of *DIAPH2* and *NBN* was significantly higher in the embryos of PCOS patients that resulted in implantation, biochemical and clinical pregnancies as well as live birth compared with embryos that were negative for these outcomes ($P < 0.01$). However, in the tubal factor group, *NBN* gene expression was significantly higher in embryos resulting in biochemical pregnancy, clinical pregnancy and live birth ($P < 0.01$) only. Multivariate logistic regression analysis showed that tPNf together with *DIAPH2* gene expression were independent prognostic factors of clinical pregnancy rate and live birth in both groups.

**Conclusions:** Some time-lapse embryo parameters may be related to cumulus gene expression and clinical outcome. Furthermore, the expressions of cumulus cell genes involved in mitotic divisions are significantly associated with ICSI outcome using Day 3 embryo transfer.

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## KEYWORDS

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Embryo morphokinetics
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Pregnancy outcome
Time lapse

## INTRODUCTION

Embryo evaluation *in vitro* and selecting viable embryos for transfer is an essential step in assisted reproductive technology (ART). Identifying a high-quality embryo that has the highest implantation potential in a cohort is critical to maximizing the probability of pregnancy. Conventionally, selection of embryos for transfer is based on morphological criteria. Recently, function of the cumulus cells that surround the oocyte has been considered as a non-invasive alternative for embryo selection (*Assou et al., 2008; Feuerstein et al., 2007*). Because there is bilateral dialogue between the oocyte and the cumulus cells, these cells can reveal and control oocyte function as well as subsequent embryo development competence (*Anderson et al., 2009; Fragouli et al., 2014*). Some studies provide evidence that gene expression pattern in cumulus cells is a potential biomarker for predicting embryo development and pregnancy outcome (*Braga et al., 2016; Wathlet et al., 2012*).

Furthermore, embryo evaluation based on conventional methods is not instructive for discerning exact embryo kinetics and morphological events that occur between two microscopic observations. Moreover, classic embryo assessment causes frequent embryo exposure to fluctuations in temperature, humidity and to the gas composition outside the incubator. Introduction of the time-lapse system (TLS) as a non-invasive tool to ART clinics produces additional information for both morphologic evaluation and on the timing of cell division 'morphokinetic variables' (*Meseguer et al., 2011*), which improves ART outcome compared with a standard incubator (*Fishel et al., 2017; Kirkegaard et al., 2013; Pribenszky et al., 2017; Rubio et al., 2014*). Several data indicate that timing of particular events such as pronuclear formation, syngamy, early cleavage divisions, cell cycle intervals, synchronicity of cell divisions and initiation of blastulation are good indicators for embryo development as well as clinical outcomes (*Adamson et al., 2016; Conaghan et al., 2013; Fishel et al., 2017; Kirkegaard et al., 2013; Milewski et al., 2016; Rubio et al., 2014*).

Furthermore, one of the main causes of female infertility is polycystic ovary syndrome (PCOS), which affects 5–10% of women of reproductive age (*Wissing et al., 2014*). Increased ART cancellation rate and decreased fertilization rate (*Heijnen et al., 2006*) in these women may be the result of a different gene expression pattern in these women (*Huang et al., 2013; Wei et al., 2011*), which is strongly related to the defects in meiosis (*Wood et al., 2007*) and plays a key role in disrupted embryo development in PCOS women.

Therefore, this current research assessed differently expressed cumulus cell genes in PCOS women. These genes include: diaphanous-related formin 2 (*DIAPH2*), which is involved in spindle dynamics; nibrin (*NBN*), which is implicated in chromosomal alignment; and NIMA-related protein kinase (*NEK4*), which is related to centrosome function. The study aimed to determine whether early embryo morphokinetics assessed by TLS is related to cumulus gene expression in PCOS women. The study also investigated the relationship between cumulus cell gene expression, as well as embryo morphokinetics, and ART outcome.

## MATERIALS AND METHODS

This experimental prospective study was approved by the Ethics Committee of Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.RSI.REC.1396.26) on 9 November 2015. The study was conducted in accordance with the Declaration of Helsinki. A written consent form was signed by all participants.

### Patients

The study included 100 consecutive intracytoplasmic sperm injection (ICSI) cycles in which embryo development was monitored by a time-lapse imaging system. Only ICSI cases were included to avoid the detrimental effects of poor-quality oocytes from PCOS patients on embryo development. All the women who underwent ICSI treatment were screened between April 2016 and April 2017 to meet the inclusion criteria and reach the number of samples in each group. Fifty women with PCOS and 50 women who were categorized as tubal factor infertility were recruited. Patients were required to be less than 43 years of age, planned Day 3 embryo transfer, had fewer than three failed IVF/ICSI cycles and at least one zygote (two-pronuclear [2PN]) available on Day 1 for image analysis. PCOS diagnosis was based on the criteria in accordance with the Rotterdam consensus (*Rotterdam, 2004*). Tubal factor infertility refers to women who had fallopian tube(s) removed because of tubal pregnancy and proximal tubal obstruction that was confirmed by hysterosalpingogram or laparoscopy. Exclusion criteria were diagnosis of severe male factor (total motile spermatozoa <1 million) and endometriosis.

Serum levels of anti-Müllerian hormone (AMH) and oestradiol were measured in a venous blood sample collected from all participants. Oestradiol level was determined by electrochemiluminescence immunoassay kit (ECLIA; Roche Diagnostics GmbH, Mannheim, Germany) on an Elecsys immunoassay analyser and AMH level was measured using a commercial ELISA kit (AMH/MSI ELISA; AnshLabs, TX, USA).

### Ovarian stimulation

Women mostly (90%) used an antagonist protocol (*Eftekhar et al., 2013*), an agonist protocol (3%) (*Nikmard et al., 2016*) or a microdose flare protocol (7%) (*Davar et al., 2010*). Follicular growth was monitored by transvaginal ultrasound. An intramuscular injection of 10,000 IU of human chorionic gonadotrophin (HCG) (Pregnyl®; Organon, Oss, the Netherlands) was administered as the minimum three follicles reached a diameter of ≥18 mm. Ovum pickup was performed by the ultrasound guide 36 h after the HCG injection.

### Laboratory procedure

Upon retrieval, oocytes were incubated in culture medium (G-IVF; VitroLife, Kungsbacka, Sweden) covered with mineral oil (Ovoil; VitroLife) at 37°C, with 6% $CO_2$ for 2–3 h. Hyaluronidase (80 IU/ml) (Sigma Co., USA) was used to help in denudation of cumulus cells. Mature (metaphase II [MII]) oocytes were injected using the husband's prepared spermatozoa. The injected oocytes were washed twice and cultured overnight individually in a standard incubator at 37°C with 6% $CO_2$ in fresh droplets of G1 (VitroLife) covered with mineral oil (Reploline Co., Germany). A nine-well embryo culture slide (Primo Vision dish, VitroLife) was prepared with 40 µl G1+ medium, and covered with 3 ml of mineral oil and equilibrated overnight

for culture of zygotes the next day. Fertilization was assessed 16–18 h after insemination by the presence of the 2PN and two polar bodies. Normally fertilized zygotes were transferred to the pre-equilibrated Primo Vision dish for culture within the Embryoscope (Primo Vision, VitroLife). The culture slide was placed in a time-lapse microscope at 37°C, 5% $O_2$ and 6% $CO_2$ for 3 days without media exchange or refreshment.

### Time-lapse imaging system

Images were acquired for each embryo every 10 min in seven focal planes. Primo Vision Embryo Viewer Software was used to recognize the precise timing from the point of ICSI: time to pronuclear fading (tPNf), time to 2 cells (t2), 3 cells (t3), 4 cells (t4), 5 cells (t5), 6 cells (t6), 7 cells (t7) and 8 cells (t8). Additional kinetic variables were calculated: duration of the second cell cycle (cc2 = t3 – t2), third cell cycle (cc3 = t5 – t3) and time to complete first, second and third synchronous divisions, s1 (t2 – tPNf), s2 (t4 – t3) and s3 (t8 – t5). Two observed cleavage anomalies were reverse cleavage (where a blastomere was reabsorbed after cleavage) and direct cleavage (when a single blastomere divided directly from 1 to 3 cells in less than 5 h). The presence of multinucleation (more than one nucleus in a blastomere) was also noted.

### Embryo selection and transfer

Embryo selection for transfer on Day 3 was based on morphologic scores with additional data provided by time-lapse imaging. The 8-cell embryos that had the best morphology as well as high scores with time lapse were chosen for transfer. Two or three embryos (in the case of poor quality or the patient's preference) were transferred using an embryo transfer Labotect catheter (Labor-Technik-Göttingen GmbH, Gottingen, Germany). Other good-quality embryos that were not selected for transfer were cryopreserved. Moreover, 400 mg progesterone suppositories (Cyclogest®; Cox Pharmaceuticals, Barnstaple, UK) were administered vaginally, twice daily from the day of oocyte retrieval until the observation of fetal heart activity by ultrasound in the 8th week.

### Cumulus cell collection

The denuded oocytes were transferred to their injection dish and held individually throughout the culture period. Cumulus cells surrounding a single oocyte were collected in separately labelled sterile 1.5 ml microtubes (Eppendorf), washed in phosphate-buffered saline (Sigma-Aldrich, Auckland, New Zealand) twice and centrifuged at 5000*g* for 1 min. Finally, the pellet was stored at –80°C using the appropriate volume of RNA*later* RNA Stabilization Reagent (Qiagen Europe) until RNA extraction.

### Cumulus cell RNA extraction and cDNA synthesis

RNA was isolated from cumulus cells using the QuantiTect®, RNeasy Micro kit (Qiagen Europe) according to the manufacturer's guide. The RNA concentration was determined by NanoDrop spectrophotometer and adjusted to a concentration of 1000 ng/µl. Then cDNA was synthesized using a RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc.) on the same day, according to the manufacturer's instructions. The reverse transcription was performed in 20 µl reactions for 60 min at 42°C, followed by 70°C for 5 min to inactivate the reverse transcriptase. The reverse transcription reaction product was directly used in quantitative polymerase chain reaction (qPCR) in a separate step to amplify the targets.

### Quantitative real-time PCR

Using specified primers, relative expressions of *DIAPH2*, *NBN* and *NEK4* were evaluated by quantitative real-time PCR (qRT-PCR). The *GAPDH* gene was used as an internal control. The PCR run was performed according to QuantiTect SYBR Green RT-PCR kit (Applied Biosystems UK, Lot no:1201416) on an ABI 7500 RT-PCR system (Applied Biosystems) using the subsequent program: stage 1: 95°C for 10 min, stage 2: 95°C for 10 s, 58°C for 20 s, 72°C for 30 s for a total of 40 cycles. This was continued by a melt curve step at 95°C for 15 s, 58°C for 1 min, and 95°C for 15 s. All samples were run in duplicate to minimize the sampling error and the mean value of the duplicates was used for all additional calculations. Reverse transcriptases minus samples as well as no template controls were run together with the main samples. Verification of Amplicon specificity and size was performed by a 2% agarose gel, product length and using a melting curve analysis. The output data were transferred to Microsoft Excel for analysis. The relative expression ratios were calculated by a mathematical model that comprised an efficiency correction for real-time PCR efficiency of the individual transcripts (*Pfaffl, 2001*) as follows: ratio = $(E_{target})^{\Delta Ct_{target}}$ (control – sample)/$(E_{ref})^{\Delta Ct_{ref}}$ (control – sample). The relative expression ratio of a target gene was defined from the real-time PCR efficiency ($E$) and the threshold cycle difference for an unknown sample versus a control (Ct control – sample). For each gene, cDNA dilution curves were created and used to calculate the individual real-time PCR efficiencies [$E$ = 10(–1/slope)]. The geometric mean of the two internal reference genes was used to correct the raw values for the genes of interest.

### Outcome measures

Cumulus gene expression and their association with early embryo morphokinetics were investigated as mentioned above. Biochemical pregnancy was defined by β-HCG >50 IU/l on Day 14 after embryo transfer and clinical pregnancy was confirmed by observation of fetal heart activity by transvaginal ultrasonography 2–3 weeks after positive β-HCG. Transfers with known implantation data (KID), where all transferred embryos implanted or failed to implant, were also analysed in detail.

### Statistical analysis

Quantitative variables were expressed as mean ± SD and compared with Student's t-test and Mann–Whitney U-test according to their distribution pattern assessed by Kolmogorov–Smirnoff test. Qualitative variables were presented as percentages and compared by chi-squared test. A Spearman's rank correlation was used to analyse relationships between cumulus cell gene expression and early cleavage timing in the developing embryos. Logistic regression analysis was performed to generate a model to control for potential confounding factors. Independent predictors of clinical pregnancy or live birth in the model included factors supposed to be clinically relevant and those found to be statistically significant during univariate analysis. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. All analyses were performed using the Statistical Package for the Social Sciences, Version 20 for Windows (IBM Corp., USA). To avoid the risk raised by multiple testing at the conventional significance level of 0.05, the level of significance was determined as 0.01. Therefore, erroneous influences would be expected to occur with a probability of 0.01.

**TABLE 1 PATIENT DEMOGRAPHICS AND CYCLE CHARACTERISTICS IN THE TWO STUDY GROUPS**

| Variable | PCOS (*n* = 50) | Tubal factor (*n* = 50) | *P*-value |
|---|---|---|---|
| Age (years) | 30.04 ± 4.59 | 31.40 ± 4.99 | NS[a] |
| Body mass index (kg/m$^2$) | 26.06 ± 4.03 | 25.55 ± 3.73 | NS[a] |
| Length of infertility (years) | 6.92 ± 4.69 | 7.54 ± 4.67 | NS[b] |
| Ovarian stimulation protocol (*n*, %) | | | |
| GnRH antagonist | 48 (96) | 42 (84) | NS |
| GnRH agonist | – | 3 (6) | |
| Microdose flare | 2 (4) | 5 (10) | |
| AMH (ng/ml) | 6.10 ± 4.39 | 3.97 ± 3.38 | 0.008[b] |
| Oestradiol on the day of HCG injection (pg/ml) | 2400.72 ± 1662.35 | 2101.90 ± 1615.09 | NS[b] |
| Oocytes retrieved | 13.12 ± 7.80 | 9.46 ± 5.19 | 0.0098[b] |
| Mature metaphase II oocytes | 10.82 ± 6.95 | 8.06 ± 4.77 | NS[b] |
| Mean embryos transferred | 2.16 ± 0.61 | 2.00 ± 0.57 | NS[b] |

Data are presented as mean ± SD unless otherwise stated.

AMH = anti-Müllerian hormone; GnRH = gonadotrophin-releasing hormone; HCG = human chorionic gonadotrophin; NS = non-significant; PCOS = polycystic ovary syndrome.

[a] PCOS vs tubal factor group using Student's t-test.

[b] PCOS vs tubal factor group using Mann–Whitney U-test; ovarian stimulation protocol was compared between groups using chi-squared test.

## RESULTS

This study evaluated embryos from 100 ICSI treatment cycles. All embryos were obtained after fertilization by ICSI: 289 embryos in the PCOS group and 258 embryos in the tubal factor infertility group. Patient demographics and cycle characteristics are listed in TABLE 1. There were no significant differences between the two groups in these respects. As expected, serum AMH level ($P = 0.008$) and number of retrieved oocytes ($P = 0.0098$) were significantly higher in PCOS women compared with the tubal factor group. From the 100 women included in the ultimate analysis, there were a total of 1129 oocytes retrieved with 944 mature oocytes. Biochemical pregnancy, clinical pregnancy and live birth rates were not significantly different between the PCOS women and the control group with tubal infertility (data not shown).

### Embryo morphokinetics between groups

The average timing of tPNf, t2 to t8, together with cc2, cc3 and s1 to s3 for the PCOS and tubal factor groups are presented in TABLE 2. The mean timings of all mentioned events were extended in the PCOS group compared with the women with tubal factor infertility. The differences were statistically significant (all $P < 0.001$) except for the cc2, cc3, s1 and s2 categories. The prevalence of morphological events including multinucleation, reverse cleavage and direct cleavage was similar between the two groups (FIGURE 1).

### Cumulus gene expression between groups

With regard to cumulus gene expression, *NBN* gene expression was significantly different between PCOS and tubal factor women ($P < 0.01$), whereas expression of *DIAPH2* and *NEK4* was not statistically significant between the two groups (FIGURE 2A).

### Cumulus gene expression and embryo morphokinetics

Correlation between cumulus cell gene expression and embryo kinetic parameters were considered to assess the relationship between these two variables in the embryo development. Cumulus gene expression of *DIAPH2* and *NBN* were negatively correlated with the cc3 (correlation coefficient [rs] = –0.15 and rs = –0.02, respectively, both $P < 0.01$) among PCOS women. Furthermore, *NBN* gene expression exhibited a negative correlation with t5 (rs = –0.17, $P < 0.01$). Cumulus gene expression had no correlation with embryo kinetic timing in infertile women with tubal factor infertility (TABLE 3).

Expression of the three mentioned cumulus cell genes was also investigated in relation to embryo morphological abnormalities. The results indicated that expression levels of *DIAPH2*, *NBN* and *NEK4* are similar between embryos with and without reverse cleavage as well as direct cleavage and multinucleation in both groups (FIGURE 3).

### Cumulus gene expression and reproductive outcome

Regarding the association of cumulus gene expression with the reproductive outcome in the tubal factor women, *NBN* gene expression in cumulus cells was significantly higher when at least some of the transferred embryos resulted in a biochemical pregnancy, clinical pregnancy and live birth ($P < 0.01$; FIGURE 2C,2E and 2G, respectively). Significantly increased expressions of *DIAPH2* and *NBN* genes were also detected in implanted versus non-implanted embryos, as well as in the transferred embryos for cases in which not all embryos resulted in biochemical and clinical pregnancy as well as live birth among PCOS women (all $P < 0.01$); but the difference for *NEK4* gene expression was not statistically significant (FIGURE 2B,2D,2F and 2H, respectively).

### Embryo morphokinetics and reproductive outcome

Embryo kinetics in PCOS and tubal factor groups were compared for all transferred embryos according to the pregnancy outcome. FIGURE 4 shows the kinetic events that are statistically different between the groups. KID were available for 40 PCOS women with 81 transferred embryos that either implanted ($n = 8$) or failed to implant ($n = 73$). Timing of embryo kinetics for KID embryos is presented in FIGURE 4B. tPNf and s1 in PCOS women

**TABLE 2 KINETIC DATA OF ALL EMBRYOS IN TWO STUDY GROUPS**

| Kinetic marker (hours post-ICSI) | PCOS (*n* = 289) | Tubal factor (*n* = 258) | *P*-value[a] |
|---|---|---|---|
| tPNf | 24.55 ± 1.09 | 23.00 ± 1.46 | <0.001 |
| t2 | 27.94 ± 0.67 | 26.32 ± 0.68 | <0.001 |
| t3 | 37.25 ± 1.06 | 34.76 ± 1.58 | <0.001 |
| t4 | 41.26 ± 0.87 | 38.92 ± 0.64 | <0.001 |
| t5 | 49.17 ± 0.60 | 47.14 ± 0.47 | <0.001 |
| t6 | 54.23 ± 0.59 | 50.15 ± 1.38 | <0.001 |
| t7 | 55.10 ± 0.41 | 52.42 ± 0.51 | <0.001 |
| t8 | 60.70 ± 0.80 | 56.16 ± 0.21 | <0.001 |
| cc2 | 9.05 ± 2.90 | 9.02 ± 1.77 | NS |
| cc3 | 11.91 ± 1.25 | 12.38 ± 1.61 | NS |
| s1 | 3.39 ± 1.31 | 3.31 ± 1.66 | NS |
| s2 | 4.01 ± 1.39 | 4.15 ± 1.51 | NS |
| s3 | 11.53 ± 0.99 | 9.01 ± 0.51 | <0.001 |

Data are presented as mean ± SD.

ICSI = intracytoplasmic sperm injection; PCOS = polycystic ovarian syndrome; tPNf = time to pronuclear fading; t2 = time to 2 cells; t3 = time to 3 cells; t4 = time to 4 cells; t5 = time to 5 cells; t6 = time to 6 cells; t7 = time to 7 cells; t8 = time to 8 cells; cc2 = duration of the second cell cycle (t3 – t2); cc3 = duration of the third cell cycle (t5 – t3); s1, s2 and s3 = complete first, second and third synchronous divisions: s1: (t2 – tPNf), s2 (t4 – t3), and s3 (t8 – t5); NS = non-significant.

[a] PCOS vs tubal factor group using Mann–Whitney U-test.

(FIGURE 4A), as well as tPNf in the tubal factor group (FIGURE 4D), were significantly different between all transferred embryos for cases of positive and negative biochemical pregnancy ($P < 0.01$). tPNf was significantly decreased and s1 was significantly increased in transferred embryos of biochemical pregnancy cases. With regard to clinical pregnancy, however, tPNf was significantly decreased; cc2 and s1 were significantly delayed in all transferred embryos of cases with positive clinical pregnancy compared with negative clinical pregnancy among PCOS women (FIGURE 4C). However, in the tubal factor group tPNf was significantly shorter in all transferred embryos of pregnant women with detected clinical pregnancy (FIGURE 4F) ($P < 0.01$). In the PCOS group, tPNf was significantly shorter in all transferred embryos of women who had live births; nevertheless, all transferred embryos of cases resulting in live birth had significantly delays in cc2 and s1 when compared with cases without live birth ($P < 0.01$) (FIGURE 4E). None of the kinetic timings was significantly different for all transferred embryos regarding live birth in the tubal factor group.

Three morphological abnormalities detected by TLS comprising of multinucleation, reverse cleavage and direct cleavage were no different between positive and negative biochemical and clinical pregnancies as well as live birth in both PCOS and tubal factor groups (data not shown).

### Logistic regression analysis

A multivariate logistic regression analysis was applied to reproductive outcome of all transferred embryos including the following variables: selection group, embryo morphokinetics and cumulus gene expression. Furthermore, female age, AMH and number of transferred embryos were recognized by univariate analysis as confounding variables that significantly influence clinical pregnancy and live birth ($P < 0.01$). They were therefore combined to create the predictive model. According to analysis, tPNf significantly impacted clinical pregnancy and live birth (both $P < 0.01$). In addition, results showed that cumulus *DIAPH2* gene expression was an independent prognostic factor of clinical pregnancy rate and live birth and that respective odds increased 3.5-fold, 4.5-fold for *DIAPH2* gene expression ($P < 0.01$; TABLE 4).

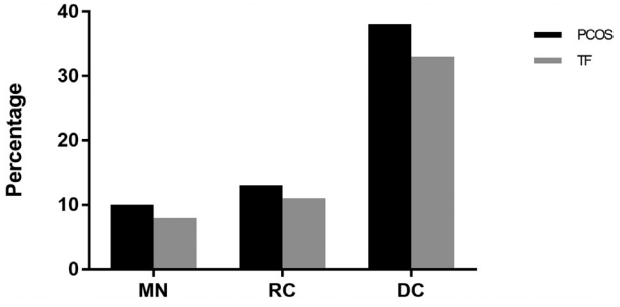


**FIGURE 1** Prevalence of three morphological abnormalities detected by time lapse in two groups. Polycystic ovarian syndrome patients (PCOS, 50 women, 289 embryos); tubal factor (TF, 50 women, 258 embryos); multinucleation (MN); direct cleavage (DC); and reverse cleavage (RC).

## DISCUSSION

This is the first study that prospectively evaluates the association of cumulus gene expression with both early embryo morphokinetics and ART outcome in PCOS women. The results showed that embryo kinetic markers reflect cumulus cell gene expression and that this could be used as a predictor for pregnancy and live birth. To date there have been a number of publications assessing the possible use of embryo morphokinetics to predict implantation potential by using either early (*Adamson et al., 2016; Wong et al., 2010*) or late (*Conaghan et al., 2013; Dal Canto*

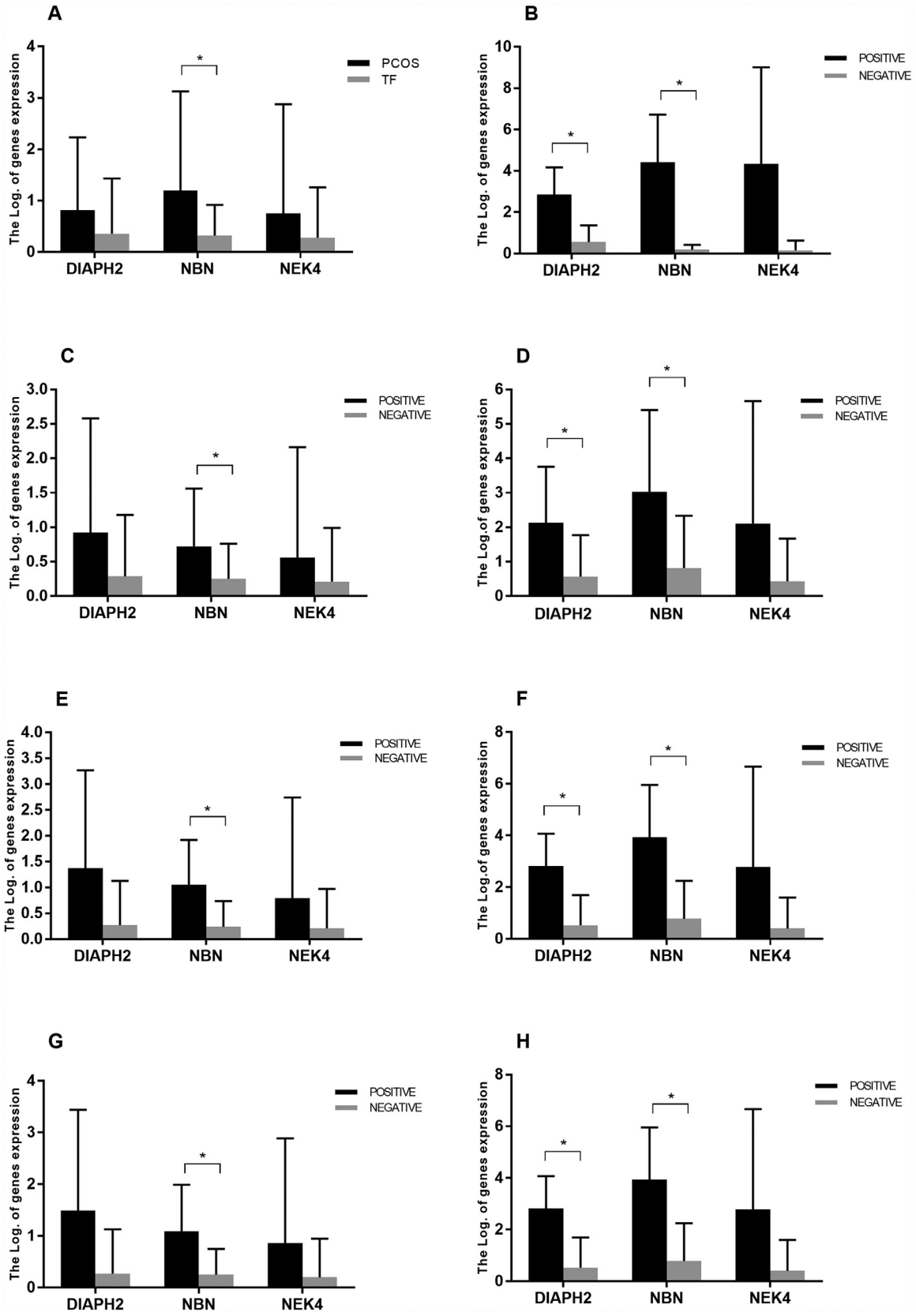


**FIGURE 2** Cumulus cell gene expression of *DIAPH2*, *NBN*, *NEK4*. (A) Gene expression between polycystic ovarian syndrome patients ($n$ = 50) (PCOS) with 289 embryos and tubal factor (TF) infertility group ($n$ = 50) with 258 embryos, significant differences between groups regarding expression of *NBN*; $P < 0.01$. (B) Gene expression in embryos with known implantation data in PCOS group (81 embryos), significant differences in *DIAPH2* and *NBN* gene expression between implanted (8 embryos from 4 women) and non-implanted embryos (73 embryos from 36 women);

*(continued)*

**TABLE 3 SPEARMAN'S RANK CORRELATIONS ANALYSING RELATIONSHIP BETWEEN CUMULUS GENE EXPRESSION AND EMBRYO KINETICS OF INDIVIDUAL EMBRYOS IN THE TWO GROUPS**

| Kinetic marker (hours post-ICSI) | PCOS (*n* = 289) | | | Tubal factor (*n* = 258) | | |
|---|---|---|---|---|---|---|
| | *DIAPH2* | *NBN* | *NEK4* | *DIAPH2* | *NBN* | *NEK4* |
| | rs | rs | rs | rs | rs | rs |
| tPNf | 0.02 | 0.00 | 0.02 | 0.04 | 0.01 | −0.05 |
| t2 | 0.00 | 0.04 | 0.08 | −0.01 | −0.08 | 0.10 |
| t3 | 0.07 | 0.06 | 0.02 | −0.05 | 0.00 | 0.04 |
| t4 | −0.03 | −0.03 | 0.01 | −0.02 | −0.07 | −0.00 |
| t5 | −0.12 | −0.17 | −0.11 | −0.06 | 0.01 | 0.05 |
| t6 | −0.03 | 0.00 | 0.07 | −0.04 | −0.09 | −0.07 |
| t7 | −0.01 | −0.02 | −0.10 | 0.06 | 0.04 | −0.02 |
| t8 | −0.00 | −0.07 | −0.05 | −0.06 | −0.10 | 0.02 |
| cc2 | 0.01 | −0.00 | −0.02 | −0.03 | 0.04 | −0.01 |
| cc3 | −0.15 | −0.20 | −0.15 | 0.05 | 0.04 | 0.03 |
| s1 | −0.01 | 0.01 | 0.02 | −0.04 | −0.03 | 0.08 |
| s2 | −0.09 | −0.09 | −0.05 | 0.06 | −0.02 | −0.04 |
| s3 | 0.10 | 0.12 | 0.08 | −0.02 | −0.05 | −0.03 |

t5 was negatively correlated with *NBN* gene expression in the PCOS group ($P = 0.003$); cc3 was negatively correlated with *DIAPH2* and *NBN* gene expression in the PCOS group ($P = 0.007$ and $P = 0.001$, respectively)

*DIAPH2* = diaphanous related formin 2; *NBN* = nibrin; *NEK4* = NIMA-related protein kinase; rs = correlation coefficient; other abbreviations as in TABLE 2.

*et al., 2012; Motato et al., 2016*) different time-lapse parameters.

According to the results of this study, the mean timing of all events was longer in the PCOS group compared with the controls. The findings are in line with the *Wissing et al. (2014)* study that showed embryos from hyperandrogenic PCOS women are significantly delayed in terms of developmental timings. With regard to reproductive outcomes, biochemical pregnancy, clinical pregnancy and live birth rates were not significantly different in the PCOS group compared with the control group with tubal infertility. This shows that embryo developmental delay in PCOS women did not influence embryo implantation potential. In accordance with our findings, *Bellver et al. (2013)* indicated that embryos from obese women, including PCOS, cleaved significantly more slowly than embryos from non-obese fertile women. This supports the concept that maternal metabolic and hormonal pattern influence embryo development *in vitro* (*Bellver et al., 2013; Wissing et al., 2014*). Neither in this study nor in the Bellver or Wissing studies were implantation and pregnancy rates affected by delayed embryo cleavage. tPNf was previously considered a good parameter for embryo selection. In a prospective study of 159 embryos, no live birth was achieved by embryos with tPNf less than 20 h and 45 min, which suggests a relationship between the two. Furthermore, the PNf time of embryos resulting in live birth was significantly longer than the PNf time of the no live birth group (*Azzarello et al., 2012*). In this study tPNf was significantly shorter in implanted embryos in PCOS patients as well as in all transferred embryos of cases resulting in biochemical pregnancy, clinical pregnancy and live birth compared with those failing to achieve pregnancy and live birth in either group. Similarly, *Goodman et al. (2016)* reported a significantly shorter tPNf in implanted embryos compared with embryos that did not implant. Recently, in a retrospective observation, 500 microinjected oocytes were assessed by TLS, and the results showed that time intervals between distinct fertilization events were highly correlated with embryo quality on Day 3. This study stated that longer intervals between the fading of the cytoplasmic halo and PN breakdown were strongly predictive of decreased blastomere number and increased embryo fragmentation (*Coticchio et al., 2018*). In our study, for PCOS women the cleavage variables cc2 and s1 were also significantly different

**FIGURE 2** (Continued) $P < 0.01$. (C) Gene expression in all transferred embryos (100 embryos) regarding biochemical pregnancy in the TF group, significant differences in *NBN* gene expression between positive (18 embryos from 18 women) and negative (82 embryos from 32 women) biochemical pregnancy; $P < 0.01$. (D) Gene expression in all transferred embryos (106 embryos) regarding biochemical pregnancy in PCOS group, significant differences in *DIAPH2* and *NBN* gene expression between positive (24 embryos from 20 women) and negative (82 embryos from 30 women) biochemical pregnancy; $P < 0.01$. (E) Gene expression in all transferred embryos regarding clinical pregnancy in the TF group, significant differences in *NBN* gene expression between positive (12 embryos from 12 women) and negative (88 embryos from 38 women) clinical pregnancy; $P < 0.01$. (F) Gene expression in all transferred embryos regarding clinical pregnancy in PCOS group, significant differences in *DIAPH2* and *NBN* gene expression between positive (18 embryos from 14 women) and negative (88 embryos from 36 women) clinical pregnancy; $P < 0.01$. (G) Gene expression in all transferred embryos regarding live birth in the TF group, significant differences in *NBN* gene expression between positive (11 embryos from 11 women) and negative (89 embryos from 39 women) live birth; $P < 0.01$. (H) Gene expression in all transferred embryos regarding live birth in the PCOS group, significant differences in *DIAPH2* and *NBN* gene expression between positive (18 embryos from 14 women) and negative (88 embryos from 36 women) live birth; $P < 0.01$. Values shown as mean ± SD.

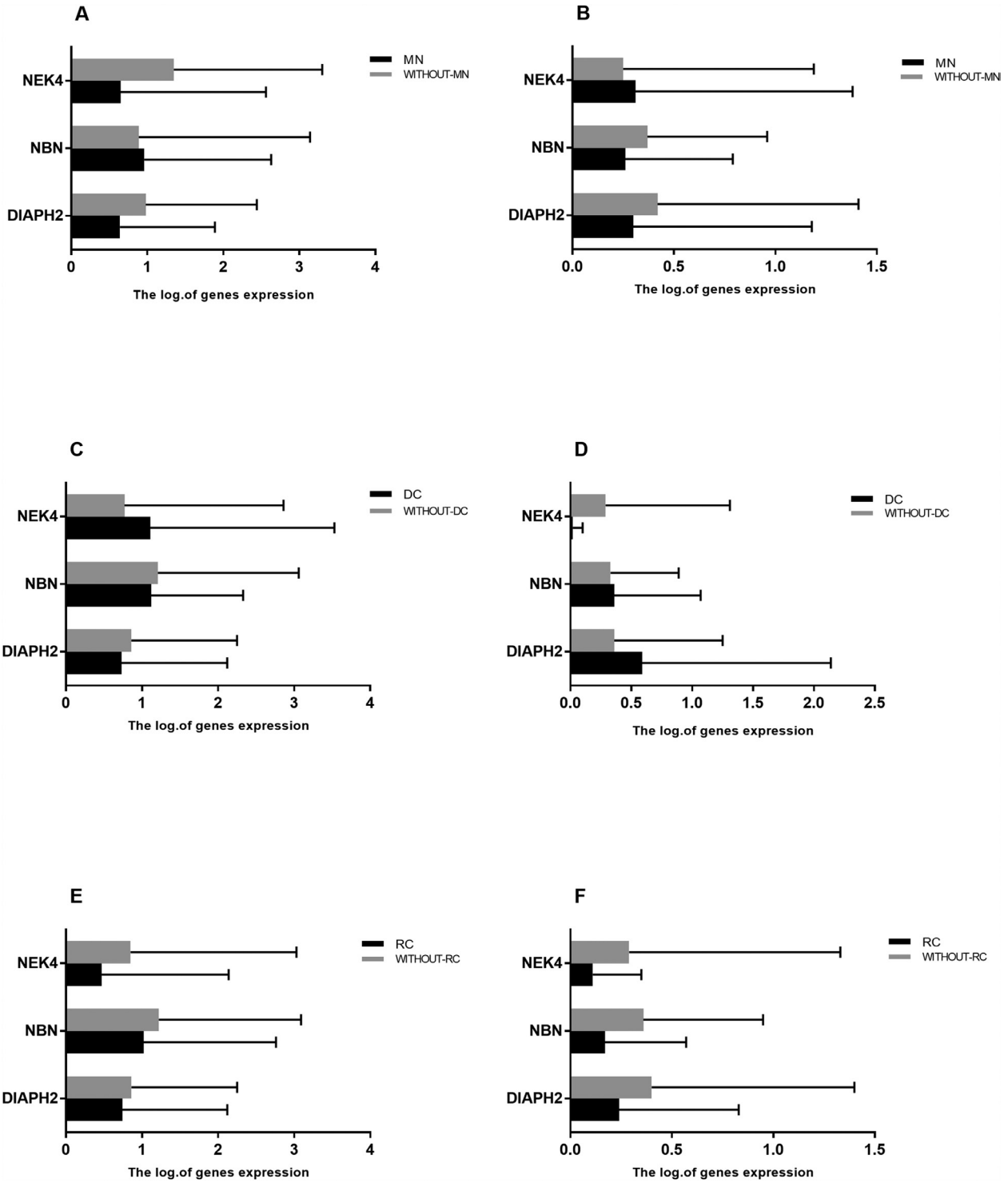


**FIGURE 3** Cumulus cell gene expression of *DIAPH2*, *NBN and NEK4* regarding embryo morphology events in all embryos: multinucleation (MN), direct cleavage (DC) and reverse cleavage (RC). (A) Gene expression in embryos with MN (112 embryos) and without MN (177 embryos) in PCOS patients. (B) Gene expression in embryos with MN (86 embryos) and without MN (172 embryos) in tubal factor (TF) patients. (C) Gene expression in embryos with DC (28 embryos) and without DC (261 embryos) in PCOS patients. (D) Gene expression in embryos with DC (20 embryos) and without DC (238 embryos) in TF patients. (E) Gene expression in embryos with RC (38 embryos) and without RC (251 embryos) in PCOS patients. (F) Gene expression in embryos with RC (30 embryos) and without RC (228 embryos) in TF patients. There are no significant differences among the above analyses. Values shown as mean ± SD.

between positive and negative outcomes for implantation, clinical pregnancy and live birth. Desai and colleagues showed a significant difference in s1 between embryos developing to blastocysts versus those that failed to blastulate (*Desai et al., 2014*). *Meseguer et al. (2011)* indicated significant differences between implanted embryos and those

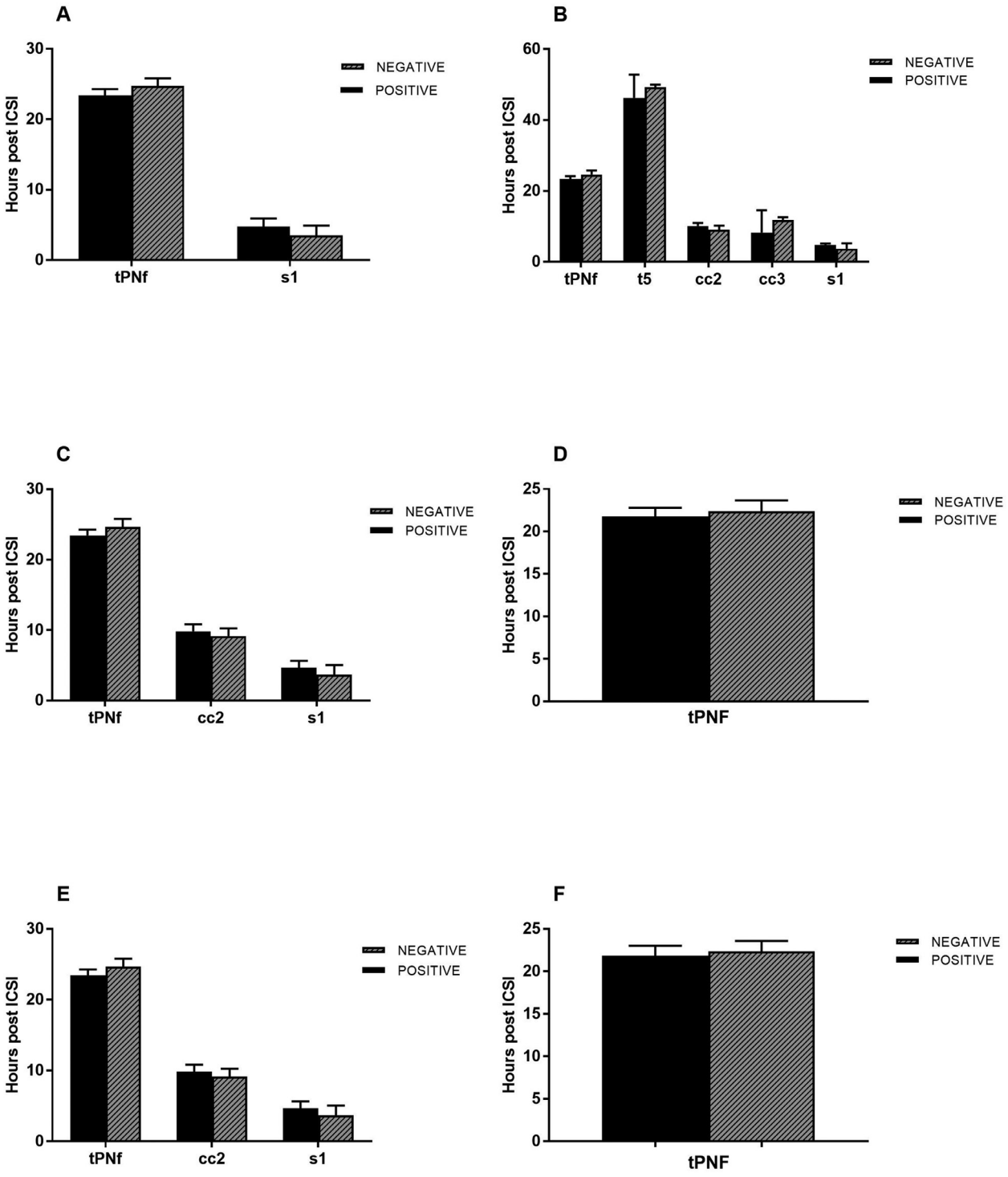


**FIGURE 4** Early embryo cleavage timing and reproductive outcome. (A) Significant differences between embryo cleavage timing in all transferred embryos and biochemical pregnancy status; positive for 20 women (24/106 embryos transferred) and negative for 30 women (82 embryos) in polycystic ovarian syndrome patients (PCOS); $P < 0.01$. (B) Significant differences between embryo cleavage timing and embryos with known implantation status; 8 embryos implanted from 4 women and 73 non-implanted embryos from 36 women in PCOS patients; $P < 0.01$. (C) Significant differences between embryo cleavage timing in all transferred embryos and clinical pregnancy status; positive for 14 women (18/24 embryos implanted) and negative for 36 women (88 embryos) in PCOS patients; $P < 0.01$. (D) Significant differences between embryo cleavage timing in all transferred embryos and biochemical pregnancy status; positive for 18 women (18/100 embryos transferred) and negative for 32 women (82 embryos) in tubal infertility patients; $P < 0.01$. (E) Significant differences between embryo cleavage timing in all transferred embryos and live birth status; positive for 14 women (18/24 embryos implanted) and negative for 36 women (88 embryos) in PCOS patients; $P < 0.01$. (F) Significant differences between embryo cleavage timing in all transferred embryos and clinical pregnancy status; positive for 12 women (12/18 embryos implanted) and negative for 38 women (88 embryos) in tubal factor patients; $P < 0.01$.

**TABLE 4 LOGISTIC REGRESSION MODEL FOR CLINICAL PREGNANCY AND LIVE BIRTH**

| Variable | Live birth | | Clinical pregnancy | |
|---|---|---|---|---|
| | OR (95% CI) | *P*-value | OR (95%CI) | *P*-value |
| tPNf | 0.21 (0.10–0.43) | <0.01 | 0.19 (0.086–0.42) | <0.01 |
| t2 | 0.77 (0.32–1.89) | NS | 0.82 (0.30–2.36) | NS |
| t3 | 0.49 (0.22–1.19) | NS | 0.53 (0.22–1.23) | NS |
| t4 | 1.12 (0.44–2.80) | NS | 0.89 (0.35–2.26) | NS |
| t5 | 0.95 (0.66–1.39) | NS | 0.91 (0.52–1.59) | NS |
| t6 | 1.99 (0.54–7.35) | NS | 4.47 (1.05–19.03) | NS |
| t7 | 2.01 (0.54–7.50) | NS | 3.84 (0.91–16.24) | NS |
| t8 | 1.10 (0.37–3.26) | NS | 0.92 (0.27–3.16) | NS |
| MN | 0.95 (0.23–3.87) | NS | 1.76 (0.38–8.19) | NS |
| RC | 1.64 (0.22–12.01) | NS | 0.97 (0.12–7.58) | NS |
| DC | 1.00 (0.99–1.00) | NS | 1.00 (0.99–1.00) | NS |
| Group (PCOS vs TF) | 1.33 (0.76–2.35) | NS | 1.41 (0.84–2.35) | NS |
| Patient age (years) | 0.96 (0.83–1.10) | NS | 0.90 (0.76–1.05) | NS |
| AMH (ng/ml) | 1.13 (0.97–1.31) | NS | 1.18 (1.00–1.38) | NS |
| No. of embryos transferred | 0.99 (0.70–1.41) | NS | 0.89 (0.60–1.33) | NS |
| *DIAPH2* gene expression | 3.47 (1.67–7.18) | <0.01 | 4.57 (1.89–11.03) | <0.01 |
| *NBN* gene expression | 1.00 (0.46–2.20) | NS | 0.77 (0.31–1.90) | NS |
| *NEK4* gene expression | 1.63 (1.06–2.50) | NS | 1.82 (1.13–2.94) | NS |

Data are presented as mean ± SD.

AMH = anti-Müllerian hormone; CI = confidence interval; *DIAPH2* = diaphanous related formin 2; MN = multinucleation; *NBN* = nibrin; *NEK4* = NIMA-related protein kinase; NS = non-significant; OR = odds ratio; RC = reverse cleavage; other abbreviations as in **TABLE 2**.

that did not for timing of t2, t3, t4, t5, cc2 and s2; they also suggested t5 and cc2 as two main parameters for prediction of implantation. Likewise, two randomized control trials proposed cc2 as a main prediction factor in the morphokinetic algorithm for embryo selection (*Goodman et al., 2016; Rubio et al., 2014*). *Kirkegaard et al. (2013)* claimed that embryo development to high-quality blastocyst could be expected by the presence or absence of direct cleavage. Similarly, other studies reported the negative impact of direct cleavage on implantation rate (*Goodman et al., 2016; Rubio et al., 2012*). However, we found no significant difference between embryos with or without direct cleavage regarding reproductive outcome. We also evaluated two other morphological abnormalities were evaluated in the embryos in this study. According to our results, multinucleation and reverse cleavage were similar in women with negative and positive biochemical pregnancy, clinical pregnancy and live birth. It was specified that the presence of multinucleation in embryos was negatively associated with implantation and birth outcome (*Desai et al., 2016; Desch et al., 2017; Goodman et al., 2016*). Nevertheless, it has been shown that most multinucleation embryos have the ability to self-correct during the early cleavage divisions and can progress into euploid blastocysts (*Desai et al., 2014*) or achieve live birth (*Balakier et al., 2016*). In line with the findings of *Goodman et al. (2016)*, we did not find any association between reverse cleavage and reproductive outcome. *Desai et al. (2014)* also reported that more than 40% of embryos with reverse cleavage can be considered for freezing. In the current study, a logistic regression model showed that tPNf could significantly predict pregnancy and live birth. However, the three morphological events evaluated in this study could not be considered as predictors of pregnancy and live birth. In this study, the rate of morphological abnormalities was low among transferred embryos due to discarding during time-lapse selection. Therefore, it is difficult to show a statistically significant difference between these abnormalities and clinical outcome. In our opinion the different findings between studies are due to the individual difference in the cohort of embryos studied as well as in study design. In addition, most of the studies combined both Day 3 and Day 5 embryo transfer (*Goodman et al., 2016; Rubio et al., 2014*) and the main difference in reproductive outcome seemed to apply to the Day 5 transfer patients. Furthermore, it is reported that different stimulation protocols (*Gurbuz et al., 2016*), as well as altered culture media, may impact embryo development (*Hardarson et al., 2015*).

This study also investigated whether cumulus cell gene expression and time-lapse parameters of the related embryo could be combined to better predict reproductive outcome. Several studies have tried to detect candidate genes expressed in cumulus cells that may be used as predictors for embryo quality (*Anderson et al., 2009; Devjak et al., 2016*) or implantation rate (*Borup et al., 2016; Gebhardt et al., 2011*). However, there is no uniformity in the proposed and studied genes. Assou and colleagues reported a significantly higher implantation and ongoing pregnancy rate while using cumulus cell gene expression as the embryo selection method (*Assou et al., 2010*). In two separate studies, *Wathlet et al. (2012)* showed the prognostic ability of *CAMK1D* and *EFNB2* as well as *SDC4* and *VCAN* for the prediction model

of pregnancy (*Wathlet et al., 2011*). In contrast, a recent study using microarray could not find any significant difference in gene expression in cumulus cells and granulosa cells between non-implanted and implanted embryos (*Burnik Papler et al., 2015*). We found that *NBN* gene expression is higher in PCOS women compared with controls. The result partially confirmed our hypothesis based on the previous study that some genes involved in the meiotic and/or mitotic cell cycle are highly expressed in the PCOS oocytes (*Wood et al., 2007*). Similarly, *NBN* and *DIAPH2* gene expression in cumulus cells were higher for implanted embryos, as well as in all transferred embryos of cases resulting in biochemical and clinical pregnancy and live birth. Application of a logistic regression model to our results showed *DIAPH2* gene expression to be independently associated with increased pregnancy and live birth (3.4-fold and 4.5-fold, respectively).

The current study also revealed correlations between cumulus cell gene expression and time-lapse parameters of the associated embryo. Higher expression levels of *DIAPH2* and *NBN* (which are involved, respectively, in spindle dynamics and chromosome alignment in cumulus cells of PCOS women) correlated with faster cleavage to 5-cell stage and decreased duration of the third cell cycle. Moreover, these two time-lapse parameters are significantly shorter for implanted embryos of PCOS women. This means that implanted embryos exhibit faster cleavage to 5-cell stage and decreased duration of the third cell cycle. *Wong et al. (2010)* evaluated the expression of nine genes involved in cytokinesis including *DIAPH2* and reported that arrested 2-cell embryos, which exhibited prolonged cytokinesis, displayed a significantly lower cumulus cell expression of all mentioned genes compared with normally developed embryos. Taken together, these results and our findings suggest the importance of mitotic genes in cumulus cells for prediction of the development pattern in early embryos and of clinical outcome. Recently, a single study revealed that 11 genes were involved in energy metabolism related to embryo developmental events detected by time lapse. However, logistic regression of the combination of gene expression and time-lapse parameters did not recognize any significant prediction of embryo quality (*Hammond et al., 2015*). The study only reported embryo quality on Day 5 and was unclear on reproductive outcomes. However, their finding on the expression levels of mitochondrial and glycolytic genes in combination with our results suggest that the metabolic status of cumulus cells plays a key role in embryo cleavage events. Similarly, our findings showed higher cumulus cell expression of the *DIAPH2* gene for the transferred embryos of PCOS women with positive compared with negative reproductive outcome. The *DIAPH2* gene was categorized previously as a maternally inherited transcript (*Wong et al., 2010*). Former studies have shown that time-lapse embryo cleavage timelines reveal the gene expression pattern of early developing embryos which could be inherited from the related oocytes (*Hammond et al., 2015; Wong et al., 2010*). Our study supports this theory but also delivers novel insight into how cumulus cell gene expression impacts early embryo developmental kinetics as well as pregnancy and live birth in PCOS patients as a large subgroup of infertile women.

Several reports have shown that age as well as AMH is a good independent predictor of pregnancy and live birth in ART cycles (*Broer et al., 2013; Gleicher et al., 2010; Khader et al., 2013; La Marca et al., 2011; Mutlu et al., 2013*). Surprisingly, in the present study, it was found that age was not an independent parameter for predicting clinical pregnancy rate and live birth. In a recent study, *Ramezanali et al. (2016)* evaluated IVF/ICSI outcomes in different PCOS phenotypes including A, B, C and D. They reported that women's age is the significant predictor for live birth in phenotypes C and D but not in phenotypes A and B. The authors concluded that in different subgroups of PCOS, the factors influencing the chance of pregnancy and live birth have different predictive values (*Ramezanali et al., 2016*); however, we did not perform analysis according to the different PCOS phenotypes. On the other hand, another study assessed the prediction value of ovarian reserve tests and indicated that AMH and age were the exclusive predictive elements of live birth among women >35 years, while the number of good-quality embryos was the sole marker for prediction of live birth in women <35 (*Lee et al., 2009*). In the current study neither age nor the number of transferred embryos were significant predictors of clinical pregnancy and live birth. However, only 16% of women were aged over 35 years. Furthermore, we did not classify patients according to their age. Moreover, it should be noted that various factors such as sample size, number of retrieved oocytes, transfer technique and endometrial receptivity define the probability of achieving pregnancy in ART cycles (*Boomsma and Macklon, 2007*); this may explain why the aforementioned tests are not sensitive enough to predict ART outcome.

The main limitation of this study is its small sample size, which may affect the predictive power in the multivariable regression model. The other limitation is the number of genes investigated as well as category of the studied population. The current study could serve as a prospective pilot study and may aid estimation of the size and design of future studies.

In conclusion, for the first time, this study demonstrated the correlation between cumulus cell gene expression and the time-lapse parameters of early developing embryos in PCOS women. These findings support the concept that cumulus cell genes involved in cytokinesis are influenced by the ovarian microenvironment in metabolic disorders such as PCOS. The current study also revealed that tPNF, as well as expression of the *DIAPH2* gene, could independently predict clinical pregnancy and live birth in associated embryos. In this innovative and developing field, larger studies are required to confirm these results and transform these basic findings to clinical application.

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