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ASSISTED REPRODUCTION TECHNOLOGIES

Cumulus cell pappalysin-1, luteinizing hormone/choriogonadotropin receptor, amphiregulin and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 mRNA levels associate with oocyte developmental competence and embryo outcomes

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Abstract

Purpose To determine whether a selected set of mRNA biomarkers expressed in individual cumulus granulosa cell (CC) masses show association with oocyte developmental competence, embryo ploidy status, and embryo outcomes.

Methods This prospective observational cohort pilot study assessed levels of mRNA biomarkers in 163 individual CC samples from 15 women stimulated in antagonist cycles. Nineteen mRNA biomarker levels were measured by real-time PCR and related to the development of their corresponding individually cultured oocytes and subsequent embryos, embryo ploidy status, and live birth outcomes.

Results *PAPPA* mRNA levels were significantly higher in CC from oocytes that led to euploid embryos resulting in live births and aneuploid embryos compared to immature oocytes by ANOVA. *LHCGR* mRNA levels were significantly higher in CC of oocytes resulting in embryos associated with live birth compared to immature oocytes and oocytes resulting in arrested embryos by ANOVA. Using a general linearized mixed model to assess ploidy status, CC *HSD3B* mRNA levels in oocytes producing euploid embryos were significantly lower than other oocyte outcomes, collectively. When transferred euploid embryos outcomes were analyzed by ANOVA, *AREG* mRNA levels were significantly lower and *PAPPA* mRNA levels significantly higher in CC from oocytes that produced live births compared to transferred embryos that did not form a pregnancy.

Conclusions Collectively, *PAPPA*, *LHCGR*, and *AREG* mRNA levels in CC may be able to identify oocytes with the best odds of resulting in a live birth, and *HSD3B1* mRNA levels may be able to identify oocytes capable of producing euploid embryos.

Keywords Cumulus cells · Real-time PCR · mRNA levels · Oocyte developmental competence · Euploid embryo

Introduction

Successful in vitro fertilization (IVF) is highly dependent upon the quality of the oocytes harvested and the subsequent quality of embryos used for uterine transfer [1]. Several studies have shown that use of preimplantation genetic testing for aneuploidy (PGT-A) to select euploid embryos for uterine transfer increases implantation, ongoing pregnancy, and delivery rates [2–4], whereas other studies showed PGT-A decreased pregnancy rates [5, 6]. Conflicting PGT-A findings might indicate that other methods beyond genetic screening are needed to improve IVF pregnancy rates. The differing PGT-A study results may also reflect the invasive methodology of PGT-A, which requires the removal of 3–10 cells from the trophectoderm. Increased numbers of trophectoderm cells per biopsy have been correlated with decreased implantation rates [7]. Therefore, a method for identifying oocytes that will produce euploid embryos with the greatest chances of leading to a live birth that does not require embryo manipulation would be advantageous.

Some non-invasive methods for assessing oocyte and embryo live birth potential include preimplantation embryo

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metabolomics and proteomics of secreted products [8, 9] and granulosa cell (GC) mRNA biomarkers [10]. The use of GC biomarkers is based on knowledge that cumulus granulosa cells (CCs) and mural granulosa cells (MGCs) interact with oocytes and can reflect the health and maturational status of the oocyte [1, 11]. GCs are obtained as a by-product of oocyte retrieval and can be rapidly assessed for mRNA levels by reverse transcription and quantitative polymerase chain reaction (PCR). Several studies suggest that CC biomarker mRNA levels from individual oocytes can be used to generate models capable of predicting pregnancy and live birth [12–14].

Approaches to evaluate differentially expressed genes in CCs have employed large-scale microarray, transcriptome deep sequencing, PCR arrays, and quantitative PCR [10]. In most cases, the ploidy status of the embryos was not known, which could have altered the study outcomes. Only a handful of studies have examined CC mRNA levels in conjunction with oocyte or embryo ploidy status [15–17]. Taken together, a focused quantitative PCR analysis of CC target genes selected from prior studies combined with the ploidy status of the embryos should yield valuable information regarding mRNA biomarker usefulness in predicting live birth.

Many oocytes fail to fertilize, or when fertilized, arrest before reaching the blastocyst stage, or fail to develop into euploid blastocysts. We hypothesize that while many mature oocytes appear morphologically normal, the actual developmental competence of those oocytes is variable and will be reflected by varying mRNA levels of specific biomarkers in their associated CCs. Furthermore, we propose that a CC mRNA biomarker model could potentially indicate whether a mature oocyte, when fertilized, will result in a euploid embryo. In addition, we hypothesize that certain CC mRNA biomarkers and their levels will associate with oocytes that are capable of producing viable embryos which result in live births.

A comprehensive review of the human literature for potential CC candidate mRNA biomarkers was performed [10], and genes were selected based on their functions. In some cases, the genes selected were based on data in non-human animals [18–24]. Gene products associated with oocyte maturation included calmodulin 1 (*CALM1*), gap junction alpha-4 protein (*GJA4*), gremlin (*GREM1*), and inhibitor of growth protein 1 (*ING1*). Genes associated with cumulus mass expansion included amphiregulin (*AREG*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and versican (*VCAN*). Genes encoding proteins mediating de novo steroidogenesis, estrogen, and progesterone production included aromatase (*CYP19A1*), cholesterol side-chain cleavage enzyme (*CYP11A1*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*), and steroidogenic acute regulatory protein (*STARD1*). Genes related to hormone modulation included insulin-like growth factor 2 (*IGF2*), insulin-like growth factor binding protein 5 (*IGFBP5*), and inhibin beta A subunit (*INHBA*), and pappalysin-1 (*PAPPA*). Gene encoding hormone receptors included follicle-stimulating hormone receptor (*FSHR*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*), progesterone receptor (*PGR*), and progesterone receptor, membrane component 1 (*PGRMC1*). The primary goal of this study was to determine if a model of specific CC mRNA biomarker levels could identify a mature oocyte's ability to produce euploid embryos. The secondary goal of this study was to determine if specific CC mRNA biomarker levels could indicate oocytes capable of leading to euploid embryos with live birth outcomes.

Materials and methods

Study population, participants, and CC isolation

This study is a prospective observational cohort pilot study. In total, 164 individual CC masses were harvested from their corresponding oocytes from 15 patients undergoing infertility treatment with PGT-A at Advanced Fertility and Reproductive Endocrinology Institute (Columbia, SC, USA) from February 2015 through June 2016. Patients either electively desired PGT-A or it was recommended based on the patients' diagnosis (Table 1). Patients with endometriosis or diminished ovarian reserve (DOR) were not included as prior work indicates these conditions alter GC gene expression [25–28]. Patient demographics and cycle information (Table 1), blastocyst development, PGT-A results, and live birth data (Supplemental Table 1), as well as CC mRNA levels were collected. Each patient's ovaries were stimulated using an antagonist protocol until at least one follicle at 15 mm was present when a GnRH antagonist (Cetrotide, EMD-Serono, USA, or Ganirelix, EMD-Serono, USA) was administered. When two follicles reached 18 mm or 50% of the follicles were ≥ 15 mm by ultrasound, an ovulatory dose of human chorionic gonadotropin (hCG) (Pregnyl, EMD-Serono, USA) or Lupron (Leuprolide Acetate, Sandoz, USA) was delivered. Thirty-six hours later, patients underwent oocyte retrieval. Each CC mass was mechanically separated from its oocyte and rinsed in medium (HTF-HEPES, Irvine, USA) to remove debris. Each CC mass and its associated oocyte/embryo were kept separate throughout the entire process. A summary flowchart of the fate of the collected oocytes is shown in Fig. 1. CC masses were snap frozen and maintained in liquid nitrogen until they were transported to the University of South Carolina School of Medicine for further processing.

Embryo culture and ploidy assessment

Mature oocytes were inseminated via intracytoplasmic sperm injection. Fertilization was confirmed by the presence of two pronuclei and two polar bodies 16–18 h after insemination.

Table 1 Patient demographics and cycle information

Pt#	Age	BMI	Diagnosis/PGT-A reason	Cycle #	Day 3 FSH level (IU/ml)	E2 per oocyte (pg/ml)	# oocytes	# mature	# fertilized	# embryos biopsied	Trigger
1	27.8	25.3	Sex selection	3	5.4	141.9	8	8	8	4	hCG
2	36.4	26.4	Male factor	1	7.2	86.6	8	7	6	4	hCG
3	39.7	25	AMA	1	12	100.4	7	4	3	3	hCG
4	30	25.6	Donor	1	6	147.4	10	9	7	6	hCG
5	32.6	26.4	Translocation w/PGT-A	2	9.2	131.0	5	5	5	5	Lupron
6	35.9	21	Idiopathic	1	8.3	163.3	4	4	4	2	Lupron
7	39.7	38.8	AMA	1	11	145.5	9	9	6	5	Lupron
8	40	32	AMA + male factor	1	12	31.4	7	5	5	4	Lupron
9	33	18.8	Male factor	1	10	194.7	11	10	10	4	Lupron
10	42	20.3	AMA	1	6.6	108.2	5	5	5	3	hCG
11	36.1	29.2	Tubal factor	1	5.2	179.5	23	23	18	12	Lupron
12	37	20.7	AMA	1	11	142.2	16	15	14	8	Lupron
13	33.9	35	Male factor	2	10	183.1	11	10	8	2	hCG
14	28	28.4	PCO	1	5.9	184.2	24	6	5	4	hCG
15	39.7	31	AMA	1	7.5	132.6	16	14	14	7	Lupron

AMA advanced maternal age, *PCO* polycystic ovaries, *hCG* human chorionic gonadotropin

Oocytes and embryos were individually cultured in 20 μL media drops (Global total, Global, USA) under oil (Ovoil, Vitrolife, USA) at 37 °C, 6% CO_2, 5% O_2, 89% N_2 in a humidified atmosphere until day 5 or day 6 post retrieval. Blastocyst morphologies were assessed using Gardner's blastocyst grading scale [29], and 3–7 trophectoderm cells were

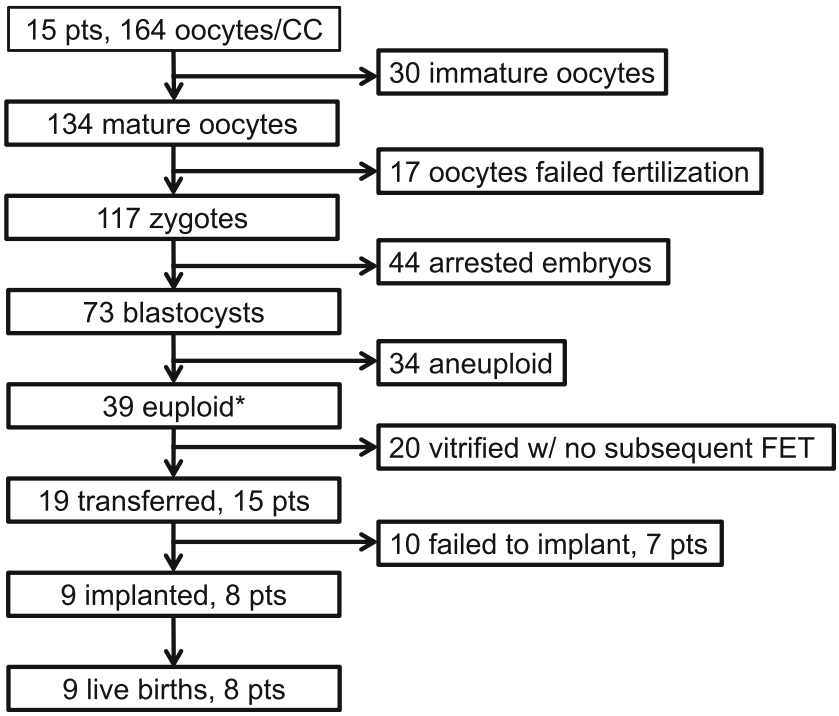

Fig. 1 Flow diagram summarizing the study population from oocyte retrieval to final outcome. Oocytes (164) were retrieved from 15 patients and individually cultured. Individual CC masses from each oocyte were collected and mRNA was harvested from each mass. Of the 164 oocytes, 134 were mature and 30 were immature. All 134 were injected with sperm. Seventy-three of the fertilized oocytes became blastocysts and were biopsied for PGT-A testing and all were vitrified. PGT-A results indicated that 39 embryos were euploid and 34 were aneuploid. Nineteen of the euploid embryos were transferred and 20 remained in cryostorage. Ten embryos from 7 patients failed to implant while 9 embryos from 8 patients implanted and resulted in live births. *The control TBP mRNA did not amplify in one CC sample from an oocyte yielding a euploid embryo and was not included in the data analysis. RNA analyses were performed on $n = 163$ individual cumulus masses

biopsied and sent to Igenomix (Miami, FL, USA) for PGT-A using next-generation sequencing. Fifteen frozen embryo transfers (FETs) were performed using the highest quality euploid embryos (19 embryos total) for each patient. Live birth outcomes were obtained from the patient's obstetrician including delivery dates and if there were any maternal or neonatal interventions or complications.

RNA isolation and cDNA synthesis

RNA was isolated from each CC mass using the Direct-zol MiniPrep Kit with DNase treatment (Zymo Research, Irvine, CA, USA). RNA concentration and purity were assessed at the wavelengths of 260 and 280 nm using a spectrophotometer with a 2 mm lid (NanoDrop 2000C, Thermo Scientific, USA). Sample amounts varied between 80 and 360 ng RNA per individual sample. Each sample was reverse transcribed into cDNA using the Bio-Rad iScript kit (Hercules, CA, USA). The reverse transcription reaction was carried out in an Eppendorf thermocycler, for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C.

Quantitative real-time PCR

Gene primer sequences, annealing temperatures, and amplicon sizes can be found in Table 2. Synthesized primers were cartridge purified (Life Technologies, Carlsbad, CA, USA). PCR reactions were run with two or more wells per sample for 45 cycles and threshold cycle (Ct) values were averaged. The reaction included 2 μL of cDNA (6–27 ng starting RNA), 300 nM of each upstream and downstream primer, and 10 μL 2X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and sterilized PCR-grade water to a final volume to 20 μL per well. PCR-grade water was substituted for the cDNA as a negative control. PCR amplification was performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad, USA). TATA-box binding protein (*TBP*) mRNA was used as an internal control [30]. Single amplicons of correct size were verified using agarose gels and by the presence of single melt-curve peaks. mRNA quantities were derived from a standard curve for each mRNA made using serial dilutions of its purified amplicon. *TBP* has extensively been tested as a control gene in our laboratory with human granulosa cells under multiple conditions or treatments and has been found not to be regulated under any circumstances. Target mRNA values were expressed relative to values of the *TBP* mRNA control.

Statistical analysis

The mRNA levels were normalized using log2 transformation. Principal component analysis (PCA) was conducted to explore the mRNA level correlation patterns between the CC samples. CC mRNA levels were compared between different oocyte and subsequent embryo developmental outcomes using repeated measures analysis of variance (ANOVA) to accommodate for the variable number of oocytes per patient followed by Tukey's post hoc tests for pairwise comparisons. The CC samples used for these studies were divided into the following categories: immature oocytes, mature oocytes that failed fertilization, oocytes that produced embryos that arrested and were not appropriate for biopsy, oocytes that produced biopsied blastocysts that were aneuploid, and oocytes that produced biopsied blastocysts that were euploid. Oocytes that produced euploid embryos were further subdivided into embryos that resulted in live birth, embryos that resulted in a negative pregnancy, and embryos that were vitrified and not transferred. Each figure states which categories were used for the specific statistical analysis.

Statistical models

Models were estimated to predict multiple dichotomous oocyte developmental outcomes using generalized linear mixed models (GLMMs) fitted by maximum likelihood using the R package lme4 [31]. The GLMMs included the normalized gene expressions as variables and used patients' demographics (age and body mass index (BMI) as controlling factors and adjusted for two ovulation induction or trigger medications). Unstructured correlation patterns were used in fitted GLMMs to capture the correlation among the repeated observations from same individual. The statistical models aimed to include as many patients as possible. However, some of the genes had high proportion of missing values for some patients because of limited RNA amounts that were obtained from CC masses. To ensure maximum utilization of the data, we started the modeling by including all the genes and proceeded backward dropping the genes one-by-one with highest proportion of missing observations until the fitted mixed-effects model attained computational convergence successfully. Since different patients had missing observations on different genes, exclusion of the missing values eventually reduced the number of patients and number of observations in the sample used to fit the model. The analysis sample size differed from model-to-model (for different response variables) as the maximum possible data points were used for a model to converge. Statistical significance in all analyses was determined at a 0.05 significance level. All the inference *P* values were reported after adjusting for multiple comparisons [32]. The predictive performances of the fitted models were assessed using receiver operating characteristics (ROC) curves and area under ROC curves (AUC). The AUC values of the models used in this study ranged from 0.75 to 1.00 implying the models exhibited good/very good to excellent predictive accuracy. Statistical analysis was performed using the statistical software R 3.4.4 (http://www.r.project.org/).

Table 2 Primer set information

Gene	Primer sequences 5′–3′	Annealing temperature (°C)	Amplicon size (base pairs)	Reference or NCBI no.
AREG	For TGGACCTCAATGACACCTACTCTG Rev GGGCTTAACTACCTGTTCAACTCTG	62°	251	[44]
CALM1	For TACTTCGTGTGCTCCGACCCAT Rev AGTCCACAGCCACAGCCTACTC	62	231	[51]
CYP11A1	For GCAACGTGGAGTCGGTTTATGTC Rev GTGCAGGACACTGACGAAGTC	60	269	[52]
CYP19A1	For GCACATCCTCAATACCAGGTC Rev TTTGAGGGATTCAGCACAGAC	60	380	[38]
FSHR	For ACCAAGCTTCGAGTCATCC Rev CATCTGCCTCTATCACCTCC	58	103	[53]
GJA4	For CATCTCCCACATCCGCTACT Rev GAAGCCTGCCTCTAGCACAC	58	295	[54]
GREM1	For CGCCGCACTGACAGTATGAG Rev ACCTTGGGACCCTTTCTTTTTC	62	108	NM_013372.7
HSD3B1	For TGTGCCAGTCTTCATCTACAC Rev TGTTTTCCAGAGGCTCTTCTTC	60	101	[38]
IGF2	For TTCCGCAGCTGTGACCTGGC Rev CCTCGAGCTCCTTGGCGAGC	62	229	NG_008849.1
IGFBP5	For AAGAAGCTGACCCAGTCCAA Rev GAATCCTTTGCGGTCACAAT	60	201	[55]
ING1	For GCCTGGTGTGAGGAGGACAA Rev CCCTATGAAAGGAATGGTTCCTT	62	124	[56]
INHBA	For AAGTCGGGGAGAACGGGTATGTGG Rev TCTTCCTGGCTGTTCCTGACTCG	62	123	[57]
LHCGR	For TGGAGAAGATGCACAATGGA Rev GGCAATTAGCCTCTGAATGG	60	122	[58]
PAPPA	For GTCATCTTTGCCTGGAAGGGAGAA Rev AGGGCTGTTCAACATCAGGATGAC	62	129	[61]
PGR	For GTCATAGACCCCCGTTGCTA Rev GCTAAGCCAGCAAGAAATGG	60	124	[60]
PGRMC1	For TCTGGACTGCACTGTTGTCCTTG Rev GCAAACACCTGTTCCTATTCTG	60	290	[61]
PTGS2	For GCTTTATGCTGAAGCCCTATGA Rev TCCAACTCTGCAGACATTTCC	60	70	[45]
STARD1	For TACGTGGCTACTCAGCATCG Rev ACAGCAGGCTGGTCTTCAAC	60	157	[62]
TBP	For CACGGCACTGATTTTCAGTTC Rev TCTTGCTGCCAGTCTGGACT	62	79	[63]
VCAN	For GCACCTGTGTGCCAGGATA Rev CAGGGATTAGAGTGACATTCATCA	60	70	[45]

Results

In total, 164 CCs were harvested from 15 patients undergoing infertility treatment with PGT-A. Fifteen FETs were performed after PGT-A results were obtained. Eleven patients had single embryo transfers and four had double embryo transfers. In the four cases where two embryos were selected, one embryo of each gender was transferred. Eight patients gave birth to nine healthy children with no complications. One hundred sixty-three of 164 CC samples ($n = 15$ patients) analyzed contained a sufficient amount of RNA to detect the endogenous control house-keeping gene, *TBP*. Due to limited starting sample RNA amounts, not all samples were able to be tested for each biomarker and the variations in sample size are noted in figures.

PCA was utilized to test the hypothesis that several of the CC genes we examined would be correlated with one another. The correlations of the CC mRNA levels of 151 observations from 14 patients were examined by creating a biplot within the multivariable dataset that contained the most biomarkers and individual patient observations that fit the model (Fig. 2). The biplot is based on the first and second principal components (PC1 and PC2) of the log2-transformed mRNA levels of each individual CC mass. PC1 and PC2 explain 35.7% and 22.9% of the variability of the model, respectively. All genes shown were positively correlated to PC1. *HSD3B1*, *IGFBP5*, *PAPPA*, and *PGR* were all positively correlated with PC2 while *CYP11A1*, *CYP19A1*, and *INHBA* are negatively correlated with PC2. In addition, a significant ($P < 0.001$) strong positive correlation was observed between *CYP19A1* and *CYP11A1*

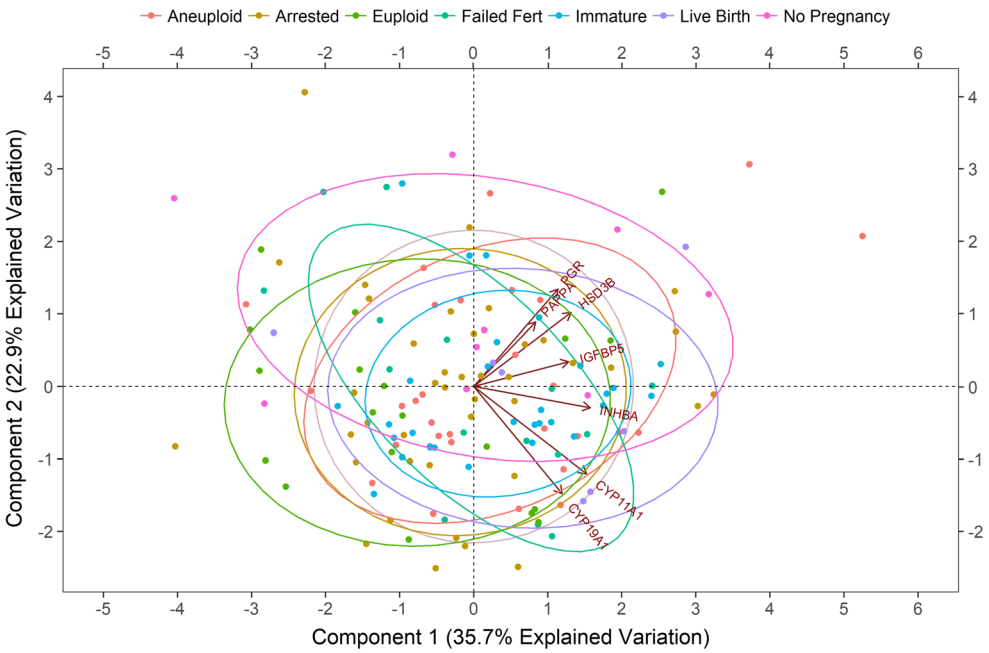

Fig. 2 Principal component analysis biplot for CC mRNA expression. Biplot of the log2-transformed mRNA levels for 7 genes from 14 patients along the first and second principal components (PC1 and PC2). PC1 accounted for 35.7% of the observed variance while PC2 accounted for 22.9% of the variance. Each data point represents an individual CC mass mRNA level associated with an individual oocyte ($n = 151$ oocytes). The biplot demonstrates the relationship between individual CC mRNA level patterns and the correlation between the different genes. The closer the data points are to each other, the more similar the normalized CC gene expression patterns. The closer the arrows are to each other, the higher the correlation between the normalized CC gene expressions. Groups represent CC mRNA from oocytes with the following descriptions: aneuploid = mature oocytes resulting in aneuploid embryos; arrested = mature oocytes resulting in embryos that did not reach the blastocyst stage; euploid = mature oocytes resulting in euploid blastocysts that were not transferred; failed fert = mature oocytes that did not fertilize; immature = immature oocytes that were not fertilized; live birth = oocytes that resulted in euploid embryos that resulted in live births; no pregnancy = oocytes that resulted in euploid embryos that did not result in a pregnancy

($r = .68$), and significant ($P < 0.001$) moderate positive correlations were observed for *PGR* and *HSD3B* ($r = .59$), *CYP11A1* and *INHBA* ($r = .50$), *PGR* and *IGFBP5* ($r = .41$), and *PAPPA* and *INHBA* ($r = .41$).

We next wanted to test the hypothesis that the CC mRNA levels of the selected genes would vary by oocyte developmental competence and resulting embryo endpoint. To test this hypothesis, we analyzed all the CC mRNA level data by the resulting developmental outcome for each group of oocytes by repeated measures ANOVA followed by Tukey's post hoc test (adjusted *P* values). *PAPPA* mRNA levels in CCs were higher in oocytes that resulted in euploid embryos that led to a live birth compared to immature oocytes and in oocytes that resulted in aneuploid embryos compared to immature oocytes, when comparing all groups ($P < 0.05$) (Fig. 3). *LHCGR* mRNA levels in CCs were higher for oocytes that resulted in embryos which led to a live birth compared to immature oocytes and oocytes that resulted in arrested embryos compared to immature oocytes, when comparing all groups ($P < 0.05$). Other CC biomarkers showed no statistical differences between groups and their expression profiles are shown in Supplemental figure 1.

We also wanted to test the hypothesis that certain CC biomarker mRNA levels associated with oocytes giving rise to euploid embryos would be distinct from those giving rise to aneuploid embryos and those oocytes that resulted in arrested embryos or those that failed fertilization. Using a GLMM, the best fitting model examining whether CC biomarker mRNA expression associated with oocytes that became euploid embryos included 11 patients ($n = 78$ CC masses) and *CYP11A1*, *CYP19A1*, *HSD3B1*, *IGFBP5*, *PAPPA*, *PGR*, *PGRMC1*, *ING1*, *LHCGR*, and *STARD1* (Fig. 4). CCs associated with oocytes that produced euploid embryos exhibited significantly ($P < 0.05$) lower CC *HSD3B1* mRNA levels than those oocytes that led to mature oocytes resulting in aneuploid blastocysts, oocytes producing embryos that arrested and did not reach the blastocyst stage, and mature oocytes that failed to fertilize, collectively (abnormal group). CC *ING1* mRNA levels associated with oocytes that produced euploid embryos trended toward being lower than CCs from oocytes that gave rise to the abnormal outcomes, collectively ($P = 0.061$).

A critical hypothesis we tested was that certain CC mRNA biomarker levels of oocytes giving rise to euploid embryos would be associated with live birth outcomes. CC mRNA levels from all 15 patients ($n = 19$ CC masses) were assessed for differences between oocytes yielding transferred embryos that resulted in a live birth and those

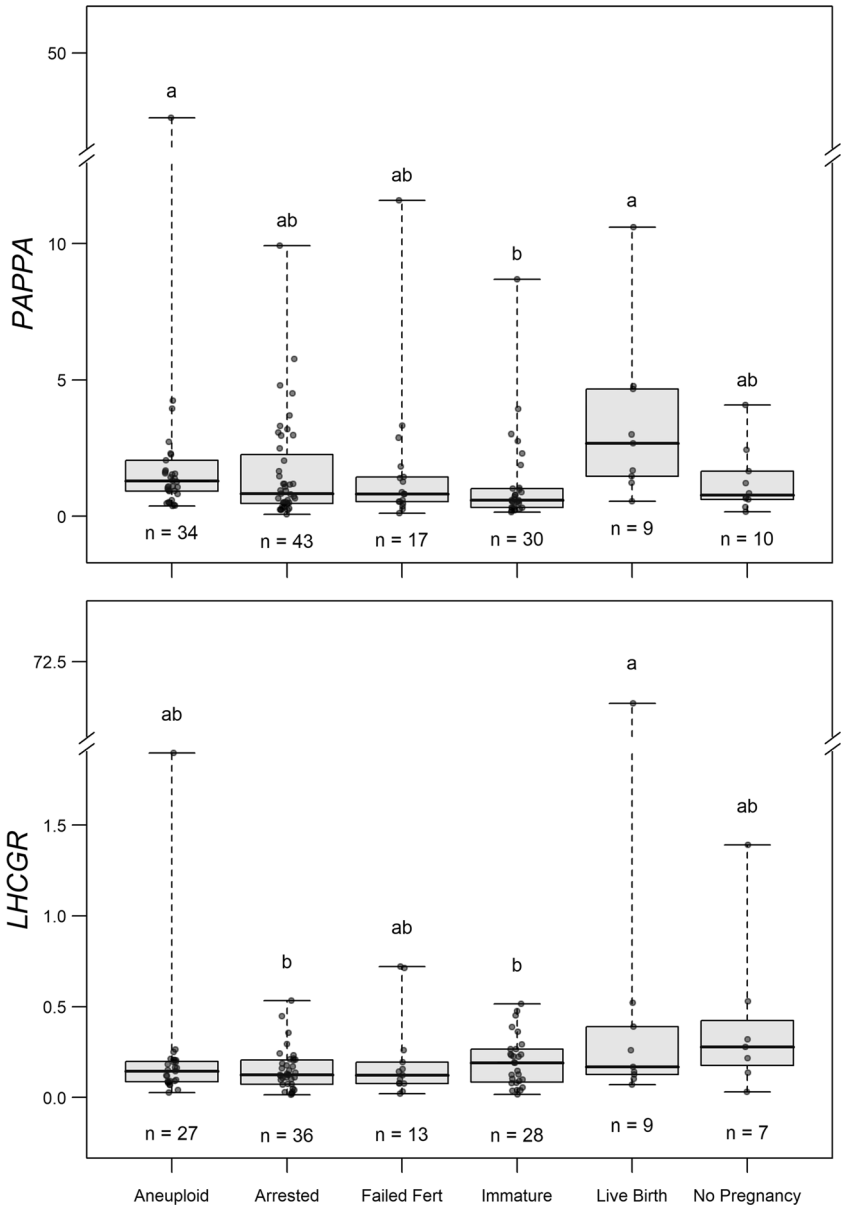

Fig. 3 Biomarkers for CC mRNA expression associated with mature oocyte competence and embryo outcomes. To determine the differences in CC mRNA between groups where oocytes had different developmental and embryo outcomes, target mRNA levels were compared using repeated measures ANOVA followed by Tukey's post hoc test (adjusted *P* values) for pairwise comparisons. Groups represent CC mRNA from oocytes with the following descriptions: aneuploid = mature oocytes resulting in aneuploid embryos; arrested = mature oocytes resulting in embryos that did not reach the blastocyst stage; failed fert = mature oocytes that did not fertilize; immature = immature oocytes that were not fertilized; live birth = oocytes that resulted in transferred euploid embryos that resulted in live births; no pregnancy = oocytes that resulted in transferred euploid embryos that did not result in a pregnancy. Data are presented as the median copy number (line inside box), first and third quartile (bottom and top of box), and highest and lowest data points (top and bottom of whiskers). Groups with different letters (a and b) exhibit significant differences ($P < 0.05$)

that did not form a pregnancy, using repeated measures ANOVA followed by Tukey's post hoc test (adjusted *P* values). *AREG* mRNA levels were significantly lower in CCs from oocytes that resulted in live births ($n = 8$ from 8 patients) compared to the no pregnancy group ($n = 7$ from 5 patients) ($P < 0.05$) (Fig. 5). *PAPPA* mRNA levels were higher in CCs from oocytes producing euploid embryos that resulted in live births ($n = 9$ from 8 patients) compared to those that produced no pregnancy ($n = 10$ from 7 patients) ($P < 0.05$). The other biomarkers showed no statistical differences between groups.

Using a GLMM, we also compared the CC mRNA biomarkers associated with oocytes that resulted in euploid embryos producing a live birth compared to oocyte outcomes not producing a live birth (non-viable group), collectively. The non-viable group included immature oocytes, mature oocytes resulting in aneuploid blastocysts, oocytes producing embryos that did not reach the blastocyst stage, and mature oocytes that failed to fertilize. The best fitting model for this comparison included 110 CC samples from 14 patients and included mRNA levels for *CYP11A*, *CYP19A1*, *IGFBP5*, *PAPPA*, *PGRMC1*, and *STARD1* (Supplemental Figure 2). As revealed in the fitted model, higher CC *PAPPA* mRNA levels ($P < 0.05$) significantly increased the odds of an oocyte producing an embryo that resulted in a live birth (OR = 4.591, 95% CI 1.098 to 19.201). The other five CC mRNA biomarkers did not significantly associate with live births.

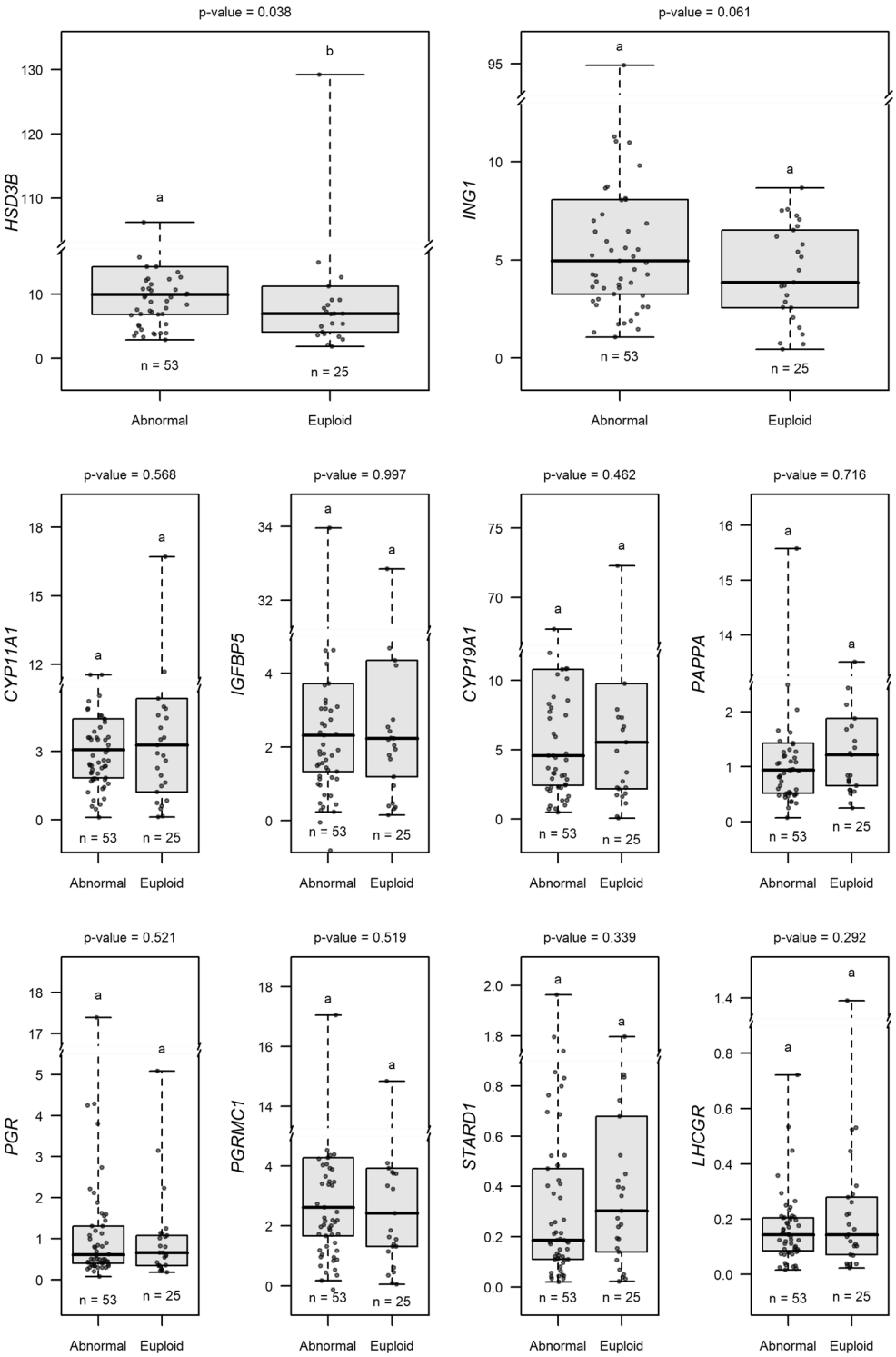

Fig. 4 Biomarkers from the GLMM model associated with oocytes producing euploid embryos versus mature oocytes with other outcomes. To evaluate CC biomarker mRNA level association with oocytes capable of producing euploid embryos, a model was fit with 78 CC samples from 11 patients and included the biomarkers: *CYP11A1*, *CYP19A1*, *HSD3B*, *IGFBP5*, *PAPPA*, *PGR*, *PGRMC1*, *ING1*, *LHCGR*, and *STARD1*. Higher *HSD3B* mRNA level significantly decreased the odds of an oocyte resulting in a euploid embryo (OR = 0.408, 95% CI 0.175 to 0.953). Higher *ING1* mRNA levels marginally decreased the odds of an oocyte resulting in a euploid embryo (OR = 0.552, 95% CI 0.297 to 1.027). Groups represent CC mRNA from oocytes with the following descriptions: abnormal = mature oocytes resulting in aneuploid blastocysts, oocytes producing embryos that did not reach the blastocyst stage, and mature oocytes that failed to fertilize; euploid = mature oocytes that resulted in euploid embryos regardless of whether they were transferred or remained vitrified. Data are presented as stated in Fig. 3 legend

Discussion

The key findings of this research were that higher *PAPPA*, higher *LHCGR*, and lower *AREG* mRNA levels in CCs were associated with oocytes that produced embryos capable of yielding live birth outcomes. In addition, lower CC *HSD3B1* mRNA was associated with oocytes that resulted in euploid embryos compared to other outcomes. Our premise was that although many retrieved oocytes in our study appeared mature, several oocytes would have mRNA expression profiles that were similar to those of immature oocytes. Aberrant mRNA levels would cause them to be incapable of forming euploid embryos and leading to live births. Therefore, an individual oocyte's developmental competence would be

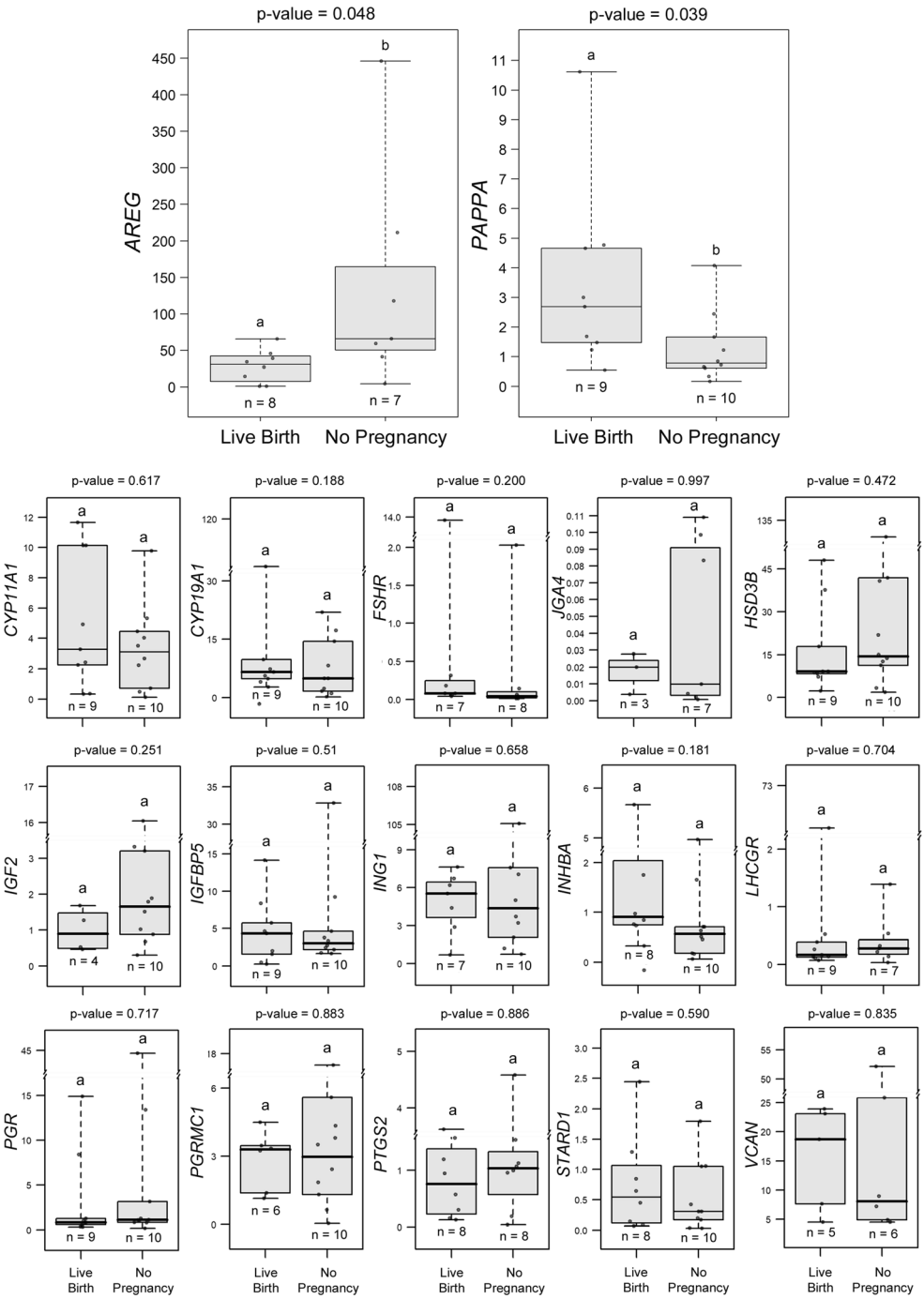

Fig. 5 CC biomarkers associated with oocytes giving rise to euploid embryos with live birth or no pregnancy. Transferred embryos ($n = 19$) from all 15 patients were assessed for mRNA level differences between those oocytes yielding embryos that resulted in a live birth and those that did not form a pregnancy using repeated measures ANOVA followed by Tukey's post hoc test (adjusted P values) for pairwise comparisons. Of the transferred embryos, 9 resulted in live births. *AREG* mRNA levels were significantly lower in CCs from oocytes that resulted in live births ($n = 8$ from 8 patients) compared to the no pregnancy group ($n = 7$ from 5 patients) ($P < 0.05$). *PAPPA* mRNA expression was significantly increased in CCs from oocytes producing embryos that resulted in live births ($n = 9$ from 8 patients) compared to no pregnancy ($n = 10$ from 7 patients) ($P < 0.05$). *GREM1* mRNA levels ($n = 3$/group) were not significantly different and not shown. Data are presented as in Fig. 3 legend

reflected in specific mRNA levels of their CC masses. For example, we predicted that an immature oocyte CC profile would differ from that of a mature oocyte CC profile capable of giving rise to a euploid embryo resulting in a successful pregnancy. This was partly the case for CC *PAPPA* and *LHCGR* mRNA levels which were higher in CCs surrounding mature oocytes that gave rise to embryos resulting in live birth than immature oocytes (by ANOVA). However, by ANOVA, the levels of CC *PAPPA* and *LHCGR* could not distinguish between oocytes giving rise to morphologically normal embryos that were aneuploid and transferred euploid embryos that resulted in live birth or no pregnancy. When utilizing the best fit GLMM, higher CC *PAPPA* levels continued to associate with oocytes giving rise to embryos with live birth compared to transferred embryos that did not result in pregnancies as well as other oocyte outcomes, collectively. In transferred embryos, lower CC *AREG* associated with oocytes giving rise to embryos with live birth outcomes by ANOVA analysis. Combining these data with that for CC *HSD3B1*, where lower levels were associated with euploid embryos, one can envision

using these findings to create a future practical model where the CC levels of all four mRNA biomarkers could be used to predict which of a patient's cohort of oocytes and embryos will most likely be euploid and give rise to a live birth outcome. Although four biomarkers of the selected group exhibited significant differences in statistical models, this was a small pilot study and the data is limited by the small sample numbers for FETs. However, these results provide a starting point for a larger prospective future study.

Several of our analyses indicated *PAPPA* may be a reliable novel biomarker in CCs for identifying oocytes capable of leading to a live birth. *PAPPA* encodes a metalloproteinase that cleaves insulin-like growth factor binding protein 4 (IGFBP4) and IGFBP5 [33]. Increased levels of *PAPPA* mRNA and protein in peri-ovulatory follicles may be necessary to increase intrafollicular free IGFs through enhanced cleavage of IGFBPs [34]. IGFs are known to enhance gonadotropin signaling and FSH-induced LHCGR abundance [35]. We also found that immature oocytes possessed lower CC *LHCGR* mRNA levels compared to all the other groups except oocytes that resulted in arrested embryos. Similarly, others have shown that CC *LHCGR* mRNA levels were higher in mature compared to immature oocytes [36]. In addition, the ratio of CC *LHCGR* mRNA splice variants was previously reported to be higher in pregnant compared to non-pregnant patients [37].

Differential mRNA levels of steroidogenic genes in CCs may impact the fate of their associated oocytes. A previous study utilizing GCs pooled from multiple patients and follicles showed increased *CYP19A1* and *HSD3B1* mRNA levels in GCs from oocytes producing embryos that resulted in pregnancies compared to oocytes resulting in embryos that failed to develop [38]. In our study, lower CC *HSD3B1* mRNA levels associated significantly with oocytes which yielded euploid embryos. Additionally, *HSD3B1* and *PAPPA* both positively correlated with principal component 1 and 2 in our biplot analysis. The difference may have become apparent as we evaluated individual CC masses rather than pooling. Before including the trigger in our best fitting model, lower CC *CYP19A1* mRNA level was associated with live birth ($P = 0.048$, not shown), but showed only a trend when trigger medication was added to the model ($P = 0.052$; Supplemental figure 2). Given that different trigger medications can alter steroidogenic gene expression [39], caution must be used in interpreting *HSD3B1* and *CYP19A1* mRNA data. Like *LHCGR*, *CYP19A1* is induced through FSH signaling, and *CYP19A1* is higher in dominant follicles of monovulatory species [40–42].

Huang and colleagues found that higher *AREG* levels in CCs and MGCs were more likely to be associated with pregnancy [43]. This differs from our study as we saw lower *AREG* levels in CCs from oocytes associated with live birth. As above, this discordant finding may be the result of pooling CCs and MGCs and/or the use four stimulation protocols in the Huang study. Our results are consistent with Feuerstein and colleagues who showed reduced *AREG* mRNA levels for CCs of oocytes that resulted in high-quality blastocysts [44].

Two strengths of our study were that all patients received the same stimulation regimen, albeit with two differing trigger medications, and we excluded patients with endometriosis or DOR. Differing stimulation protocols have been reported to alter mRNA expression and bias results [45–47]. In previous studies, CCs and MGCs of patients with DOR [27, 28] or endometriosis [25, 26] exhibited altered mRNA expression relative to their control groups. For these reasons, we excluded patients with these conditions.

As with any patient-driven clinical study, our study had limitations. Patient heterogeneity [45, 48], patient age [48, 49], and differing trigger medications have been previously demonstrated to alter gene expression [39]. A study design including younger patients with only male factor infertility using the same trigger medication would be a more ideal group to examine. Additionally, sample size may have influenced the findings. While we were able to analyze 163 samples, the samples were collected from 15 patients. Additionally, we were not able to analyze all genes for each of the samples as RNA amounts were a limiting factor with individual cumulus masses. With more patients, other mRNA expression level differences may have been apparent, and a larger prospective study is necessary to confirm the results we found and determine the predictive value of the CC mRNA levels for oocyte endpoints such as euploidy and live birth outcomes. Moreover, an argument can be made that looking at oocyte CC biomarkers and comparing the ploidy status of embryos disregards the potential that aneuploidy may have resulted from the paternal contribution to the embryos. We feel our study is still a valid assessment as aneuploidy is predominantly due to maternal origins [50]. Finally, we cannot overlook the impact of uterine receptivity. Although we have no evidence of patient uterine receptivity issues, it is possible some non-implanting embryos were capable of leading to a live birth but were not able to implant due to a suboptimal uterine environment. A way to account for this would be to perform double embryo transfers with euploid embryos and assess CC mRNA expression for those where one implants and one does not [16].

In conclusion, taken together the ideal oocyte developmental competence needed to produce live birth may be reflected by the interplay between *AREG*, *HSD3B1*, *LHCGR*, and *PAPPA* mRNA levels and likely their resulting proteins. The current data support that CC mRNA levels of these four biomarkers may be useful in assisted reproduction. A larger prospective study will be needed in to order to corroborate the

results and to determine numerical ranges for levels of these biomarkers that could potentially have clinical value in selecting oocytes to fertilize or embryos for uterine transfer that will have the highest odds of yielding live birth.

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Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and in its later amendments or comparable ethical standards. This study was approved by the University of South Carolina Institution Review Board (IRB registration number: 00000240).

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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