Archives of Gynecology and Obstetrics (2019) 299:863–871
https://doi.org/10.1007/s00404-018-5031-y

PGR and PTX3 gene expression in cumulus cells from obese and normal weighting women after administration of long-acting recombinant follicle-stimulating hormone for controlled ovarian stimulation

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Received: 26 July 2018 / Accepted: 14 December 2018 / Published online: 4 January 2019

Abstract

Purpose The present study aimed to determine clinical IVF parameters and gene expression in cumulus cells (CCs) in obese and normal weighting women after administration of 150 mcg of corifollitropin alfa for controlled ovarian hyperstimulation (COH).

Methods 150 mcg of corifollitropin alfa and gonadotropinreleasing hormone antagonist were used for COH. Analysis of CC gene expression was performed using quantitative real-time PCR.

Results We did not find significant differences in biochemical and clinical pregnancy rates between obese and normal weighting women. Obese women required twice as much of additional gonadotropins for ovarian stimulation and had a significantly lower proportion of good quality embryos on day 5 of IVF cycle. Expression of *PGR* and *PTX3* was significantly higher in CCs of obese women.

Conclusion Obese women require significantly larger amounts of gonadotropins to achieve similar IVF success rates as normal weighting women. Differences in CC gene expression and smaller proportion of good quality embryos may imply that oocytes derived from obese women are of lower quality. Further studies are needed to evaluate whether obesity itself or the higher amount of gonadotropins used in obese women causes this effect.

Keywords Obesity · Long acting rFSH · IVF success rates · Molecular oocyte quality

Introduction

Obesity, defined as body mass index (BMI) over 30 kg/m^2 [1], is an increasing health problem in many world countries, affecting approximately 20% of women between 15 and 44 years [2]. Obese women have three times higher chances to suffer from infertility than normal weighting women and take longer time to conceive, even if they have regular menstrual cycles [3]. In addition, success rates of in vitro fertilization (IVF) cycles in obese women have been shown to be lower than in normal weighting women [4]. Shah et al. [5] found that obese women had fewer normally fertilized oocytes, lower pregnancy and live birth rates when compared to women with normal BMI, despite similar number of mature oocytes retrieved. Although several lines of evidence suggest that female obesity negatively affects reproductive functions by dysregulating

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hypothalamic–pituitary–ovarian axis [6], thus impairing oocyte quality [7] and endometrial receptivity [8], to date the exact mechanism(s) underling the correlation between increased BMI and poor reproductive outcomes is still far from being fully elucidated. Interestingly, decreased pregnancy rates were reported in obese women who had transfer of embryos derived from autologous oocytes, but not from donor oocytes [9]. Lower pregnancy and live birth rates were also reported in donor–recipient IVF cycles with increasing oocyte's donor BMI [10]. These findings may imply that obesity negatively impacts oocyte's quality and developmental potential prior to fertilization.

Cumulus cells (CC) surround the oocyte, and bi-directional communication between the oocyte and CC is essential for the development of mature and competent oocytes [11]. Recently, gene expression analysis in CC has been extensively investigated to find potential biomarkers of oocyte and embryo quality [12]. Differential expression of CC genes between oocytes of lower and higher quality has been shown, in particular, hyaluronan synthase 2 (HAS2) [13, 14], pentraxin 3 (PTX3) [13, 15, 16], glutathione peroxidase 3 (GPX3) [16, 17], versican (VCAN) [18, 19], vascular endothelial growth factor C (VEGFC) [20], serine protease inhibitor E2 (SERPINE2) [20], follicle-stimulating hormone receptor (FSHR) [20] and progesterone receptor (PGR) [21] gene expression was reported to correlate with oocyte maturity, quality and developmental potential.

Several studies found that obese women undergoing ovarian stimulation in IVF procedures require higher doses and longer periods of gonadotropin treatment than normal weighting women [22, 23]. Corifollitropin alfa is a long acting recombinant follicle-stimulating hormone (rFSH) used for controlled ovarian hyperstimulation (COH) in IVF patients [24]. As previously shown [23], a single subcutaneous injection of corifollitropin alfa on day 2 or 3 of the menstrual cycle induces and sustains development of multiple follicles during the first 7 days of COH, meaning less injections required during one treatment cycle. According to recent data [25, 26], the optimal treatment regimen with corifollitropin alfa should be 100 mcg for women who weigh less than 60 kg and 150 mcg for women who weigh more; in this regard, it has been shown that a dose of 150 mcg of corifollitropin alfa sustains ovarian response for 7 days even in women weighting over 90 kg [27]. Nevertheless, robust data about ovarian response after 7 days in obese women with the use of 150 mcg of corifollitropin alfa are still missing.

Considering these elements, the aim of the current study was to compare clinical IVF parameters between obese and normal weighting women after administration of 150 mcg of corifollitropin alfa for COH. In addition, we aimed to investigate oocyte quality from the molecular point of view, analysing CC gene expression by real-time polymerase chain reaction (qPCR), between obese and normal weighting women.

Materials and methods

Patient population, controlled ovarian hyperstimulation protocol and IVF procedure

The study was approved by the National Medical Ethics Committee of Slovenia (Approval Nr: 0120-498/2015-2). Each patient enrolled in the study signed informed consent for all the procedures and to allow data collection and analysis for research purpose. The study was not advertised, and no remuneration was offered to the patients to enter or continue the study. An independent data safety and monitoring committee evaluated the results. The design, analysis, interpretation of data, drafting and revisions conform the Helsinki Declaration, the Committee on Publication Ethics (COPE) guidelines (http://publicationethics.org/), and the strengthening the reporting of observational studies in epidemiology (STROBE) statements, available through the EQUATOR (enhancing the quality and transparency of health research) network (http://www.equator-network.org).

Patient recruitment started in March 2016 and ended in January 2017, at the Department of Human Reproduction, Division of Gynaecology of the University Medical Centre Ljubljana. Inclusion criteria for the study group were: first or second IVF cycle, BMI $\geq$ 30 kg/m^2, tubal factor of infertility, normal partner's spermiogram. Inclusion criteria for the control group were: first or second IVF cycle, normal BMI (19–24.9 kg/m^2), tubal factor of infertility and normal partner's spermiogram. Considering these criteria, we enrolled 34 consecutive women in the study group and 36 control women in the control group.

All women underwent COH by administration of 150 mcg of corifollitropin alfa (Elonva, Merck Sharp & Dohme, Hertfordshire, UK) with GnRH antagonist (Cetrotide, Asta Medica AG, Frankfurt, Germany) subcutaneously. Follicular development was followed by serial transvaginal ultrasound. If required, we have added 200 IU of daily rFSH (Puregon, Schering Plough, New Jersey, USA) to achieve optimal ovarian response. Final follicular maturation was induced by administering 5000 or 10,000 IU of human chorionic gonadotrophin (hCG) (Pregnyl, N.V. Organon, Oss, the Netherlands) when at least three follicles measured $\geq$ 17 mm in diameter. When there were more than 20 follicles in both ovaries, oocyte maturation was induced with injection of 0.6 ml of buserelin acetate (Suprefact, Aventis Pharma, UK) to minimise the risk of ovarian hyperstimulation syndrome.

Ultrasound-guided transvaginal retrieval of follicles was performed 34–36 h later. Each follicle was aspirated separately. After the oocyte retrieval, CCs of each oocyte were

immediately removed and snap frozen in liquid nitrogen. Oocytes were fertilized and cultured individually, allowing a correlation with their respective CCs. After 18–20 h, oocyte fertilization status was assessed. Oocytes were considered fertilized when expressed two pronuclei and two polar bodies. Fertilized oocytes were individually cultured for 3 or 5 days in Universal IVF Medium followed by Blast Assist System (M1 and M2, Origio, Denmark). Embryo quality was assessed on day 3 of IVF cycle and blastocyst quality on day 5. Embryo/blastocyst transfer was performed on day 3 or 5 of in vitro culture based on the total number of embryos; when one or two were available, they were transferred on day 3, whereas in other cases, the cultivation was prolonged to day 5, when top quality embryos reached the blastocyst stage. Supernumerary embryos were cryopreserved. We also cryopreserved embryos of patients with excessive number of oocytes to reduce the risk for ovarian hyperstimulation syndrome.

Biochemical pregnancy was determined by measuring serum levels of β-hCG 14 days after embryo transfer. Clinical pregnancy was determined as the presence of gestational sac and foetal heartbeat by transvaginal ultrasound scan 4 weeks after embryo transfer.

Samples collection, RNA extraction and cDNA synthesis

We have analysed 109 individual CC samples derived from 60 women using qPCR. Only CCs surrounding mature, MII, oocytes were analysed. Fifty-three (53) individual CC samples derived from 29 women in the study group and 56 individual CC samples derived from 31 women in the control group. Samples from ten women were not included in the qPCR analysis, as we did not obtain sufficient mRNA to perform the analysis. Oocytes and embryos were cultured individually to directly associate IVF clinical parameters with individual oocytes.

Immediately after oocyte retrieval, CCs were mechanically stripped from first two retrieved oocytes by a needle and a glass denudation pipette (Swemed, Sweden). CCs were then washed in phosphate-buffered saline (PBS), snap frozen in liquid nitrogen and stored at − 80 °C until RNA isolation; RNA was extracted only from CCs that surrounded mature, MII, oocytes. TRI reagent (Sigma-Aldrich, St. Louis, USA) was used for RNA extraction according to slightly modified manufacturer's instruction. Due to small sample volume, glycogen was used as a carrier to increase RNA yield. Individual CC samples were homogenized in 500 µL TRI reagent supplemented with 125 µg of glycogen (Ambion, Austin, USA). After 2 min incubation at room temperature, 100 µl chloroform was added and the sample was vortexed vigorously. RNA was precipitated from the aqueous phase with isopropanol and collected after 15 min centrifugation at 12,000×*g* and 4 °C. RNA pellet was washed three times with 75% ethanol, dried and dissolved in 15 µl of RNAse free water. The integrity of the RNA samples was assessed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, USA) and the total RNA quantity was measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). cDNA was prepared from 300 ng RNA using the SuperScript Vilo reverse transcriptase (Invitrogen, Carlsbad, USA) according to manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qPCR)

Gene qPCR was performed using TaqMan® Gene Expression pre-designed assays (Applied Biosystems, Foster City, USA). All qPCR reactions were performed in triplicates in 96-well formats in a 20 µl final volume reactions. Measurements were performed using ABI Prism 7000 Sequence detection system (Applied Biosystems). Thermal cycling conditions were as follows: an initial step at 50 °C for 2 min, denaturation at 95 °C for 10 min, amplification for 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The threshold cycle (Ct) values were then determined for each assay and data were normalized to internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was co-ran with each sample.

We performed a systematic literature search in PubMed electronic database to decide the gene expression in human CCs to investigate for assessing oocyte maturity and quality. After initial screening, we selected articles published in English from January 2005 to December 2015 which investigated human CC gene expression by qPCR, using combinations of the following MeSH terms: "cumulus cells" and "gene expression", "oocyte maturity", "oocyte quality", "non-invasive biomarker", "embryo quality". Titles and/or abstracts of studies retrieved using the search strategy, and those from additional sources were screened independently by two authors (T.B.P., N.J.). The full text of these potentially eligible studies was retrieved and independently assessed for eligibility by other two research team members (U.P.Z., A.S.L.). A standardized, pre-piloted form was used to extract data from the included studies for assessment of study quality. Any disagreement between them over the eligibility of particular studies was resolved through discussion with a third external collaborator.

Based on this systematic review, we decided to investigate the expression of the following genes, as potential/putative biomarkers of oocyte quality: PGR, PTX3, SERPINE2, HAS2, COX2, VCAN, VEGFC, FSHR.

Statistical analyses for clinical parameters

Because of the different numbers of oocytes retrieved from each participant, oocyte-level statistics could treat them as independent groups. Therefore, generalized estimating equations were used instead of more common logistic regression to model probability of each oocyte to become mature, fertilized and developed into blastocyst stage. Probability for pregnancy was modelled with logistic regression. For all statistics, an IBM SPSS Statistics version 24 was used.

Statistical analysis for qPCR

Differences in gene expression were calculated using the delta–delta Ct method, as previously described by Livak and Schmittgen [28]. We checked the normality of distribution of gene expression data with Shapiro–Wilk's test. Data for some genes were not normally distributed (GPX3, HAS2, VCAN, SERPINE2). For this reason, we used non-parametric Mann–Whitney test for all genes to detect whether there are statistically significant differences in gene expression. Graphical representation of gene expression patterns was done in R package ggplot2. For each patient in which RNA extraction was successful for at least one CC, one bar is represented on the graph. If more than one CC gave satisfactory amount of RNA, than the bar represents mean value of gene expression in all CCs per patient. For each group, mean value and 95% confidence interval was calculated.

Results

Clinical parameters

As showed in Table 1, we did not find significant differences for age ($p = 0.18$), number of retrieved oocytes per patient ($p = 0.53$), biochemical ($p = 0.41$) as well as clinical pregnancy rate ($p = 0.51$) between cases and controls; conversely, the amount of additional rFSH IU used for COH was significantly higher in the group of cases respect to controls ($p = 0.0003$). Per study protocol, mean BMI of cases was significantly higher than controls ($p \leq 0.0001$).

As far oocyte parameters are concerned (Table 2), we did not find significant differences for number of mature oocytes ($p = 0.51$), day 3 quality embryos ($p = 0.7$), fertilization rate ($p = 0.32$) and number or cryopreserved embryos ($p = 0.95$); conversely, we found a significantly lower number of day 5 quality embryos ($p = 0.04$) and higher number or degenerated oocytes ($p = 0.006$) in the group of cases respect to controls.

Table 1 Clinical IVF parameters in study and control groups

Clinical parameter	Study group ($n = 34$)	Control group ($n = 36$)	*P*
Age (years)	30.3 ± 3.9	31.5 ± 3.6	0.18
BMI (kg/m^2)	32.55 ± 3.08	22.85 ± 2.07	< 0.0001
rFSH added (*n*, %)	33 (97.1%)	29 (80.6%)	0.05
Amount of additional rFSH (IU)	649 ± 509	297 ± 210	0.0003
No. of oocytes retrieved per patient	11.3 ± 7.5	12.4 ± 7.4	0.53
Biochemical pregnancy rate (%)	40.9	32.7	0.41
Clinical pregnancy rate (%)	36.4	29.1	0.51

Continuous variables are expressed as mean and standard deviations and analysed by *t* test or Mann–Whitney test. Categorical variables are expressed as percentages and analysed by χ^2 test

BMI Body mass index, *rFSH* recombinant follicle-stimulating hormone

Table 2 Oocyte parameters in study and control groups

Oocyte parameter	Study group ($n = 381$)	Control group ($n = 444$)	*P*
Proportion of mature oocytes	0.70 ± 0.26	0.73 ± 0.22	0.51
Quality embryos day 3 (*n*, %)	121 (71.2)	139 (73.2)	0.7
Quality embryos day 5 (*n*, %)	78 (50)	79 (62.7)	0.04
Degenerated oocytes (*n*, %)	41 (10.8)	19 (4.3)	0.006
Fertilization rate (%)	76.3	72.6	0.32
Cryopreserved embryos (*n*, %)	45 (73.8)	49 (74.2)	0.95

Continuous variables are expressed as mean and standard deviations and analysed by *t* test. Categorical variables are expressed as percentages and analysed by χ^2 test

qPCR analyses

PGR and *PTX3* gene expression was significantly higher ($p < 0.001$) in CCs surrounding mature oocytes from cases respect to controls (Table 3, Fig. 1). Conversely, we did not find significant differences for *HAS2*, *COX2*, *VCAN*, *VEGFC, SERPINE2* and *FSHR* gene expression between CCs surrounding mature oocytes from cases respect to controls (data not shown).

Table 3 Relative mRNA expression of PGR and PTX3 genes

Gene	Study group	Control group	*P*
PGR	2.53 ± 1.73	1.31 ± 0.9	< 0.001
PTX3	1.49 ± 1.37	0.63 ± 0.58	< 0.001

Data are expressed as means and standard deviations, and compared by Mann–Whitney test

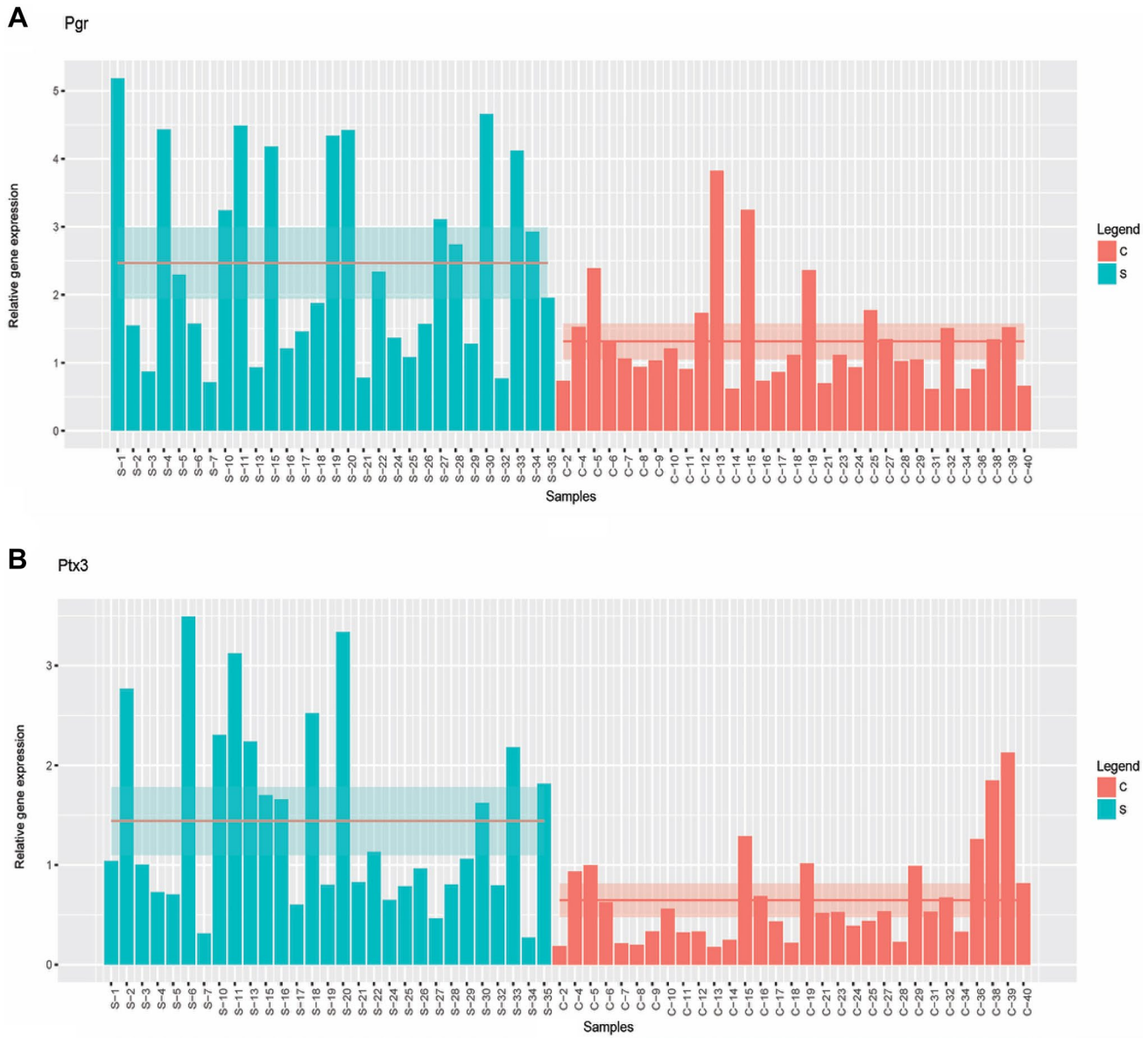

Fig. 1 Gene expression level of PGR (**a**) and PTX3 (**b**) as measured by qPCR in samples of individual patients. *S* Study group, *C* control group. Red line represents mean value for each group and the shadow shows 95% confidence interval

Discussion

Clinical outcomes of IVF procedure in obese and normal weighting women

Recent data suggest that a single subcutaneous injection of corifollitropin alfa, long acting rFSH, replaces first seven daily injections of gonadotropins: in particular, this treatment regimen has turned out to be equally effective in terms of clinical IVF parameters as daily administration of gonadotropins for COH [29]. According to this scheme, women weighting less than 60 kg receive 100 mcg of corifollitropin alfa on day 2–3 of menstrual cycle, whereas women weighting more than 60 kg receive 150 mcg. In our study, the clinical efficacy of 150 mcg of corifollitropin alfa used for COH was compared between obese and normal weighting women. Considering that average age of women in our study did not differ between obese and normal weighting women, this parameter is not likely to have influenced the subsequent data analysis.

We did not find significant differences regarding the number of oocytes retrieved and the proportion of mature oocytes obtained between obese and normal weighting women. Nevertheless, a significantly higher proportion of obese women required additional daily rFSH administration to achieve optimal ovarian response. In addition, the dose of additional rFSH administered in obese women was significantly higher than in normal weighting women. These findings are in line with previous reports where overweight and obese women required higher amounts of daily rFSH for COH [22, 30–32]: in the studies of Souter et al. [30], Ozekinci et al. [31] and Esinler et al. [32] obese women required approximately 33–36% higher doses of rFSH when compared to normal weighting women. The amount of rFSH used in obese women in the study of Balen et al. [22] was approximately 60% higher than in normal weighting women. In our study, obese women required roughly 110% higher dose of rFSH, compared to normal weighting women. The reason for this difference between the above mentioned studies and our investigation could be due to the different protocol used for COH, since corifollitropin alfa was used for the first 7 days of COH only in our study.

As far as the number of mature oocytes retrieved is concerned, we did not find a significant difference between obese and normal weighting women. Nevertheless, in the group of obese women there was a higher rate of degenerated oocytes, which are incapable of fertilization and are therefore discarded in IVF procedures. Our findings are in agreement with results of studies on mice, where more degenerated oocytes were obtained after superovulation in obese mice fed with high fat diet [33, 34].

Fertilization rates in the present study did not differ between obese and normal weighting women, which are in line with previous studies [32, 35]. On the contrary, other studies [36, 37] have shown that obese women have significantly lower fertilization rates as compared to normal weighting women.

In IVF cycles, the general aim is to obtain high quality embryos, which have the highest chance of achieving pregnancy. According to our data analysis, quality of embryos on day 3 after oocyte retrieval did not differ between obese and normal weighting women. On day 5, however, significantly less high quality embryos were obtained in the obese group of women, as was previously found elsewhere [7, 38, 39, 40]. Furthermore, it has been shown that early embryo development in obese mice is disturbed, with delayed progression to all developmental milestones [35]. In contrast, other studies found no differences in the number of good quality embryos between obese and normal weighting women [34, 36].

At this moment, we cannot explain why obese women produce less high quality embryos during IVF cycles: on the one hand, this could be due to the intrinsic oocyte defects that occur during maturation process; on the other hand, the reason could lie in the large amounts of gonadotropins used in obese women, as it has been shown that ovarian stimulation itself could have detrimental effects on oocyte maturation and embryo quality [41]. Nonetheless, in our study, the lower number of good quality embryos obtained on day 5 after oocyte retrieval did not affect pregnancy rates in obese women with respect to normal weighting women. These results may suggest that, although fewer good quality embryos are obtained during IVF procedures in obese women, those that develop to the blastocyst stage are equally competent to implant as embryos of normal weighting women. This speculation is in line with a previous report, where after the transfer of top quality embryos no adverse effect of BMI on pregnancy rates was found [42]. In contrast, several other studies reported lower pregnancy rates in obese women when compared to normal weighting women [5, 43].

Although not statistically significant, obese women had fewer embryos suitable for cryopreservation, despite the fact they used twice as much gonadotropins as normal weighting women. This may underlie that obese women will need a new ovarian stimulation for IVF cycle sooner than normal weighting women: in this scenario, a new IVF procedure with the use of gonadotropins means a bigger health burden for them as COH leads to a non-physiological state of super-ovulation. It also means a bigger financial burden for them as well as the healthcare system in countries where IVF cycles are reimbursed.

Obesity and cumulus cell gene expression

Our analysis has shown that *PGR* and *PTX3* are significantly higher expressed in CCs of obese as compared to normal weighting women.

PTX3 plays a crucial role in cumulus matrix expansion prior to ovulation and maintains its stability [44]. Previous study showed that *PTX3* mRNA expression in CCs is modulated by FSH and hCG [45]: in mouse model, *PTX3* expression in CCs starts 2 h after hCG administration for final oocyte maturation, reaches the maximum level after 6 h and decreases thereafter, suggesting a tight time-dependent regulation [46]. In mice, exposure of cumulus–oocyte complexes to high levels of lipids (imitating the lipotoxic effect of obesity) during in vitro maturation resulted in reduced levels of PTX3 protein and poor CC matrix formation [47]. In contrast, expression of *PTX3* was significantly higher in CCs of obese women in our study. Unfortunately, we did not perform morphological evaluation of CC expansion in the present study, so we are not able to correlate this element to *PTX3* expression and to compare it between obese and normal weighting women. The difference observed between our data analysis and previous studies on animal models could be due, at least in part, to the fact that studies in mice cumulus–oocyte complexes were performed in in vitro condition, whereas our study was performed on CCs surrounding in vivo matured oocytes. The other possible explanation could again be that although we analysed CC gene expression surrounding morphologically mature oocytes, the process of CC matrix formation and oocyte maturation was not yet finished at the molecular level at the time of oocyte retrieval in obese women. This could be the reason for higher number of degenerated oocytes and lower number of quality embryos obtained in obese women.

Recent and non-recent data have already demonstrated that progesterone exerts its biological activity through progesterone receptors and induces meiotic resumption of mammalian oocytes [48, 49]. In details, expression of *PGR* in humans is significantly higher in antral, compared to pre-antral follicles [50]. In porcine cumulus–oocyte complexes, FSH and LH induce *PGR* expression and oocyte meiotic resumption [51]. As was already reported [52], the expression of *PGR* was found significantly lower in CCs surrounding mature oocytes that developed to high quality embryos on day 3 after oocyte retrieval. In our study, significantly higher expression of *PGR* was detected in CCs of obese women. These women had a significantly lower number of good quality embryos obtained on day 5 after oocyte retrieval. On day 3, the number of good quality embryos was also lower, but the difference did not reach statistical significance. Considered altogether, our findings may suggest that reduction of *PGR* expression at the time of oocyte retrieval is mandatory for oocytes to acquire full developmental competence. According to this speculation, we presume that in obese women the process of oocyte maturation is somehow prolonged and thus not yet finished at the molecular level at the time of oocyte retrieval. Based on this background, we take the opportunity to solicit further studies with larger number of samples to confirm this hypothesis.

The main limitation of our study is a relatively low number of patients and samples included, and therefore results obtained must be interpreted with caution. Nonetheless, our study has shown that 150 mcg of corifollitropin alfa for COH in obese women yields similar clinical IVF results as in normal weighting women, once adjustments are made to optimize ovarian response. These results will be of value for infertility subspecialists who decide on the optimal regimen of ovarian stimulation in obese women on a daily basis. Besides that despite the fact that we were comparing expression of CC genes surrounding morphologically mature oocytes in both groups of women, there are differences in expression of some genes involved in oocyte maturation process. Differential expression could indicate that obesity causes alterations within follicular environment at molecular level, causing intrinsic oocyte defects that lead to degeneration and/or lower embryo quality. In addition, it is possible to speculate that the higher dose of administered rFSH in obese women during stimulation may have affected the level of FSH-dependent expression of all investigated genes in CC: this point is of paramount importance and should be taken into account for a proper data interpretation. Again, further studies are needed to clarify our findings.

Besides the abovementioned data and speculative hypotheses, we take the opportunity to underline that obese women should be continuously and firmly encouraged to change their lifestyle towards regular exercise and healthier diet for weight loss prior to inclusion to IVF procedures.

Acknowledgements We thank Dr. Ivan Verdenik for performing statistical analyses and Dr. Rok Košir for performing R-based analysis.

Author contributions TBP: designed the study, contributed to data analysis, and wrote the manuscript. EVB: supervised the research project and gave final approval. UPZ: performed the lab experiments. MS: collected and analysed data. ASL: contributed to data analysis and manuscript writing. FG: edited the manuscript. NJ: contributed to study design and manuscript editing. All the authors fulfil the International Committee of Medical Journal Editors (ICMJE) criteria.

Funding The study was funded by Merck Sharp & Dohme.

Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the insti-

tutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

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