

RESEARCH

Open Access



Letrozole-associated controlled ovarian hyperstimulation in breast cancer patients versus conventional controlled ovarian hyperstimulation in infertile patients: assessment of oocyte quality related biomarkers

Oranite Goldrat^{1,2*} , Geraldine Van Den Steen², Eric Gonzalez-Merino¹, Julie Dechène², Christine Gervy³, Anne Delbaere¹, Fabienne Devreker¹, Viviane De Maertelaer⁴ and Isabelle Demeestere^{1,2}

Abstract

Background: Fertility preservation (FP) protocols in case of breast cancer (BC) include mature oocyte cryopreservation following letrozole associated controlled ovarian hyperstimulation (Let-COH). To date, the impact of Let-COH on the follicular microenvironment has been poorly investigated, although a high androgen/estrogen ratio was previously associated with low oocyte quality.

Methods: In this prospective study, follicular fluid (FF) steroid levels (estradiol, testosterone, progesterone) and cumulus cell (CC) gene expression related to oocyte quality (*HAS2*, *PTGS2*, *GREM1*) were compared between 23 BC patients undergoing Let-COH for FP and 24 infertile patients undergoing conventional COH without letrozole. All patients underwent an antagonist COH cycle, and ovulation was triggered with hCG or GnRHa in both groups.

Results: FF estradiol levels were significantly lower while testosterone levels were significantly higher in the study group compared to controls irrespective of the trigger method. However, estradiol levels increased significantly with GnRHa triggering compared to hCG in the study group (median = 194.5 (95.4–438) vs 64.4 (43.8–152.4) ng/ml, respectively, $p < 0.001$), but not in the control group (median = 335.5 (177.5–466.7) vs 354 (179–511) ng/ml, respectively). After hCG trigger, Cumulus cell (CC) gene expression was lower in the study group compared to the control group, and difference was significant for *PTGS2*. Conversely, CC gene expression of *PTGS2* and *GREM1* was significantly higher in the study group compared to controls when ovulation was triggered with GnRHa.

Conclusions: Let-COH triggered with hCG may negatively impact oocyte quality. However, ovulation triggering with GnRHa may improve the oocyte microenvironment and cumulus cell genes expression in Let-COH, suggesting a positive impact on oocyte quality in breast cancer patients.

(Continued on next page)

* Correspondence: oranite.goldrat@erasme.ulb.ac.be

¹Fertility Clinic, Department of Obstetrics and Gynecology, CUB-Hôpital Erasme, Université Libre de Bruxelles (ULB), Route de Lennik 808, Brussels, Belgium

²Research Laboratory on Human Reproduction, Université Libre de Bruxelles (ULB), Route de Lennik 808, Brussels, Belgium

Full list of author information is available at the end of the article



© The Author(s). 2019 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated.

(Continued from previous page)

Trial registration: Clinicaltrials.gov -NCT02661932, registered 25 January 2016, retrospectively registered.

Keywords: Breast cancer, Controlled ovarian hyperstimulation, Letrozole, Oocyte quality, Follicular fluid, Cumulus cells, Gene expression

Background

Recent advances in primary systemic therapy have greatly improved relapse-free survival of young breast cancer (BC) patients [1, 2], and increased survival has led clinicians to focus on long-term quality of life issues such as access to motherhood. Moreover, pregnancy after BC treatment does not increase relapse risk and may even improve overall survival [3]. Nevertheless, chemotherapy including alkylating agents may cause infertility or premature ovarian failure, reducing patient's chances to conceive [4]. Furthermore, in patients with hormone-sensitive disease, endocrine therapy is administered for several years. Consequently, aging should also be taken into consideration as a crucial fertility decline factor [5–7]. Fertility preservation (FP) has hence become a priority for patients requiring chemotherapy. Oocyte and/or embryo vitrification before treatments are usually offered to BC patients [8]. However, high estradiol level observed during controlled ovarian hyperstimulation (COH) before oocyte collection has been subject of debate regarding possible proliferative effects on the tumor. Consequently, more than a decade ago, a new COH protocol associated with letrozole, a type II nonsteroidal competitive aromatase inhibitor, was developed (Let-COH) to collect several mature oocytes while potentially avoiding negative effects of estrogens on tumor growth [9, 10]. Through its competitive action on the aromatase enzyme [11, 12], letrozole prevents the aromatization of androgens to estrogens, which may induce significant changes in the endocrine follicular environment and impact oocyte competence. However, an estrogenic environment was previously associated with better oocyte outcomes and anti-atretic effect, while elevated androgen/estrogen ratio was reported to induce granulosa cell apoptosis, associated with low quality or degenerating oocytes [13–17]. Recent studies have shown significantly improved oocyte yield in BC patients undergoing Let-COH for FP compared to conventional COH for elective oocyte cryopreservation, as well as in infertile patients undergoing IVF with Let-COH or conventional COH [18, 19].

Nevertheless, as developmental competence of frozen oocytes/embryos of large cohorts of BC patients might be known only in several years through live-birth rates, the assessment of indirect markers in the microenvironment surrounding the oocyte is an attractive approach to evaluate the oocyte quality. Several genes expressed in cumulus cells (CC) have been evaluated as potential markers of oocyte competence, offering an attractive

approach to select embryos with the highest developmental potential. Among them, Hyaluronic acid synthase 2 (*HAS2*), Prostaglandin-endoperoxide synthase-2 (*PTGS2*) and Gremlin1 (*GREM1*) have been associated with higher oocyte competence and good embryo quality, given their interaction with oocyte secreted factors and their role in CC expansion during oocyte maturation. Although not always statistically significant, a higher expression of these genes has been associated with higher oocyte competence [20–25].

The impact of Let-COH for FP in BC patients on the follicular milieu remains poorly investigated so far. The objective of this study was hence to compare the impact on follicular fluid (FF) steroid levels and CC gene expression of the Let-COH protocol for FP in BC patients and conventional COH in infertile patients.

Materials and methods

Population

BC patients were enrolled in the BROVALE trial, a prospective study conducted between December 2012 and February 2017. The study group included 23 young BC patients of 18–41 years with non-metastatic disease and basal follicle stimulating hormone (FSH) < 20 IU/L, undergoing oocyte/embryo freezing for FP with Let-COH protocol. The control group included 24 infertile women aged < 41 years, treated with first or second ICSI cycles for tubal, male, and/or idiopathic infertility, undergoing similar ovarian stimulation for intra-cytoplasmic sperm injection (ICSI), without letrozole (conventional COH). Patients with severe endometriosis, ovarian insufficiency or severe polycystic ovary syndrome (PCOS), based on Anti-Müllerian Hormone (AMH) levels < 0.5 or > 8 ng/ml respectively, were excluded from this analysis.

Controlled ovarian hyperstimulation protocols

For this study, patients in both groups underwent a gonadotropin releasing hormone (GnRH) antagonist cycle (Cetrorelix, Cetrotide® 0.25 mg, Serono, Germany) with recombinant FSH (rFSH 150–300 IU/day, Gonal-f®, Serono, Germany) and were triggered using 10,000 IU hCG (Pregnyl®, MSD, Switzerland) or 0.2 mg Triptorelin (GnRH-agonist, Decapeptyl®, Ipsen, Belgium) [26], according to local protocol. In the control group, Triptorelin was used mainly when there was a risk to develop ovarian hyperstimulation syndrome (OHSS). In the BC group, GnRHa trigger recently replaced hCG trigger in all patients, regardless of

OHSS risk, to maintain low progesterone level during luteal phase [26].

In BC group, a “standard” or “random start” COH was applied depending on the cycle phase, either early follicular or late follicular/luteal phase, respectively [27]. In standard protocol, letrozole 5 mg/day (Femara®, Novartis, Switzerland) was started on cycle day 2, and gonadotropins were administered the following day. GnRH antagonists were initiated after 5 days. In the “random start” protocol, letrozole, gonadotropins, and GnRH antagonist were often administered together during the stimulation. Letrozole administration was discontinued on the ovulation trigger day in both protocols. Follicular development was monitored by pelvic ultrasound scans and serum endocrine profile (luteinizing hormone (LH), estradiol, and progesterone) and ovulation was triggered as soon as at least 2 follicles reached 19–20 mm [10].

In the control group, patients underwent a conventional antagonist COH, without letrozole. Ovulation was triggered according to usual practice, as soon as at least 3 follicles reached 17–18 mm. Oocyte pick-up (OPU) was performed 34–36 h after ovulation triggering. Follicles were aspirated via a single-lumen needle (Cook®, Australia) using transvaginal ultrasound transducer, with an aspiration pressure of 120 mmHg (Cook®, Australia). Each follicle was aspirated individually into a 5 ml Falcon tube and systematically separated from the flush medium.

Follicular fluid (FF) analysis

After removal of the cumulus-oocyte-complex (COC), each FF was recovered separately and centrifuged at 3000 RPM for 10 min. Only individual samples of > 2 ml enclosing a mature oocyte, without visible contamination of flushing media or blood were stored at –20 °C for further analysis.

Estradiol (E2), and progesterone (P) were assayed by electrochemiluminescence immunoassay using a competitive immunoassay (Modular E170 – Roche diagnostics, Mannheim, Germany). The inter-assay coefficient of variation was less than 5% for both assays. Testosterone (T) levels were measured by radioimmunoassay (DIA source, Louvain-La-Neuve, Belgium). The inter-assay coefficient of variation was less than 7%. Samples were diluted (1:1000) in Multi assay diluent (MA) for E2 and in the E2-P diluent for P. T was diluted (1:2) in study group only, in fetal calf steroid-free serum.

Cumulus cell (CC) collection and oocyte handling

Collected COC were washed and incubated individually in Fertilization medium (Cook Medical, USA) under mineral oil for 1 h before denudation. Denudation was performed in 30 µl droplets of Gamete Buffer (Cook Medical, USA) containing 80 IU hyaluronidase (HYASE,

Vitrolife, Sweden) for 30 s, and then washed in two 30 µl droplets of enzyme-free Gamete Buffer.

Whenever a BC patient had a male partner, ICSI was performed on a variable proportion of mature oocytes (MII), according to patient’s decision. Embryos obtained were vitrified at 2PN or cleavage stage. If the patient was single, all MII oocytes were directly vitrified after denudation.

In the control group, all MII oocytes were subjected to ICSI. Embryo transfer was performed on day 3 or 5 of culture and the remaining good quality embryos were vitrified according to local protocol. Oocytes and embryos were handled individually in both groups to allow CC per oocyte analysis.

RNA extraction, reverse transcription (RT) and real-time PCR

For each individual mature oocyte, CC samples were collected separately and centrifuged twice for 10 min (2000 RPM at 4 °C in PBS) to remove culture media and mineral oil.

Total RNA extraction was performed immediately, using RNAqueous®-Micro Total RNA Isolation kit (Thermo Fisher Scientific, USA) according to manufacturer’s instructions. Samples were treated with recombinant DNase I RNase-free (Thermo Fisher Scientific, USA) to remove any potential genomic DNA contamination. RNA was assessed for quantity and purity by spectrophotometry (NanoDrop 2000) and stored at –80 °C until RT. RT and qPCR were conducted on CC samples with an mRNA concentration > 5 ng/µl. Reverse transcripts of total RNA were prepared using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) with a final reaction volume of 20 µl. Negative controls were performed by replacing the enzyme with water. cDNA samples were stored at –20 °C until qRT-PCR. *HAS2*, *PTGS2*, and *GREM1* were selected as target genes. Ribosomal protein L19 (*RPL19*) and Hypoxanthine phosphoribosyltransferase-1 (*HPRT1*) were selected as housekeeping genes (validated by Genorm software). Primers were designed using the free Primer3-Plus software except for *GREM1* (commercial assay, SigmaAldrich, Austria) (Additional file 1: Table S1). Amplification efficiency ranged between 90 and 110% for each primer pair. Specificity of single PCR products was confirmed by gel electrophoresis for all genes. qPCR experiments were performed on a 7500 Cycler (Applied Biosystems). The reaction mixture contained 5 µl cDNA (1 ng), 200 nM of each primer and 10 µl PowerSYBR® Green PCR Master Mix (Thermo Fisher Scientific, USA) in a final reaction volume of 20 µl. After activation and denaturation (20 s at 50 °C and 10 min at 95 °C), the cDNA was subjected to 40 amplification cycles (15 s at 95 °C and 1 min at 60 °C). All samples were run in triplicate and a No Template Control (NTC) was included for each gene. Gene

expression levels were normalized to the geometric mean of the housekeeping genes and fold increases were calculated using the $2^{\Delta\Delta CT}$ method.

Statistics

Statistical analyses were performed using SPSS 23 (IBM, Brussels, Belgium) on Mac OS X. Mann-Whitney or Student's t-tests were performed where appropriate. Considering some patients in the study group underwent 2 cycles of Let-COH, and the number of samples per patient was variable in both groups, we performed a Two-way ANOVA with group as a fixed factor and patients as a nested within group random factor to test statistical dependencies of samples using the NCSS 10 Statistical Software 2015 LLC. Kaysville, Utah, USA, ncss.com/software/ncss). All tests were two-tailed and a *P*-value of less than 0.05 was considered statistically significant.

Results

Biomarkers data were analyzed separately for hCG and GnRHa triggers, as follicular microenvironment differs according to ovulation trigger method [28–30]. Patient and cycle characteristics are shown in Table 1.

FF endocrine profile assessment

We assessed the effect of letrozole on steroid levels in the FF to indirectly evaluate its impact on oocyte competence. To avoid potential bias related to differences in follicular size, only FFs of similar volumes were analyzed in both groups (3.4 ± 1.4 vs 3.2 ± 1 ml per follicle, $p = 0.533$). Additionally, after exclusion of contaminated samples, a total of 73 FF samples from 18 control patients, and 66 FF samples from 16 BC patients performing 18 and 20 cycles respectively, were eligible for the FF analysis. First, we confirmed that no difference

was observed in FF steroid levels between "standard" and "random start" protocols in the study group, in both ovulation trigger methods (unpublished data). Steroid levels were then compared between study and control groups.

Estradiol levels were significantly lower in the study compared to the control group, while testosterone levels were significantly higher (Fig. 1a, b). Progesterone levels were comparable between groups (Fig. 1c). Using a nested two-way ANOVA analysis, we confirmed there was no group effect regarding FF volume. However, we observed a significant effect of both patient and group factors for hormonal levels ($p < 0.001$). Interestingly, estradiol levels increased significantly after GnRHa trigger compared to hCG trigger in the study group (median = 194.5 (95.4–438) vs 64.4 (43.8–152.4) ng/ml, respectively, $p < 0.001$) but not in the control group (median = 335.5 (177.5–466.7) vs 354 (179–511) ng/ml, respectively) (Fig. 1a).

CC gene expression related to oocyte competence

A total of 19 controls (82 CC samples) and 22 BC patients (89 CC samples) performing 19 and 24 cycles respectively, were eligible for the CC analysis. To validate the analysis, we first confirmed that the expression of genes was lower in CC from unfertilized oocytes or low quality embryos compared to CC from mature oocytes resulting in top quality embryos in our control group. However, the difference reached significance only for *HAS2* and *PTGS2* after GnRHa trigger (Additional file 2: Figure S1).

In the hCG-trigger, expression of *HAS2* and *PTGS2* was lower in the study group ($n = 8$ Let-COH) compared to the control group ($n = 10$ COH), but the difference reached statistical significance only for *PTGS2* ($p = 0.015$) (Fig. 2a). Conversely, when GnRHa was used as the trigger, *HAS2* expression was comparable between groups ($n = 16$ Let-COH and 9 COH), but expression of *PTGS2* and *GREM1* was significantly higher in the study compared to the control group ($p < 0.001$ for both genes) (Fig. 2b). As for FF hormonal levels, nested Two-way ANOVA analysis showed an effect of both patient and group factors ($p < 0.05$).

Discussion

While hundreds of BC patients have cryopreserved eggs using Let-COH, to date, only two studies have reported specific outcomes of nearly 100 frozen embryo transfers. Success rates were similar to that of the general infertile population after frozen embryo transfer, with implantation and live birth rates per transfer reaching 39.7 to 40.7% and 32.3 to 45%, respectively [18, 31]. A recent study on infertile patients undergoing IVF confirmed the similar cumulative pregnancy rates between Let-COH

Table 1 Patients and cycle characteristics

	Study group	Control Group	<i>P</i> value
N patients	23	24	
N cycles	27	24	
Age (years)	30.4 ± 3.8	30.8 ± 3.9	0.71
AMH (ng/dl)	2.7 ± 2.1	3.1 ± 1.7	0.32
N stimulation days	9.9 ± 2.6	9.7 ± 1.3	0.8
Total dose of FSH used	2280 ± 1027	1629 ± 624	0.004
E2 level at triggering (pg/ml)	315 ± 225	2144 ± 915	< 0.001
Triggering methods (cycles)			0.22
hCG	9	12	
GnRHa	18	12	
Number of oocytes collected	9.5 ± 5.1	10.9 ± 6.8	0.4
Maturation rate	0.83 ± 0.19	0.87 ± 0.14	0.34

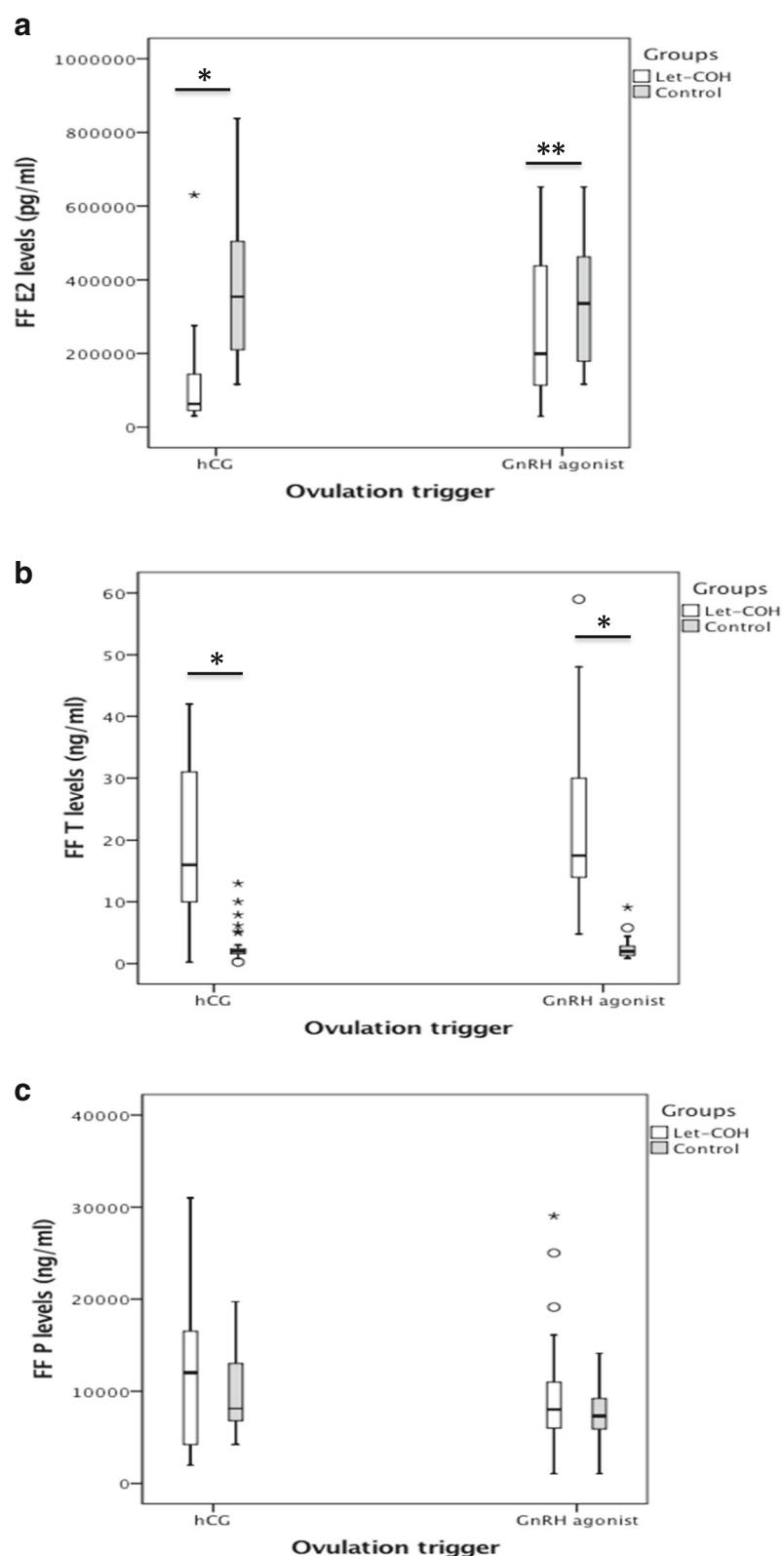
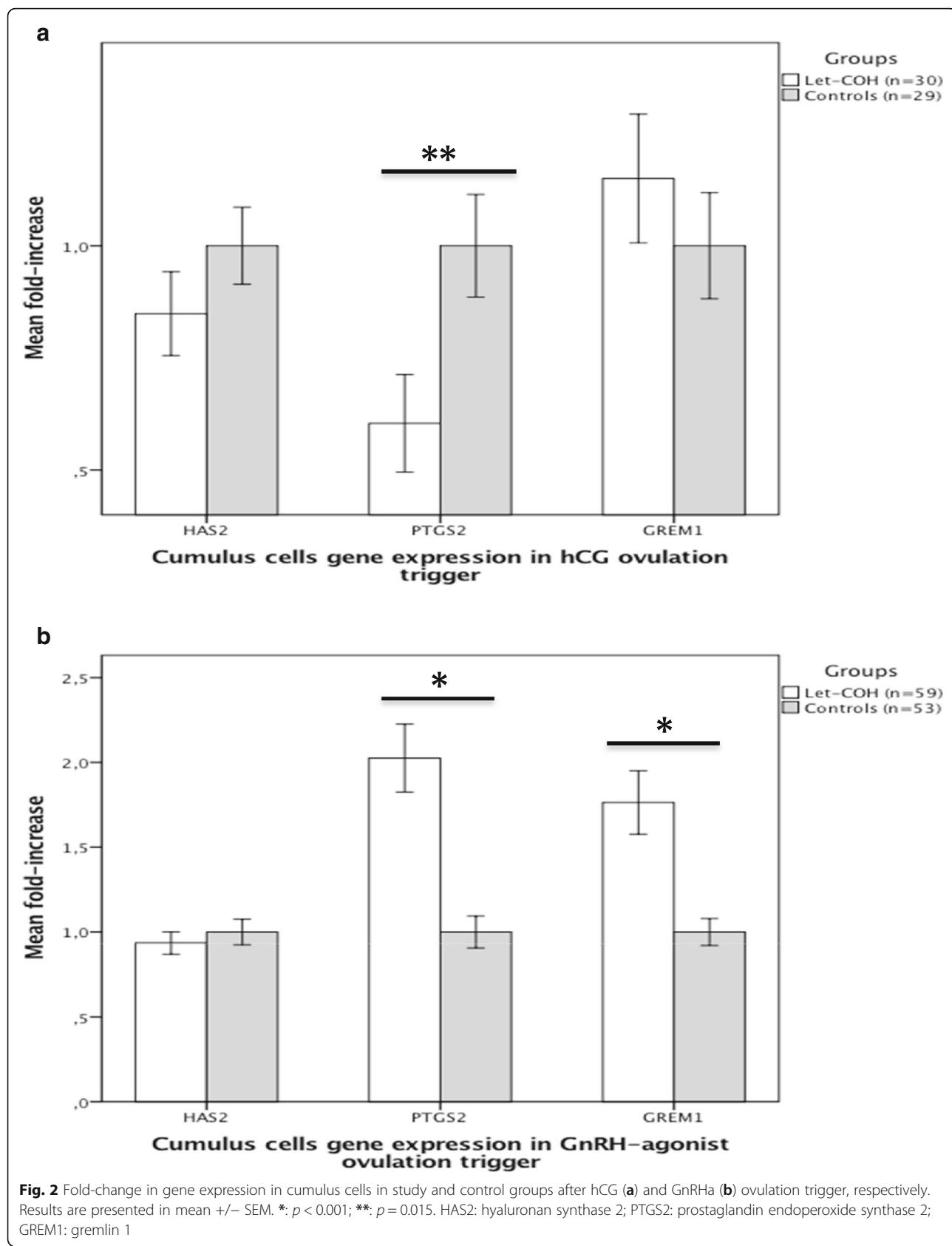


Fig. 1 Steroid levels in follicular fluid: estradiol (a), testosterone (b) and progesterone (c) concentrations in study and control groups. Boxplots represent the median, 25th, and 75th percentiles. The whiskers represent 1.5 times the interquartile range, and outliers are identified by circles (out-values) and stars (extreme values). *: p < 0.001; **: p = 0.011



and conventional COH groups (58.3% vs 65.2% respectively) [19]. Notwithstanding these promising results, oocyte quality has been poorly investigated so far in BC patients undergoing Let-COH for FP. Our study aimed to compare indirect markers potentially related to oocyte quality, following Let-COH in BC patients and conventional COH in infertile patients. Ovulation was triggered with either hCG or GnRHa in both groups, and considering the impact of ovulation triggering on the microenvironment surrounding the oocyte [32], we compared results between groups according to final oocyte maturation trigger methods. In the control group, we observed stable FF steroid levels regardless of ovulation trigger method. Conversely, in the study group triggered with GnRHa, E2 levels increased significantly as compared to hCG trigger, although T levels remained comparably high. Similarly, results of CC gene expression revealed a less favorable profile in the study compared to the control group triggered with hCG, although only difference in *PTGS2* expression reached statistical significance. On the contrary, the expression of *PTGS2* and *GREM1* was significantly improved in the study compared to the control group when triggered with GnRHa. Decreased estradiol/testosterone ratio in Let-COH triggered with hCG may actually generate a suboptimal FF environment. This observation is in accordance with previous studies on infertile patients, in which aromatase inhibitors were used for androgen priming before standard COH to increase follicular sensitivity to FSH. In these studies, high FF testosterone level was associated with reduced oocyte fertilization rates but good embryo quality [33, 34]. The authors suggested a dual effect of androgens on granulosa cell function, as they increase FSH receptors and stimulate steroidogenesis in small antral follicles but may be detrimental in later stages of follicular development. Similarly, in a mouse model, addition of a high dose of an aromatase inhibitor during follicular culture improved maturation rates compared with controls. However the fertilization rates were decreased (45% vs 76%) while blastocyst/2-cell embryo ratios were similar between groups [35].

In our study, increased E2 levels following GnRHa trigger in the Let-COH group induced a high *PTGS2* expression, which may be beneficial in terms of oocyte quality. Indeed, estradiol has been previously shown in aromatase knockout mice to be mandatory for *PTGS2* induction and ovulation [36]. We hence hypothesize that the FSH surge induced by agonist trigger at the time of letrozole interruption may stimulate aromatase activity to increase estradiol production in the pre-ovulatory follicle. This rise in estradiol level may positively influence oocyte

maturity and quality, regardless of follicular testosterone level [37].

Our results are also in accordance with a recent study that showed comparable pregnancy rates in normal responder infertile patients undergoing Let-COH or conventional COH. The authors observed significantly lower E2 and significantly higher T levels in the FF [38].

Our study has several limitations. Follicular size limit determined for ovulation trigger was different between groups. Indeed, more than a decade ago, Oktay et al. showed lower maturation rates when Let-COH was triggered as soon as follicles reached 17–18 mm as in conventional COH. This observation led to a timing modification of ovulation triggering in Let-COH and maturation rate improvement [10]. In our study however, maturation rates were comparable between groups regardless of ovulation trigger, and only FFs of similar size were analyzed to avoid possible bias related to follicular size.

FF steroid concentrations should be interpreted with caution despite the significant difference observed between groups, as high variability of FF steroid concentrations within and between subjects were previously reported; testosterone and progesterone showed higher inter-subject variability while estradiol showed higher intra-subject variability, suggesting that estradiol may be a better marker to indirectly assess oocyte quality [39]. Comparison of CC gene expression between groups should also be interpreted with caution since they remain merely indirect markers of oocyte competence. Moreover, the use of predictive gene panel of oocyte quality expressed in CC has not been validated yet in clinical practice. Finally, difference between high and low quality embryos didn't reach significance in the hCG triggered control cohort. However, as all our BC patients are now triggered with GnRHa, CC gene expression analysis in the latter was more clinically relevant.

In conclusion, clinical results of Let-COH efficiency in terms of oocytes quality and pregnancy outcomes in BC patients are still limited, and large data will probably be available in several years. We evaluated for the first time Let-COH impact on oocyte microenvironment in BC patients. Our results suggest that GnRHa-trigger may improve oocyte quality in this population.

Additional files

Additional file 1: Table S1. Primer sequences for housekeeping and target genes. (DOCX 14 kb)

Additional file 2: Figure S1. Fold-change in gene expression in cumulus cells of good quality embryos compared with poor quality embryos and unfertilized oocytes in the control group, after hCG (a) and GnRHa (b) ovulation trigger, respectively. Results are presented in mean +/- SEM. * $p = 0.004$; ** $p = 0.036$. HAS2: hyaluronan synthase 2; PTGS2: prostaglandin endoperoxide synthase 2; GREM1: gremlin 1. (DOCX 64 kb)

Abbreviations

AMH: Anti-müllerian hormone; BC: Breast cancer; CC: Cumulus cells; COC: Cumulus-oocyte-complex; COH: Controlled ovarian hyperstimulation; E2: Estradiol; FF: Follicular fluid; FP: Fertility preservation; FSH: Follicular stimulating hormone; GnRH: Gonadotropin releasing hormone; GREM1: Gremlin1; HAS2: Hyaluronic acid synthase 2; hCG: Human chorionic gonadotropin; HPRT-1: Hypoxanthine phosphoribosyltransferase-1; ICSI: Intracytoplasmic sperm injection; Let-COH: Letrozole associated controlled ovarian hyperstimulation; LH: Luteinizing hormone; MII: Metaphase 2 oocyte; OPU: Oocyte pick-up; P: Progesterone; PCOS: Polycystic ovary syndrome; PN: Pronuclei; PTGS2: Prostaglandin-endoperoxide synthase-2; RPL19: Ribosomal protein L19; T: Testosterone

Acknowledgements

We thank all the oncologists and gynecologists of Ambroise-Paré Hospital, Bordet Institute, Brugmann Hospital, Chirec-Edith Cavell and Sainte Anne Hospitals, Cliniques de l'Europe, Chwapi Notre Dame Hospital, EpiCura-Ath Hospital, Erasme Hospital, Haute Senne Medical Center, Ixelles Hospital, Marie-Curie Hospital, Saint Joseph Hospital, Saint Pierre Hospital and Tivoli Hospital who have referred their breast cancer patients for fertility preservation, as well as all the nurses and doctors of the Fertility Clinic of Erasme Hospital for their help and collaboration with the fertility preservation program. A special acknowledgment to R. Antonacci, J. Biramane, G. Fasano, EG. Mbongolo Mbella, TM. Uyen Nguyen, C. Vanhelleputte, A. Van Langendonck and AS. Vannin from the IVF laboratory for their tremendous help and efforts for the realization of this project. We thank M. Leprovost, MI Garcia and V. Fernandez-Vallone of the IRIBHM for their collaboration and help with qPCR analysis. We also acknowledge the contribution of a medical writer, Sandy Field, PhD, for English language editing.

Funding

This study was supported by grants from the FNRS-Télévie (Crédit n° 7.4612.13 and Crédit n°7.4528.15) and the Fonds Erasme.

Availability of data and materials

The data and materials are available from the corresponding author on reasonable requests.

Authors' contributions

OG designed the study, collected and processed samples, performed statistical analysis and wrote the manuscript. GVDS and JD collected and processed samples and participated in the writing of the manuscript. EGM helped with primers design and revised the manuscript. CG helped with the data analysis and revised the manuscript. AD and FD participated in the study design and revised the manuscript. VDM was involved with the study design and revised the statistical analysis and the manuscript. ID designed the study, interpreted results and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Erasme Hospital in Brussels. All participants signed an informed consent ([Clinicaltrials.gov](#), NCT02661932).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Fertility Clinic, Department of Obstetrics and Gynecology, CUB-Hôpital Erasme, Université Libre de Bruxelles (ULB), Route de Lennik 808, Brussels, Belgium. ²Research Laboratory on Human Reproduction, Université Libre de Bruxelles (ULB), Route de Lennik 808, Brussels, Belgium. ³Laboratory of Chemistry, CUB-Hôpital Erasme, Université Libre de Bruxelles (ULB), Route de

Lennik 808, Brussels, Belgium. ⁴IRIBHM and SBIM, Université Libre de Bruxelles (ULB), Route de Lennik 808, Brussels, Belgium.

Received: 10 November 2018 Accepted: 12 December 2018

Published online: 03 January 2019

References

- DeSantis C, Ma J, Bryan L, Jemal A. Breast cancer statistics, 2013. *CA Cancer J Clin.* 2014;64(1):52–62.
- DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, et al. Cancer treatment and survivorship statistics, 2014. *CA Cancer J Clin.* 2014;64(4):252–71.
- Hartman EK, Eslick GD. The prognosis of women diagnosed with breast cancer before, during and after pregnancy: a meta-analysis. *Breast Cancer Res Treat.* 2016;160(2):347–60.
- Tomasini N, Lamberti M, Hulsbosch S, Peccatori AF, Amant F. Strategies for fertility preservation in young early breast cancer patients. *Breast.* 2014;23(5):503–10.
- Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet.* 2013;381(9869):805–16.
- Llarena NC, Estevez SL, Tucker SL, Jeruss JS. Impact of Fertility Concerns on Tamoxifen Initiation and Persistence. *J Natl Cancer Inst.* 2015;107(10):djv202.
- Waks AG, Partridge AH. Fertility preservation in patients with breast Cancer: necessity, methods, and safety. *J Natl Compr Cancer Netw.* 2016;14(3):355–63.
- De Vos M, Smitz J, Woodruff TK. Fertility preservation in women with cancer. *Lancet.* 2014;384(9950):1302–10.
- Oktay K, Buyuk E, Libertella N, Akar M, Rosenwaks Z. Fertility preservation in breast cancer patients: a prospective controlled comparison of ovarian stimulation with tamoxifen and letrozole for embryo cryopreservation. *J Clin Oncol.* 2005;23(19):4347–53.
- Oktay K, Hourvitz A, Sahin G, Oktem O, Safro B, Cil A, et al. Letrozole reduces estrogen and gonadotropin exposure in women with breast cancer undergoing ovarian stimulation before chemotherapy. *J Clin Endocrinol Metab.* 2006;91(10):3885–90.
- Bhatnagar AS, Hausler A, Schiewek K, Lang M, Bowman R. Highly selective inhibition of estrogen biosynthesis by CGS 20267, a new non-steroidal aromatase inhibitor. *J Steroid Biochem Mol Biol.* 1990;37(6):1021–7.
- Njar VC, Brodie AM. Comprehensive pharmacology and clinical efficacy of aromatase inhibitors. *Drugs.* 1999;58(2):233–55.
- Andersen CY, Lossi K. Increased intrafollicular androgen levels affect human granulosa cell secretion of anti-Müllerian hormone and inhibin-B. *Fertil Steril.* 2008;89(6):1760–5.
- Tetsuka M, Hillier SG. Differential regulation of aromatase and androgen receptor in granulosa cells. *J Steroid Biochem Mol Biol.* 1997;61(3–6):233–9.
- Carpintero NL, Suarez OA, Mangas CC, Varea CG, Rioja RG. Follicular steroid hormones as markers of oocyte quality and oocyte development potential. *J Hum Reprod Sci.* 2014;7(3):187–93.
- Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol.* 2009;7:40.
- Sanchez F, Smitz J. Molecular control of oogenesis. *Biochim Biophys Acta.* 2012;1822(12):1896–912.
- Pereira N, Hancock K, Cordeiro CN, Lekovich JP, Schattman GL, Rosenwaks Z. Comparison of ovarian stimulation response in patients with breast cancer undergoing ovarian stimulation with letrozole and gonadotropins to patients undergoing ovarian stimulation with gonadotropins alone for elective cryopreservation of oocytes. *Gynecol Endocrinol.* 2016;32(10):823–6.
- Haas J, Bassil R, Meriano J, Samara N, Barzilay E, Gonen N, et al. Does daily co-administration of letrozole and gonadotropins during ovarian stimulation improve IVF outcome? *Reprod Biol Endocrinol.* 2017;15(1):70.
- Gebhardt KM, Feil DK, Dunning KR, Lane M, Russell DL. Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. *Fertil Steril.* 2011;96(1):47–52 e2.
- McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, et al. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod.* 2004;19(12):2869–74.

22. Assou S, Haouzi D, De Vos J, Hamamah S. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Mol Hum Reprod.* 2010;16(8):531–8.
23. Anderson RA, Sciorio R, Kinnell H, Bayne RA, Thong KJ, de Sousa PA, et al. Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. *Reproduction.* 2009;138(4):629–37.
24. Assou S, Haouzi D, Dechaud H, Gala A, Ferrieres A, Hamamah S. Comparative gene expression profiling in human cumulus cells according to ovarian gonadotropin treatments. *Biomed Res Int.* 2013;2013:354582.
25. Cillo F, Brevini TA, Antonini S, Paffoni A, Ragni G, Gandolfi F. Association between human oocyte developmental competence and expression levels of some cumulus genes. *Reproduction.* 2007;134(5):645–50.
26. Goldrat O, Gervy C, Englert Y, Delbaere A, Demeeestere I. Progesterone levels in letrozole associated controlled ovarian stimulation for fertility preservation in breast cancer patients. *Hum Reprod.* 2015;30(9):2184–9.
27. Sonmezler M, Turkcuoglu I, Coskun U, Oktay K. Random-start controlled ovarian hyperstimulation for emergency fertility preservation in letrozole cycles. *Fertil Steril.* 2011;95(6):2125 e9–11.
28. Borgbo T, Povlsen BB, Andersen CY, Borup R, Humaidan P, Grondahl ML. Comparison of gene expression profiles in granulosa and cumulus cells after ovulation induction with either human chorionic gonadotropin or a gonadotropin-releasing hormone agonist trigger. *Fertil Steril.* 2013;100(4):994–1001.
29. Haas J, Ophir L, Barzilay E, Machtinger R, Yung Y, Orvieto R, et al. Standard human chorionic gonadotropin versus double trigger for final oocyte maturation results in different granulosa cells gene expressions: a pilot study. *Fertil Steril.* 2016;106(3):653–9 e1.
30. Andersen CY, Humaidan P, Ejdrup HB, Bungum L, Grondahl ML, Westergaard LG. Hormonal characteristics of follicular fluid from women receiving either GnRH agonist or hCG for ovulation induction. *Hum Reprod.* 2006;21(8):2126–30.
31. Oktay K, Turan V, Bedoschi G, Pacheco FS, Moy F. Fertility preservation success subsequent to concurrent aromatase inhibitor treatment and ovarian stimulation in women with breast Cancer. *J Clin Oncol.* 2015;33(22):2424–9.
32. Fauser BC, de Jong D, Olivennes F, Wramsby H, Tay C, Itskovitz-Eldor J, et al. Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for in vitro fertilization. *J Clin Endocrinol Metab.* 2002;87(2):709–15.
33. Lossi K, Andersen CY, Loft A, Freiesleben NL, Bangsbøll S, Andersen AN. Short-term androgen priming by use of aromatase inhibitor and hCG before controlled ovarian stimulation for IVF. A randomized controlled trial. *Hum Reprod.* 2008;23(8):1820–9.
34. Lossi K, Andersen AN, Loft A, Freiesleben NL, Bangsbøll S, Andersen CY. Androgen priming using aromatase inhibitor and hCG during early-follicular-phase GnRH antagonist down-regulation in modified antagonist protocols. *Hum Reprod.* 2006;21(10):2593–600.
35. Hu Y, Cortvrindt R, Smitz J. Effects of aromatase inhibition on in vitro follicle and oocyte development analyzed by early preantral mouse follicle culture. *Mol Reprod Dev.* 2002;61(4):549–59.
36. Toda K, Ono M, Yuhki K, Ushikubi F, Saibara T. 17beta-estradiol is critical for the preovulatory induction of prostaglandin E(2) synthesis in mice. *Mol Cell Endocrinol.* 2012;362(1–2):176–82.
37. McNatty KP, Smith DM, Makris A, Osathanondh R, Ryan KJ. The microenvironment of the human antral follicle: interrelationships among the steroid levels in antral fluid, the population of granulosa cells, and the status of the oocyte in vivo and in vitro. *J Clin Endocrinol Metab.* 1979;94(6):851–60.
38. Haas J, Bassil R, Gonen N, Meriano J, Jurisicova A, Casper RF. The VEGF and PEDF levels in the follicular fluid of patients co-treated with LETROZOLE and gonadotropins during the stimulation cycle. *Reprod Biol Endocrinol.* 2018;16(1):54.
39. Carpintero NL, Suarez OA, Varea CG, Rioja RG, Mangas CC. Variability between the follicular steroid hormone levels in different follicles of the same patient and between patients. *J Hum Reprod Sci.* 2015;8(1):37–42.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

