Journal Pre-proof


# Cumulus cells microRNA expression when LH is added to controlled ovarian stimulation protocol: a pilot study

Francieli Maria Vigo MD, PhD , Renato Fraietta , Fernanda Rodrigues , Cristina V Carvalho , Tatiana CS Bonetti , Eduardo LA Motta

PII: S1472-6483(21)00355-2
DOI: https://doi.org/10.1016/j.rbmo.2021.07.014
Reference: RBMO 2750

To appear in: *Reproductive BioMedicine Online*

Received date: 18 February 2021
Revised date: 1 July 2021
Accepted date: 6 July 2021

Please cite this article as: Francieli Maria Vigo MD, PhD , Renato Fraietta , Fernanda Rodrigues , Cristina V Carvalho , Tatiana CS Bonetti , Eduardo LA Motta , Cumulus cells microRNA expression when LH is added to controlled ovarian stimulation protocol: a pilot study, *Reproductive BioMedicine Online* (2021), doi: https://doi.org/10.1016/j.rbmo.2021.07.014

# Cumulus cells microRNA expression when LH is added to controlled ovarian stimulation protocol: a pilot study

Francieli Maria Vigo[1], Renato Fraietta[2], Fernanda Rodrigues[1,3], Cristina V Carvalho[1], Tatiana CS Bonetti[1], Eduardo LA Motta[1,3]

1. Departamento de Ginecologia, Escola Paulista de Medicina da Universidade Federal de São Paulo (UNIFESP-EPM). Rua Napoleão de Barros, 608 Vila Clementino, São Paulo – SP. CEP: 04024-002, Brazil
2. Setor Integrado de Reprodução Humana – Departamento Cirurgia. Universidade Federal de São Paulo - Escola Paulista de Medicina (UNIFESP-EPM). Rua Botucatu, 725. Vila Vila Clementino. São Paulo - SP. CEP: 04024-002, Brazil
3. Huntington - Medicina Reprodutiva. Av. República do Líbano, 529 Ibirapuera. São Paulo – SP. CEP: 04501-000, Brazil

**Corresponding Author**

Francieli Maria Vigo, MD, PhD

Address: Germano Arduino Toniolo, 196 Caxias do Sul- RS, CEP 95012-346, Brazil

Phone: +55 54 99962-8722

Email: francieli.vigo@gmail.com

## ABSTRACT

**Research Question**: Recombinant FSH administration in controlled ovarian stimulation for *in vitro* fertilization (IVF) is a standard procedure, while the role of LH is controversial. MicroRNAs participate in the regulation of gene expression of gonadotrophin functions. We aimed to study the possible role of miRNAs in ovarian follicular development in groups having different ovarian stimulation protocols. We aimed to identify miRNA expression in cumulus cells of infertile women undergoing *IVF* by comparing two distinct protocols; to study if there are different miRNA expression profiles in these protocols, and to analyse the regulated pathways. **Design**: This prospective observational study included 13 patients following the inclusion criteria: < 38 years of age, a tubal infertility factor, a male factor, or idiopathic infertility. This is a pilot study in which the patients were aleatory enrolled into two groups: 7 in FSH group (recombinant FSH, 225 IU) and 6 in FSH + LH group (recombinant FSH, 150 IU + recombinant LH, 75 IU). The granulosa cells obtained from the follicular ovarian pick-up were analysed using PCR. Results were analysed using DIANA Tools, an online bioinformatics tool. **Results**: Among the 84 microRNAs evaluated, 11 were differentially expressed between the groups, all of which were overexpressed in the FSH + LH group, compared to the FSH group. Differentially expressed miRNAs profiles are related to oestrogen signalling, oocyte meiosis, and pluripotent cells regulation. **Conclusion**: miRNA overexpression in the FSH + LH group is consistent with the independent and fundamental role of LH in folliculogenesis, leading to a distinct molecular response between groups.

**Keywords:** ovarian stimulation, recombinant LH, microRNA, PCR *array*

## INTRODUCTION

Folliculogenesis is a dynamic and highly regulated process that requires coordinated actions of multiple systems to develop a viable gamete. In infertile women, follicle-stimulating hormone (FSH) is commonly used for ovarian stimulation; however, the role of supplementary luteinising hormone (LH) is still controversial. Studies about patients with worst prognosis, especially patients with advanced maternal age shows some benefits in ovarian stimulation. LH has several actions in the intrafollicular environment, that can vary considerably due to different gonadotropin protocols. (Kovacs, et al. 2010, Westergaard, et al. 2011).

The granulosa cells surround the oocyte by multiple somatic layers, named cumulus cells (CCs). CCs are highly specialised cells with trans-zonal cytoplasmic projections, which form gap junctions to create the cumulus–oocyte complex (COC) (Abd El Naby, et al. 2013). Disruption or deregulation of the COC interaction may affect oocyte quality, and consequently, embryo development (Huang and Wells 2010, Patrizio, et al. 2007, Vanderhyden and Armstrong 1989).

It is believed that CC gene expression might vary according to the stimulation protocol for ovarian stimulation in in vitro fertilization (IVF) cycles, affecting the stages of oocyte nuclear maturation, embryo development, and pregnancy outcome (Assou, et al. 2013). In addition to mRNA, microRNAs (miRNAs) are small RNA molecules consisting of noncoding RNAs that control

mRNA expression and proteins, and are believed to be involved in post-transcriptional regulation of spatial and temporal gene expression (Carthew and Sontheimer 2009).

Numerous miRNAs have been identified in CC with potential regulatory functions during folliculogenesis. For example, the expression of three miRNA (miR-21, -132, and -212) has been found to be increased in granulosa cells following the LH surge ( Fiedler, Carletti et al. 2008). Despite of mechanisms and genes regulated by these miRNA remain to be determined, several of them have been shown to exhibit differential expression in response to hormonal stimulation.(Christenson 2010).

Based on this, we hypothesised that supplementation of LH to FSH during ovarian stimulation could modulate gene expression in CC through miRNA control. Thus, the aim of the present study was to identify miRNA profiles in CCs isolated from periovulatory follicles of patients undergoing controlled ovarian stimulation (COS) with recombinant follicle stimulating hormone (rFSH), in conjunction with recombinant luteinizing hormone (rLH), compared to the conventional protocol that uses only rFSH.



## MATERIALS AND METHODS

This prospective study included 13 women who were enrolled in the assisted reproduction program at the Human Reproduction Section of the Federal University of São Paulo (UNIFESP). This is a pilot study, that was approved by the UNIFESP Institutional Review Board (reference no. 1545/11 and no 196/96), and all the couples read and signed an informed consent form.

The inclusion criteria for the enrolment of patients were: (i) age under 38 years; (ii) hormonal measurements considered normal (FSH ≤ 14 UI/mL, LH ≤ 12 UI/mL, TSH ≤ 3.0 mcUI/mL and prolactin < 31 mg/mL); (iii) eumenorrheic (25 - 35 days of cycle length); (iv) no hormonal contraceptive in the last 3 months; (v) tubal factor infertility, unexplained infertility, or male factor infertility. We excluded women presenting (i) uni or bilateral hydrosalpinx, (ii) severe endometriosis (stages III or IV), (iii) polycystic ovary syndrome, (iv) ovarian failure, or (v) any ovarian or intrauterine anatomical alteration.

**Ovarian stimulation protocol and study groups**

The routine of the service perform the controlled ovarian stimulation by using human recombinant FSH (Gonal® Merck) or recombinant FSH associated to recombinant LH (Pergoveris® Merck) associated to GnRH antagonist to pituitary supression. Patients included in this study were prospectively enrolled to receive one of those two protocols for ovarian stimulation. The inclusion of patients in each group was aleatory in a 1:1 ratio alternating one for rFSH protocol and one for rFSH+rLH protocol.

Briefly, ovarian stimulation was initiated on menstrual cycle day 2 or 3 by using 225 IU of human recombinant FSH (Gonal® Merck) or 150 IU of recombinant FSH + 75 IU of recombinant LH (Pergoveris® Merck). When, at least, one follicle exceeded a mean diameter of 14 mm or, on the 8$^{th}$ day of the menstrual cycle, pituitary suppression was achieved by daily administration of 0.25 mg of cetrorelix acetate (Cetrotide, Merck). Oocyte maturation was triggered by the administration of 250 mcg of recombinant human Chorionic Gonadotropin (rhCG) (Ovitrelle, Merck). Transvaginal oocyte retrieval was

performed 36 hours after hCG administration and follicles with more than 10mm were punctured. Immediately after oocyte recovery, CCs were cut from all oocytes collected for each of the patients and frozen at -80°C until the analysis.

Patients who had received r-FSH (Gonal® - Merck) (FSH group) and those who had received rFSH + rLH (Pergoveris® Merck) (FSH + LH group) received the same amount of 225 IU of gonadotropins, independent of gonadotropin formulation, according to Stelman-Poolman's theory. The number of patients included were six patients in the FSH group and seven patients in the FSH + LH group.

**miRNA expression analysis**

The miRNA expression profile was determined by the miScript™ – miRNA PCR Array - MIHS-001ZC-24 (Qiagen) including 84 of the most well-known human miRNAs. The analysis were performed individually for each patient. Briefly, the CC RNA purification was performed using the miRNeasy mini Kit (Qiagen) according to the manufacturer's instructions and total RNA concentration was measured using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific). The RNA samples were reverse transcribed to cDNA using the miScript II RT Kit (Qiagen) and miRNA expression using the miScript™ – miRNA PCR Array - MIHS-001ZC-24 (Qiagen) followed the manufacturer's Protocol. The reactions were carried out using the StepOne Plus™ Real Time PCR System and analysed using Sequence Detection Software (SDS) v1.3 (Applied Biosystems, Thermo Fisher Scientific). The detected miRNAs were expressed as measured by using the quantitative expression protocol ΔΔCt by GeneGlobe Data Analysis Center (Qiagen). The relative expression of miRNAs between the study groups was compared using a

t-test and expressed as a fold change of each miRNA from FSH+LH groups over FSH group, considering a significant fold-change of ≥ 2.0, ≤ 2.0, and a $p \leq 0.05$.

About the analysis of demographic and clinical characteristics of patients, we used a SPSS V.21 (IBM SPSS Software, USA) and the non-parametrical test (Mann-Whitney) due to small number of samples per group. We described the results as mean ± standard deviation (SD) and considered $p \leq 0.05$ statistically significant.

**Data analysis**

Differentially expressed miRNAs were then subjected to a second step analysis to evaluate the pathways associated with their function, using an online bioinformatics tool (DIANA: http://diana.imis.athena-innovation.gr/DianaTools/index.php). The DIANA provides algorithms for interpretation and archiving data in a systematic framework to annotate miRNA regulatory elements and targets along with the interpretation of their role in various diseases and pathways. The software is capable of identifying potentially altered molecular pathways by the expression of single or multiple miRNAs.



**RESULTS**

**Profiling of patients and miRNAs in the study groups**

Patient demographics and the clinical profiles of patients are presented in Table 1. The age, basal hormones measurements and number of MII oocytes

collected were similar between FSH and FSH + LH groups. The mean of total number of oocytes/COC collected per patient were 14.9±1.5 in the FSH and 13.1±2.0 in the FSH+LH groups (p=0.429) and the MII oocyte rate was similar between groups (58.2% versus 55.5%, p=0.820). Despite it was not an objective of our study, we had four pregnancies (26.7%), as three in the FSH and one on the FSH+LH group.

The miRNA expression analysis showed that all of them were expressed in the CCs of both groups. However, all 84 analysed miRNAs were upregulated in the FSH + LH group, when compared to the FSH group (Figure 1) and 33 miRNAs were significantly upregulated (fold change ≥ 2.0 and p ≤ 0.05) in the FSH + LH group, compared to the FSH group (Table 2).

**Bioinformatics analysis**

For a better understanding of the more pronounced miRNA upregulation in the FSH + LH group, we performed a bioinformatics analysis to obtain insights into the possible functions and processes involving those miRNAs. For this analysis we considered to be extremely up-regulated (fold change ≥ 3.0 and p ≤ 0.01) 11 miRNAs, which are highlighted in Table 2. Those 11 miRNAs selected for bioinformatics analysis were submitted to the DIANA tools individually or combined within the same miRNA family. The pathways associated to miRNA function were analysed.

Three important pathways possibly associated to folliculogenesis and oocyte maturation were identified. The hsa-miR-26a, hsa-miR-26b, hsa-miR-200c, hsa-miR-181b, hsa-let-7a, and hsa-let-7e were associated with genes

that regulate oocyte meiosis (Figure 2). The miRNA hsa-miR-181a was associated to oestrogen signalling pathway (Figure 3) and hsa-let-7e, hsa-miR-181a, and hsa-miR-181b regulate genes of the stem cell pluripotency signalling pathway (Figure 4).

**DISCUSSION**

We compared the expression of miRNA in cumulus cells of patients undergoing in vitro fertilization (IVF) under two different ovarian stimulation protocols: recombinant FSH alone or the combination of recombinant FSH + recombinant LH (2:1 proportion). Our findings demonstrated that all miRNAs studied were overexpressed when LH was added. Moreover, super expressed miRNAs (fold change ≥ 3.0 and $p \leq 0.01$) were related to cumulus cell gene expression with oestrogen production, oocyte meiosis, and stem cell activity, all crucial steps for a successful fertilisation and subsequent embryonic development.

Ovarian stimulation for the IVF cycles could be achieved with different gonadotropin regimens; however, since the mid-90s, when DNA techniques were designed to produce recombinant FSH with many potential advantages, such as batch-to-batch consistency, no contamination, among others, those benefits have been postulated with the use of FSH. However, from an individualised perspective, the addition of LH during ovarian stimulation in women with a worst prognosis, especially women with advanced maternal age, has demonstrated some benefits (Alviggi, et al. 2006, Bosch, et al. 2011, Hill, et al. 2012 ). Urinary gonadotropins were initially used in ovarian stimulation protocols. With the availability of recombinant LH, new stimulation protocols

haver incorporated its use and could confirm its beneficial actions in this group of patients (Hill, et al. 2012).

In clinical practice, the LH is added in ovarian stimulation since it could supposedly repair the internal mechanisms of the oocyte apparatus, aiming to improve potential of oocyte development (Hill, et al. 2012). Clinical data have already proven that LH is essential for oocyte maturation at the end of the follicular period in the natural cycle and that low levels of LH may be associated with worse clinical outcomes (Fleming, et al. 1998, Hillier 2013, von Wolff, et al. 2014). In addition, supplementation of recombinant LH to ovarian stimulation could decrease the levels of apoptosis in cumulus cells (Fischer 2007). However, clinical results are extremely difficult to prove, mainly in severe situations, opening the window for molecular studies, such as mRNA in CCs.

Our study aimed to evaluate how the addition of LH to ovarian stimulation affects miRNA expression in granulosa cells, as those molecules are post-transcriptional gene regulator and refers to many different regulatory events. Despite of functions of miRNAs are not completely known, it is recognised that all types of ovarian cells express miRNAs and regulate ovarian function for gamete signalling (Maalouf, et al. 2016). Several authors have already shown the involvement of miRNAs in reproductive physiology, including folliculogenesis, spermatogenesis, and embryonic development itself (Bernstein, et al. 2003, Dunning, et al. 2012, Otsuka, et al. 2008). Apoptosis pathways have been demonstrated to play a regulatory role in miRNAs during folliculogenesis (Assou, et al. 2013).

Interestingly, all miRNA studied here were overexpressed in CC when LH was added in the ovarian stimulation protocol, what makes us believe in the paramount importance of its action in molecular events of folliculogenesis and oocyte quality establishment. It is well established that cumulus cells proliferate during folliculogenesis and their essential activity is related to oocyte maturation and quality (Assou, et al. 2013). We identified overexpressed miRNAs linked to two important folliculogenesis pathways: oestrogen signalling (miRNA hsa-miR-181a) and activation of meiosis (miRNAs hsa-miR-26a, hsa-miR-26b, hsa-miR-200c, hsa-miR-181b, hsa-let-7a, and hsa-let-7e). These pathways are interconnected and despite the CCs are not directly responsible for the meiosis process, improvement in oestrogen synthesis could be related to an enhanced signalling and a better substrate to activate meiotic competence, since it clearly depends on an adequate follicular maturation. Studies in mouse follicles in which granulosa cells were transfected with miRNA showed that miRNA in granulosa cells can modify the competence of oocyte meiosis, leading to a better ovulation process (Kim, et al. 2013).

The let-7 miRNAs family, which were overexpressed in our study, have already been studied by other authors with similar results, demonstrating its possible role in ovarian steroidogenesis and follicular growth (Velthut-Meikas, et al. 2013), regulation of pregnancy (Zhang, et al. 2013) and the development of ovarian cancer (Shibahara, et al. 2012). Also, studies in cattle demonstrated that the let-7 family is extremely abundant in bovine ovarian tissue, having a direct role in oocyte development through the regulation of genes which control mitotic proliferation and meiotic activity (Miles, et al. 2012, Salilew-Wondim, et al. 2014), corroborating our findings regarding its involvement in oocyte meiosis.

Moreover, the overexpression of hsa-let-7e, hsa-miR-181a, and hsa-miR-181b, when LH was added, is associated with genes linked to cellular pluripotency, suggesting that folliculogenesis optimisation would, consequently, have a crucial role in embryogenesis, as the main characteristic of embryonic cells is pluripotency (Nichols and Smith 2012). We did not find other studies identifying the hsa-miR-200 family in granulosa cells.

The mechanisms by which LH contributes to a better ovarian response in stimulated cycles are so far discussed, as well as its contribution to the quality of recovered oocytes. In our study, both groups received the same dose of gonadotropins in different combinations: 225 units of FSH alone, or a combination of 150 units of FSH with 75 units of LH. It is important to highlight that both groups had the same ability to recover oocytes and to convert them to embryos, featuring a synergy between FSH and LH to promote an ovarian response. However, the molecular function involved in the process of follicle recruitment seems to be affected by LH addition, as demonstrated by the evident difference in miRNA expression in cumulus cells. The overexpression of miRNAs in the FSH + LH group is consistent with the theory that LH has an independent and fundamental role in folliculogenesis, leading to a completely distinct molecular response between groups. However, how much those molecular differences found in our study would turn in clinical benefits for patients in IVF treatments, where the overdose of FSH seems to be enough to recruit mature oocytes, cannot be determined, and our conclusions are only speculative at this point.

The small number of patients included in our study is a limitation and for that, it is a pilot study. However, this is an initial screening study in which a big

number of miRNA were evaluated in an array. The outcome found here should be validated in studies evaluating the genes regulated by the miRNA differentially expressed. Also, studies focusing the molecular mechanisms might contribute to evaluate the action of LH in the oocyte quality.

In summary, the proper activation using the LH protocol seems to produce a different link in granulosa cells that compose the *cumulus,* changing the oestrogen signalling pathways and oocyte meiosis, and for that possibly modifying oogenesis. In addition, the same signs could be speculated with embryogenesis, represented by the regulation of pluripotent cells. At this point, it is worth reflecting on the possible hypotheses on how those molecular modifications can provide an improvement or not in oocyte competence. Based in our study is possible to conclude the molecular environment in the cumulus cells is modulated when LH is added in the ovarian stimulation protocol. The over-expression of miRNA in FSH+LH group or the downregulation of miRNA in the FSH group in response to LH adding are molecular mechanisms that, can eventually change some aspects of folliculogenesis and, in the end of the day, the oocyte competence. But, how it can result in a clinical improvement, need to be studied in forward studies.



**Declaration of Interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

**Funding**

This work was supported by Sao Paulo Research Foundation (FAPESP) nº 2012/16911-0

**Author contribution statement**

FV conceived the study and wrote the paper

TCSB analyzed the data and wrote the paper

CVC performed experiments

RF supported in the clinical conduction of patients treatment

FR supported in the clinical conduction of patients treatment

ELAM conceived the study and wrote the paper

**Acknowledgements**

The authors gratefully acknowledge the Human Reproduction Section of the Federal University of São Paulo (UNIFESP) team for the assistance with patients assistance and sample collection

**References**

**Abd El Naby, WS, TH Hagos, MM Hossain, D Salilew-Wondim, AY Gad, F Rings, MU Cinar, E Tholen, C Looft, K Schellander, M Hoelker, and D Tesfaye** 2013 Expression analysis of regulatory microRNAs in bovine cumulus oocyte complex and preimplantation embryos. *Zygote* **21** 31-51.

**Al-Edani, T, S Assou, A Ferrieres, S Bringer Deutsch, A Gala, CH Lecellier, O Ait-Ahmed, and S Hamamah** 2014 Female aging alters expression of human cumulus cells genes that are essential for oocyte quality. *Biomed Res Int* **2014** 964614.

**Alviggi, C, R Clarizia, A Mollo, A Ranieri, and G De Placido** 2006 Outlook: who needs LH in ovarian stimulation? *Reprod Biomed Online* **12** 599-607.

**Assou, S, T Al-edani, D Haouzi, N Philippe, CH Lecellier, D Piquemal, T Commes, O Ait-Ahmed, H Dechaud, and S Hamamah** 2013 MicroRNAs: new candidates for the regulation of the human cumulus-oocyte complex. *Hum Reprod* **28** 3038-3049.

**Bernstein, E, SY Kim, MA Carmell, EP Murchison, H Alcorn, MZ Li, AA Mills, SJ Elledge, KV Anderson, and GJ Hannon** 2003 Dicer is essential for mouse development. *Nat Genet* **35** 215-217.

**Bosch, E, E Labarta, J Crespo, C Simón, J Remohí, and A Pellicer** 2011 Impact of luteinizing hormone administration on gonadotropin-releasing hormone antagonist cycles: an age-adjusted analysis. *Fertil Steril* **95** 1031-1036.

**Bradford, AP, K Jones, K Kechris, J Chosich, M Montague, WC Warren, MC May, Z Al-Safi, S Kuokkanen, SE Appt, and AJ Polotsky** 2015 Joint MiRNA/mRNA expression profiling reveals changes consistent with development of dysfunctional corpus luteum after weight gain. *PLoS One* **10** e0135163.

**Carthew, RW, and EJ Sontheimer** 2009 Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136** 642-655.

**Christenson, LK** 2010 MicroRNA control of ovarian function. *Anim Reprod* **7** 129-133.

**Dunning, KR, LN Watson, DJ Sharkey, HM Brown, RJ Norman, JG Thompson, RL Robker, and DL Russell** 2012 Molecular filtration properties of the mouse expanded cumulus matrix: controlled supply of metabolites and extracellular signals to cumulus cells and the oocyte. *Biol Reprod* **87** 89.

**Filicori, M** 1999 The role of luteinizing hormone in folliculogenesis and ovulation induction. *Fertil Steril* **71** 405-414.

**Fischer, R** 2007 Understanding the role of LH: myths and facts. *Reprod Biomed Online* **15** 468-477.

**Fleming, R, F Lloyd, M Herbert, J Fenwick, T Griffiths, and A Murdoch** 1998 Effects of profound suppression of luteinizing hormone during ovarian stimulation on follicular activity, oocyte and embryo function in cycles stimulated with purified follicle stimulating hormone. *Hum Reprod* **13** 1788-1792.

**Hill, MJ, G Levy, and ED Levens** 2012 Does exogenous LH in ovarian stimulation improve assisted reproduction success? An appraisal of the literature. *Reprod Biomed Online* **24** 261-271.

**Hillier, SG** 2013 IVF endocrinology: the Edwards era. *Mol Hum Reprod* **19** 799-808.

**Huang, Z, and D Wells** 2010 The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. *Mol Hum Reprod* **16** 715-725.

**Imbar, T, and I Eisenberg** 2014 Regulatory role of microRNAs in ovarian function. *Fertil Steril* **101** 1524-1530.

**Jiajie, T, Y Yanzhou, AC Hoi-Hung, C Zi-Jiang, and C Wai-Yee** 2017 Conserved miR-10 family represses proliferation and induces apoptosis in ovarian granulosa cells. *Sci Rep* **7** 41304.

**Karakaya, C, O Guzeloglu-Kayisli, A Uyar, AN Kallen, E Babayev, N Bozkurt, E Unsal, O Karabacak, and E Seli** 2015 Poor ovarian response in women undergoing in vitro fertilization is associated with altered microRNA expression in cumulus cells. *Fertil Steril* **103** 1469-1476 e1461-1463.

**Kim, YJ, SY Ku, YY Kim, HC Liu, SW Chi, SH Kim, YM Choi, JG Kim, and SY Moon** 2013 MicroRNAs transfected into granulosa cells may regulate oocyte meiotic competence during in vitro maturation of mouse follicles. *Hum Reprod* **28** 3050-3061.

**Kovacs, P, T Kovats, and SG Kaali** 2010 Results with early follicular phase recombinant luteinizing hormone supplementation during stimulation for in vitro fertilization. *Fertil Steril* **93** 475-479.

**Liu, S, X Zhang, C Shi, J Lin, G Chen, B Wu, L Wu, H Shi, Y Yuan, W Zhou, Z Sun, X Dong, and J Wang** 2015 Altered microRNAs expression profiling in cumulus cells from patients with polycystic ovary syndrome. *J Transl Med* **13** 238.

**Maalouf, SW, WS Liu, and JL Pate** 2016 MicroRNA in ovarian function. *Cell Tissue Res* **363** 7-18.

**Miles, JR, TG McDaneld, RT Wiedmann, RA Cushman, SE Echternkamp, JL Vallet, and TP Smith** 2012 MicroRNA expression profile in bovine cumulus-oocyte complexes: possible role of let-7 and miR-106a in the development of bovine oocytes. *Anim Reprod Sci* **130** 16-26.

**Nichols, J, and A Smith** 2012 Pluripotency in the embryo and in culture. *Cold Spring Harb Perspect Biol* **4** a008128.

**Otsuka, M, M Zheng, M Hayashi, JD Lee, O Yoshino, S Lin, and J Han** 2008 Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. *J Clin Invest* **118** 1944-1954.

**Ouandaogo, ZG, D Haouzi, S Assou, H Dechaud, IJ Kadoch, J De Vos, and S Hamamah** 2011 Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage. *PLoS One* **6** e27179.

**Patrizio, P, E Fragouli, V Bianchi, A Borini, and D Wells** 2007 Molecular methods for selection of the ideal oocyte. *Reprod Biomed Online* **15** 346-353.

**Salilew-Wondim, D, I Ahmad, S Gebremedhn, S Sahadevan, MD Hossain, F Rings, M Hoelker, E Tholen, C Neuhoff, C Looft, K Schellander, and D Tesfaye** 2014 The expression pattern of microRNAs in granulosa cells of subordinate and dominant follicles during the early luteal phase of the bovine estrous cycle. *PLoS One* **9** e106795.

**Shi, L, S Liu, W Zhao, and J Shi** 2015 miR-483-5p and miR-486-5p are down-regulated in cumulus cells of metaphase II oocytes from women with polycystic ovary syndrome. *Reprod Biomed Online* **31** 565-572.

**Shibahara, Y, Y Miki, Y Onodera, S Hata, MS Chan, CC Yiu, TY Loo, Y Nakamura, J Akahira, T Ishida, K Abe, H Hirakawa, LW Chow, T Suzuki, N Ouchi, and H Sasano** 2012 Aromatase inhibitor treatment of breast cancer cells increases the expression of let-7f, a microRNA targeting CYP19A1. *J Pathol* **227** 357-366.

**Vanderhyden, BC, and DT Armstrong** 1989 Role of cumulus cells and serum on the in vitro maturation, fertilization, and subsequent development of rat oocytes. *Biol Reprod* **40** 720-728.

**Velthut-Meikas, A, J Simm, T Tuuri, JS Tapanainen, M Metsis, and A Salumets** 2013 Research resource: small RNA-seq of human granulosa cells reveals miRNAs in FSHR and aromatase genes. *Mol Endocrinol* **27** 1128-1141.

**von Wolff, M, Z Kollmann, CE Flück, P Stute, U Marti, B Weiss, and NA Bersinger** 2014 Gonadotrophin stimulation for in vitro fertilization significantly alters the hormone milieu in follicular fluid: a comparative study between natural cycle IVF and conventional IVF. *Hum Reprod* **29** 1049-1057.

**Westergaard, LW, PM Bossuyt, F Van der Veen, and M van Wely** 2011 WITHDRAWN: Human menopausal gonadotropin versus recombinant follicle stimulation hormone for ovarian stimulation in assisted reproductive cycles. *Cochrane Database Syst Rev* CD003973.

**Xu, B, YW Zhang, XH Tong, and YS Liu** 2015 Characterization of microRNA profile in human cumulus granulosa cells: Identification of microRNAs that regulate Notch signaling and are associated with PCOS. *Mol Cell Endocrinol* **404** 26-36.

**Yerushalmi, GM, M Salmon-Divon, L Ophir, Y Yung, M Baum, G Coticchio, R Fadini, M Mignini-Renzini, M Dal Canto, R Machtinger, E Maman, and A Hourvitz** 2018 Characterization of the miRNA regulators of the human ovulatory cascade. *Sci Rep* **8** 15605.

**Zhang, XD, YH Zhang, YH Ling, Y Liu, HG Cao, ZJ Yin, JP Ding, and XR Zhang** 2013 Characterization and differential expression of microRNAs in the ovaries of pregnant and non-pregnant goats (Capra hircus). *BMC Genomics* **14** 157.

Author BIo

I had my degree in Medicine at Federal University of Rio Grande do Sul. I performed medical residency in Gynecology and Obstetrics at the Hospital de Clínicas de Porto Alegre. I attended residence in Human Reproduction at the Federal University of São Paulo (UNIFESP). Completed post-graduation – doctorate degree at UNIFESP. I am currently director of the Effetto assisted reproduction clinic.



Key message

This paper aim to demonstrate that miRNAs change during ovarian stimulation protocols; they were overexpressed when LH was added. Using the LH protocol seems to produce a superior link in granulosa cells that compose the *cumulus,* boosting the oestrogen signalling pathways and oocyte meiosis, and possibly enhancing oogenesis.

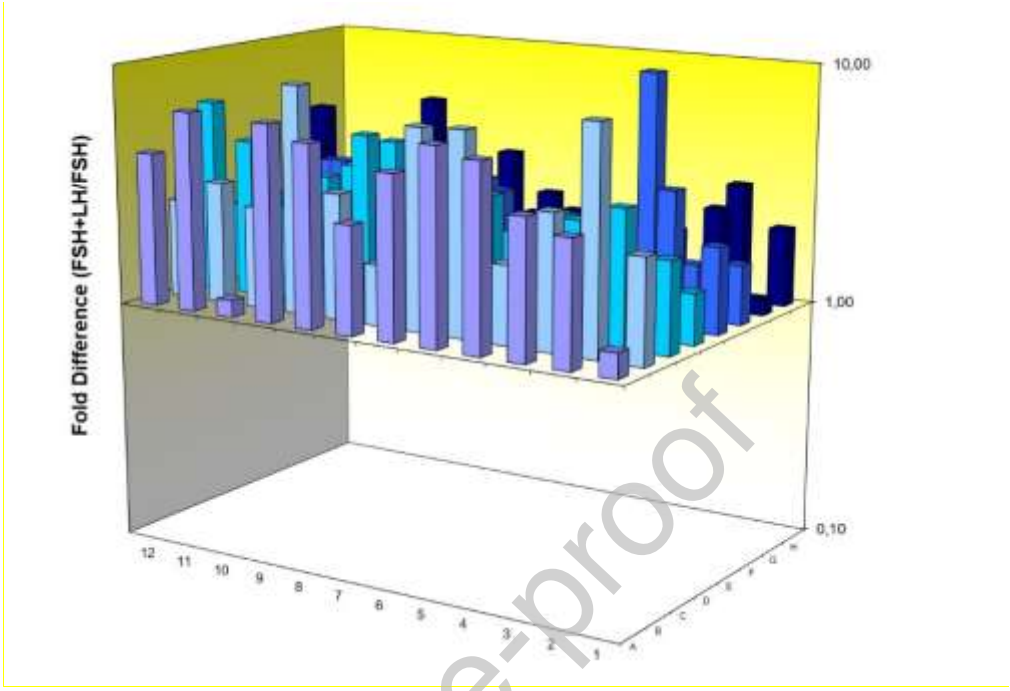


**Figure 1:** Graph representative of fold change between miRNA expression in FSH + LH vs. FSH group.

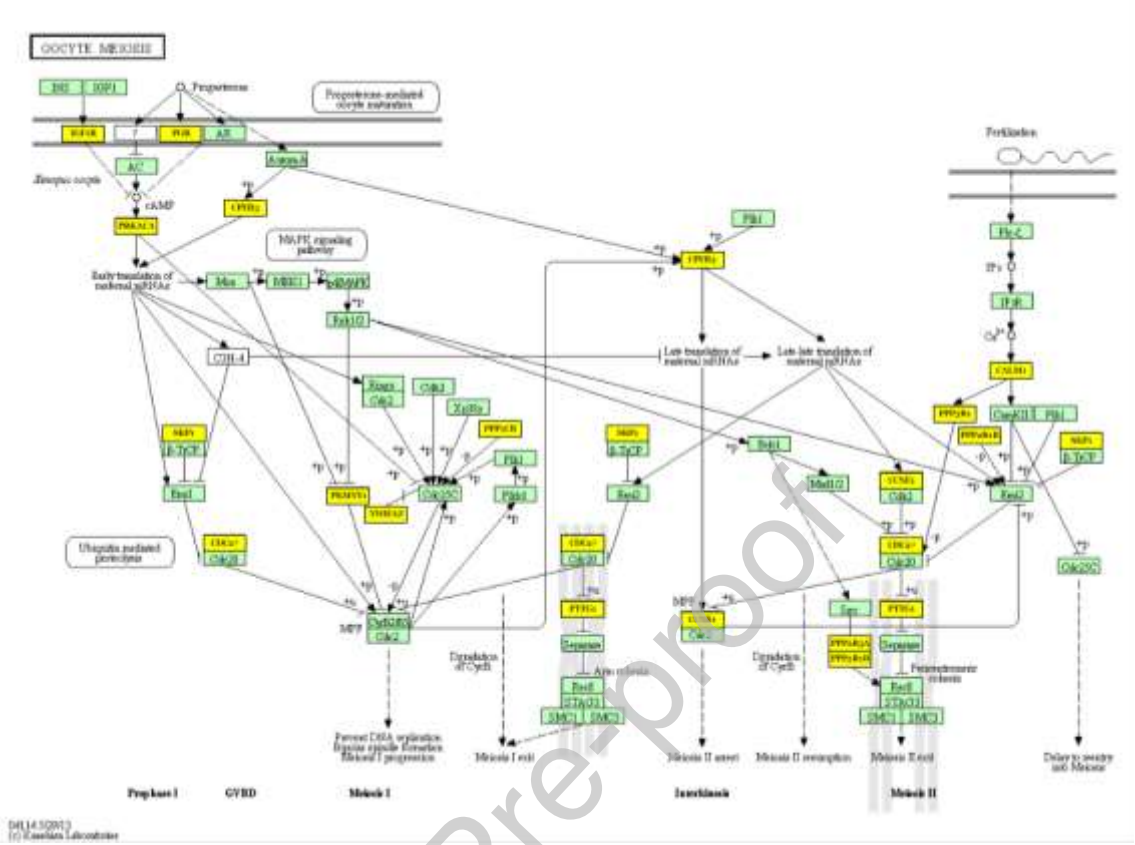

**Figure 2:** Representative figure of oocyte meiosis pathway. The genes highlighted in yellow are targets of miRNAs hsa-miR-26a, hsa-miR-26b, hsa-miR-200c, hsa-miR-181b, hsa-let-7a, and hsa-let-7e and were found to be differentially expressed between groups. (DIANA-miRPath: Summary of genes targeted by miRNAs from the oocyte meiosis pathway).

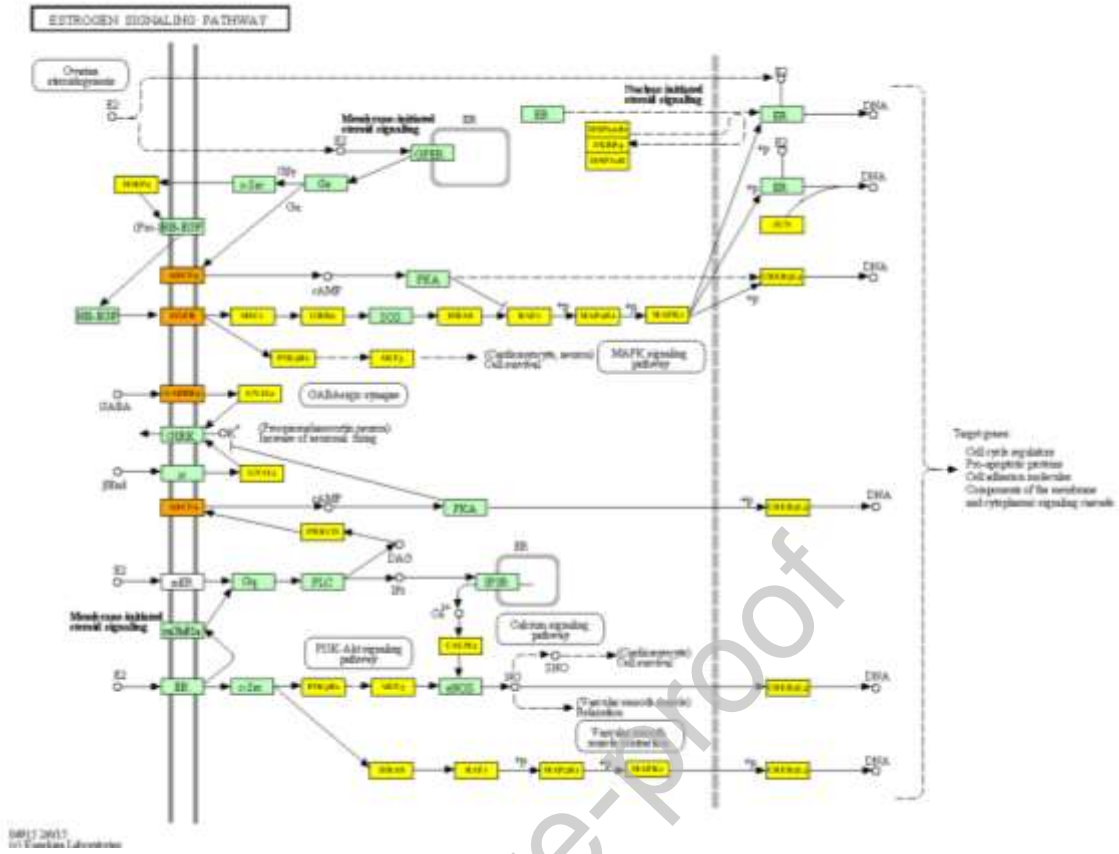


**Figure 3**: Representative figure of oestrogen signalling pathway. The genes highlighted in yellow and orange are targets of miRNA hsa-miR-181a. (DIANA-miRPath: Summary of genes targeted by miRNAs from the oestrogen signaling pathway)

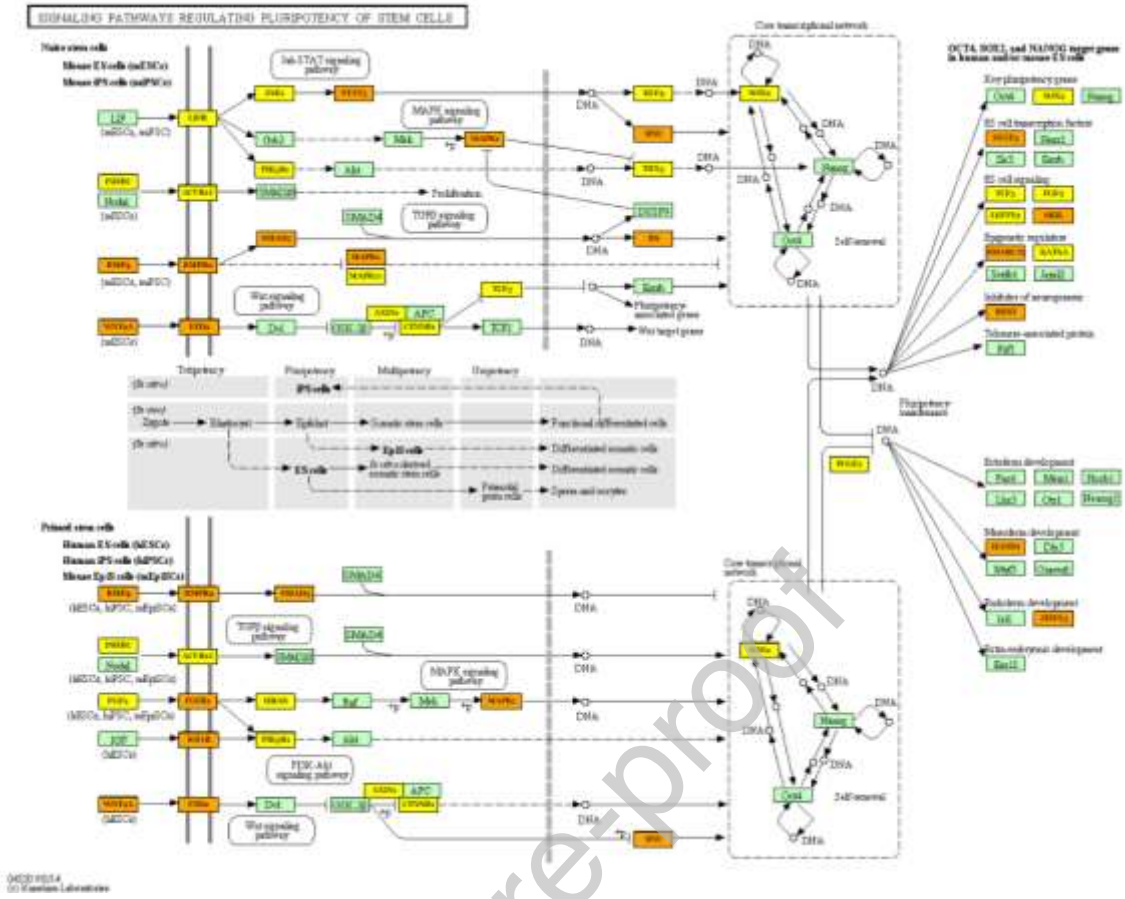


**Figure 4**: Representative figure of signalling pathways regulating the pluripotency of stem cells. The genes highlighted in yellow and orange are targets of miRNAs hsa-let-7e, hsa-miR-181a, and hsa-miR-181b. (DIANA-miRPath: Summary of genes targeted by miRNAs from the signaling pathways regulating pluripotency of stem cells)

**Table 1**: Demographic and clinical characteristics of patients included in study groups.

| | **FSH (7 patients)** | **FSH + LH (6 patients)** | **p-value** |
|---|---|---|---|
| Age (years) | 31.38 ± 3.2 | 34.25 ± 2.55 | 0.0670 |
| Body Mass Index - BMI (kg/m$^2$) | 22.37 ± 2.17 | 21.96 ± 2.94 | 0.7525 |
| Follicle Stimulating Hormone - FSH (UI/mL) | 7.05 ± 1.0 | 6.86 ± 1.3 | 0.7518 |
| Luteinizing Hormone - LH (UI/mL) | 4.52 ± 1.64 | 4.75 ± 1.25 | 0.7573 |
| Prolactin (ng/mL) | 20.13 ± 4.25 | 27.23 ± 13 | 0.1641 |
| Thyroid Stimulating Hormone - TSH (mUI/mL) | 2.07 ± 1.10 | 1.92 ± 0.86 | 0.7928 |
| Anti-Mullerian Hormone - AMH (ng/mL) | 1.52 ± 0.61 | 1.39 ± 0.64 | 0.6942 |
| Number of MII oocytes | 8.25 ± 3.54 | 6.88 ± 3.48 | 0.4463 |

**Table 2:** miRNAs differentially expressed in cumulus cells of patients on FSH + LH group vs. FSH group (Fold change ≥ 2 and p ≤ 0.05)

| miRNA ID | fold change (FSH + LH vs. FSH group) | p-value |
|---|---|---|
| hsa-let-7d | 4.68 | 0.015 |
| hsa-miR-16 | 5.63 | 0.029 |
| **hsa-miR-26a** | **6.45** | **0.003** |
| **hsa-miR-26b** | **6.64** | **0.006** |
| hsa-let-7g | 4.30 | 0.022 |
| hsa-miR-30c | 2.67 | 0.038 |
| hsa-miR-24 | 6.75 | 0.024 |
| hsa-miR-155 | 6.64 | 0.022 |
| hsa-miR-425 | 3.29 | 0.028 |
| **hsa-miR-181b** | **8.69** | **0.002** |
| hsa-miR-30b | 3.17 | 0.049 |
| **hsa-miR-200c** | **3.58** | **0.008** |
| hsa-miR-15b | 3.06 | 0.044 |
| **hsa-miR-181a** | **5.33** | **0.005** |
| **hsa-miR-125b** | **3.44** | **0.007** |
| **hsa-miR-28-5p** | **4.43** | **0.004** |
| hsa-miR-320a | 6.24 | 0.021 |
| **hsa-miR-423-5p** | **4.70** | **0.007** |
| **hsa-let-7a** | **3.58** | **0.001** |
| hsa-miR-124 | 2.83 | 0.023 |
| hsa-miR-92a | 2.48 | 0.008 |
| hsa-miR-23a | 2.13 | 0.049 |
| hsa-miR-25 | 2.26 | 0.024 |
| **hsa-let-7e** | **3.61** | **0.001** |

| | | |
|---|---|---|
| **hsa-miR-151-5p** | **3.14** | **0.002** |
| hsa-miR-195 | 3.36 | 0.028 |
| hsa-miR-302a | 9.74 | 0.028 |
| hsa-let-7b | 2.32 | 0.010 |
| hsa-miR-93 | 2.07 | 0.047 |
| hsa-miR-27a | 3.03 | 0.034 |
| hsa-let-7c | 2.62 | 0.004 |
| hsa-let-7f | 2.53 | 0.032 |
| hsa-miR-100 | 2.29 | 0.014 |