Patients with endometriosis and patients with poor ovarian reserve have abnormal follicle stimulating hormone receptor signaling pathways

Fertility and Sterility Vol. 95, No. 7, June 2011

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Introduction

- Follicle-stimulating hormone (FSH) plays a major role during folliculogenesis.
- Exogenous FSH is the major gonadotropin used for controlled ovarian hyperstimulation (COH) in assisted reproductive technology (ART)
- The FSH action is mediated by its specific receptor (FSHR) expressed in granulosa cells

- The physiological responses to FSH are modulated by the activation of multiple target genes and micro RNAs in granulosa cells, such as
  - LH receptor
  - autocrine factors
  - P450 aromatase for estrogen production (Cyp19A1)
  - pregnancy associated plasma protein-A (PAPP-A)
  - transcription factors.

- We and other investigators have previously demonstrated that response to FSH in patients undergoing IVF is positively correlated to the expression level of FSHR in granulosa-lutein (GL) cells

- Compared with patients with no ovarian factor of infertility (normoresponders), the FSHR expression level is significantly higher in patients with PCOS (hyper-responders) and lower in poor responders.

- However, alterations of the FSHR signaling pathway have not been studied previously in patients with different types of infertility undergoing IVF.
- The objective of this preliminary work:
  - possible alterations of the FSHR signaling pathway through the analysis of correlations between expression of FSHR and the FSH-regulated genes Cyp19A1 and PAPP in granulosa-lutein cells at the time of egg retrieval
  - their relationship with the infertility diagnosis.

MATERIALS AND METHODS (Patients)

- The GL cells from 117 consecutive infertile women undergoing IVF were analyzed under a protocol approved by the Ethics Committee of the Universidad de La Laguna.

- Infertile patients were classified as follows:
  - [1] *controls* (no ovarian factor, NOF) were patients with an healthy ovary and a male or tubal factor of infertility (n=35);
  - [2] *poor responders* (PR; n=28) included patients with day 3 plasma FSH >10 IU/mL and women >40 years old with less than four mature eggs (regardless of FSH level);

- [3] *endometriosis* (EM; n=32) all stages of this disease diagnosed by ultrasound and/or laparoscopy; further subdivided into
  - poor (endometriosis-A, n=16 [stage III-IV, n =12; stage I-II, n=4])
  - good responders (endometriosis-B, n=16 [stage III-IV, n =14; stage I-II, n=2])
- [4] *patients with polycystic ovary syndrome* (PCOS; n=22) as defined according to the Rotterdam criteria

- Patient's demographics and the most relevant clinical characteristics are illustrated in Table 1.

TABLE 1

Clinical variables and gene expression values of the patients studied.

	Cause of infertility				
	NOF	Poor responders	Endometriosis-A	Endometriosis-B	PCOS
No. of patients	35	28	16	16	22
FSHR	150.3 ± 0.5[b,e]	57.2 ± 13.9[a,e]	58.7 ± 31.6[e]	110.7 ± 48.1[e]	304.6 ± 57.3[a–d]
PAPP	448.2 ± 50.8	421.0 ± 64.0	504.4 ± 83.2	502.5 ± 123.9	609.2 ± 142.0
CYP19A1	16,262.1 ± 3,681.4[c,d]	9,519.2 ± 1,972.3[d]	8,088.3 ± 1,592.6[a,d]	3,374.6 ± 1,101.6[a–c,e]	11,842.9 ± 3,049.8[d]
Age	32.5 ± 1.17[b,c]	36.5 ± 0.9[a,e]	36.6 ± 1.1[a,e]	33.9 ± 0.9	32.6 ± 0.83[b,c]
Treatment days	10.1 ± 0.3	10.6 ± 0.3	11.1 ± 0.4	10.2 ± 0.4	10.5 ± 0.3
Day 3 FSH (IU)	6.8 ± 0.5[b,c]	10.4 ± 0.5[a,d,e]	9.7 ± 0.9[a,e]	7.9 ± 1.0[b]	6.3 ± 0.3[b,c]
E_2 (pg/mL)	2,507 ± 150.6[b,d]	1,820 ± 164.5[a,d,e]	2,377.6 ± 399.3[d]	3,939.2 ± 597.1[a–c,e]	2,643 ± 280.7[b,d]
Recombinant FSH (IU)	4,111 ± 333.3[b,c,e]	6,019 ± 284.4[a,d,e]	6,112.5 ± 475.4[a,d,e]	4,670.6 ± 477.1[b,c,e]	2,122 ± 284.4[a–d]
LH (IU)	1,821 ± 229.4[b,c,e]	2,860 ± 209.6[a,e]	2,971.9 ± 306.6[a,e]	2,214.8 ± 280.6[e]	673 ± 148.3[a–d]
Total oocytes	15.9 ± 1.4[b,c]	7.5 ± 0.6[a,d,e]	6.9 ± 0.5[a,d,e]	18.7 ± 2.8[b,c]	20.1 ± 2.0[b,c]
Mature oocytes	10.7 ± 1.0[b,c,e]	5.0 ± 0.5[a,d,e]	4.9 ± 0.7[a,d,e]	14.1 ± 1.8[b,c]	15.5 ± 1.7[a–c]

Note: Mean and standard error of clinical parameters and FSHR, CYP19A1, and PAPP gene expression in patients with different causes of infertility. NOF = no ovarian factor; Endometriosis-A = endometriosis with ≤10 oocytes; Endometriosis-B = endometriosis with >10 oocytes; PCOS = polycystic ovary syndrome.

Gene expression values are expressed as $\times 10^5$ relative to β-actin expression.

Results of comparisons between groups are reported as follows:

[a] Significantly different from NOF.

[b] Significantly different from poor responders.

[c] Significantly different from endometriosis-A.

[d] Significantly different from endometriosis-B.

[e] Significantly different from PCOS.

González-Fernández. Abnormal FSHR signaling in IVF patients. Fertil Steril 2011.

- Induction of ovulation → agonist downregulation (n=55) or an antagonist (n=62) protocol using recombinant FSH, combined with recombinant LH or hMG
- Ultrasound-guided egg retrieval was performed 36 hours after administration of 10,000 IU of hCG.
- In all patients the fertilization method for the retrieved oocytes was intracytoplasmic sperm injection (ICSI).
- One to three embryos were replaced in each cycle, depending on the patient's age and the embryo morphology.

(Isolation of Granulosa-Lutein Cells)

- Cells were collected from follicular fluid (FF) obtained during oocyte retrieval.
- All FF from each patient was pooled and the GL cells lightly centrifuged after removal of the oocyte.
- Cells were then washed in ‘‘isolation medium’’
- Leukocytes were separated using anti-CD45-coated magnetic beads

(Quantitative Reverse Transcriptase-Polymerase Chain reaction)

- Relative expression levels of the messenger RNA were determined in GL cells from all patients by quantitative reverse transcriptase-polymerase chain reaction (PCR) of cDNA.
- The specific primers used for each gene were:
  - b-actin (cttccttcctgggcatgg, gccgccagacagcactgt),
  - FSHR (accaagcttcgagtcatcc,catctgcctctatcacctcc),
  - CYP19A1 (agaaaaaagacgcaggatttc, ctcttgtcaggtcaccacg),
  - PPAP (gggtagagagagttgtctgcac, taattgtctcccatgaaggg).

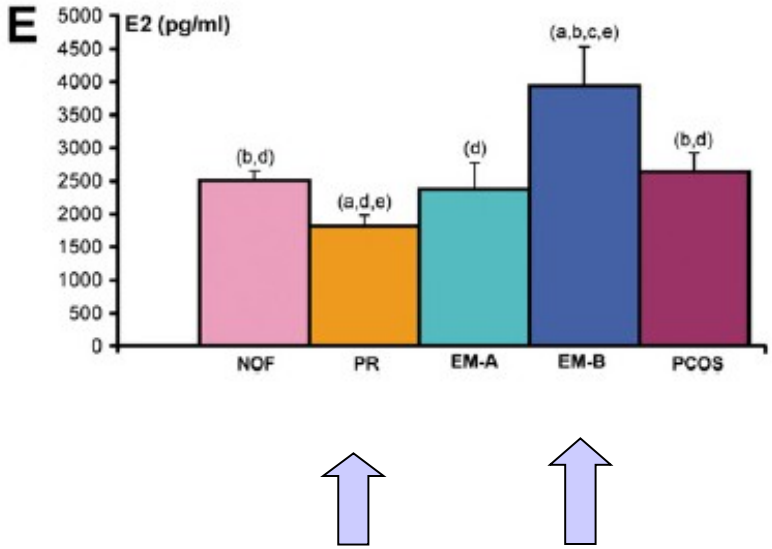
(Statistical Analysis)

- performed with the SPSS software using Pearson's correlation coefficient, Pearson's partial correlation, Student's t-test, and one-way analysis of variance (ANOVA) as appropriate.
- A *P* value of <.05 was considered statistically significant.

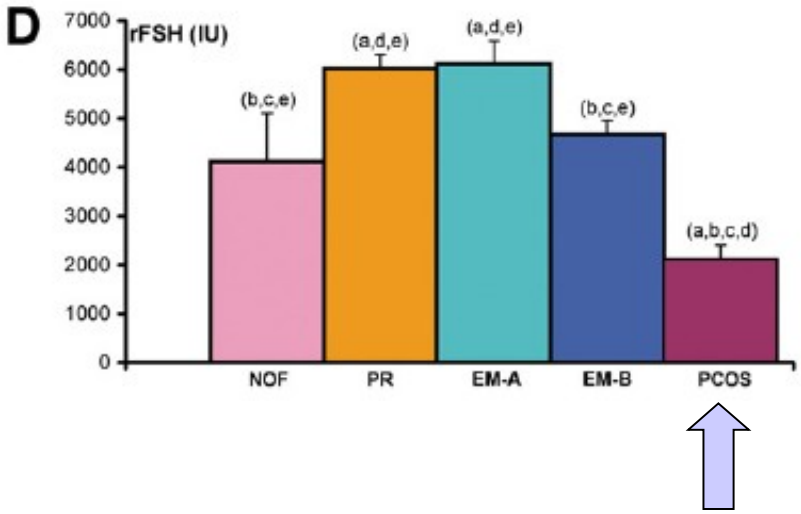
RESULTS
(Clinical Characteristics of the Different Groups of Patients studied)

- Table 1 shows the most relevant clinical variables of the IVF cycle in the patients studied.
- Agonist and antagonist cycles are equally represented in all groups.
- Within each group, no statistically significant difference in gene expression was observed between the two protocols (data not shown).

- day 3 FSH level of poor responders and patients with endometriosis-A was significantly higher than in patients with NOF and PCOS
- the E2 level on the day of hCG administration was significantly lower in poor responders than in patients with NOF, endometriosis-B, and PCOS.
- In patients with endometriosis-B, the E2 level was significantly higher than in all other groups.



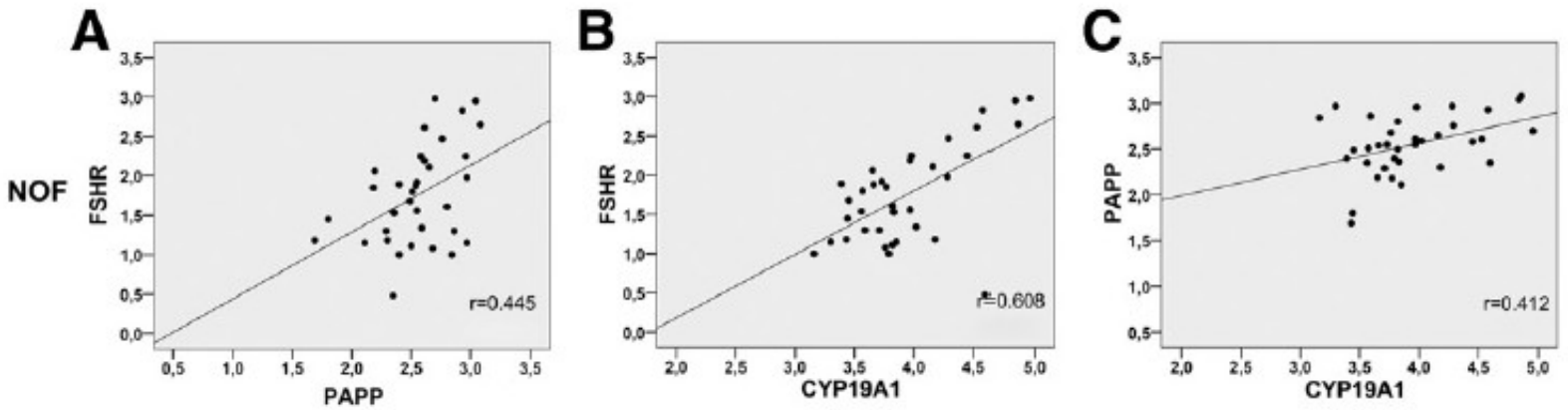
In patients with PCOS and endometriosis-A, the E2 levels were similar to patients with NOF (Fig. 1E).



doses of FSH and LH administered also differed between groups (Fig. 1D).

(Correlation of FSHR, PAPP, and Cyp19A1 Gene Expression in GL Cells From NOF Ovaries and Relationship With Clinical IVF Cycle Parameters)

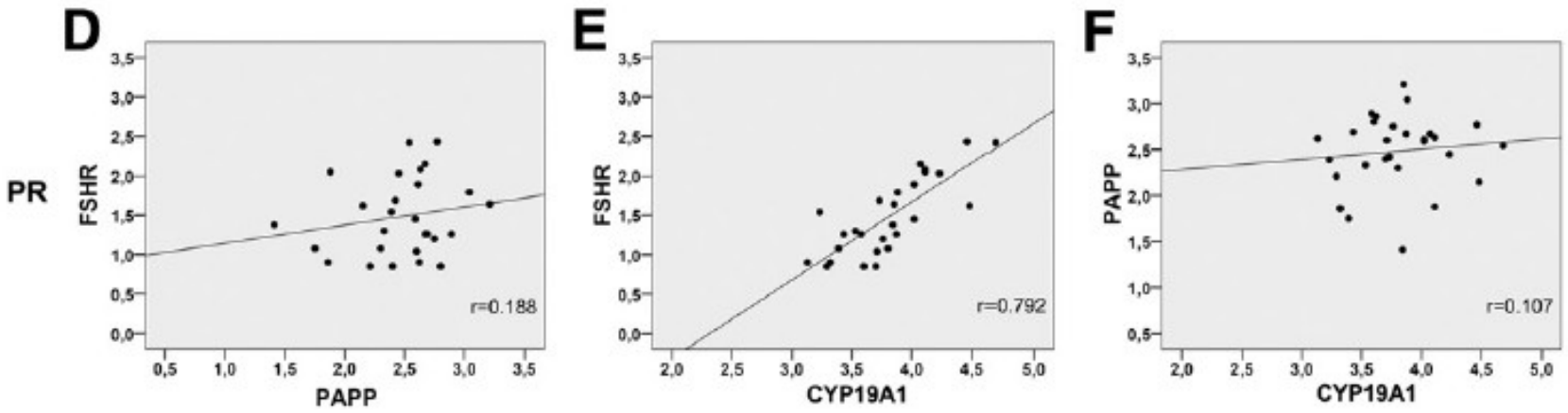
- The FSHR, PAPP, and CYP19A1 gene expression levels were positively correlated in NOF patients (Fig. 2A–2C).



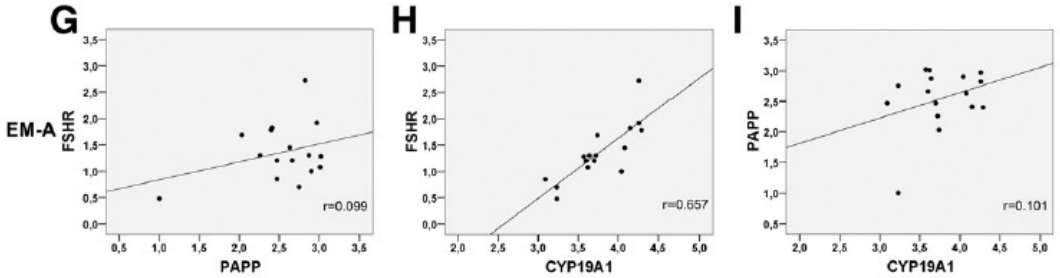
- Regarding clinical variables, a positive correlation was found only for CYP19A1 expression with the amount of gonadotropin administered and age, but not for other variables or genes.

(Correlation of FSHR, PAPP, and Cyp19A1 Gene Expression in GL Cells From Patients With Ovarian Abnormalities and Relationship With Clinical IVF Cycle Parameters)

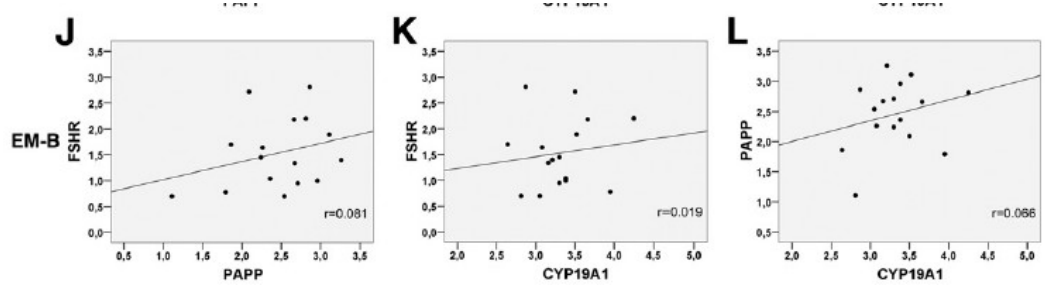
- Poor responders:
  - The FSHR expression correlates positively to Cyp19A1, but not with PAPP.
  - No correlation is observed between PAPP and Cyp19A1 (Fig. 2D–2F).
  - The only clinical correlation observed is a negative relationship between age and CYP19A1 gene expression (r=0.457, P<.05).



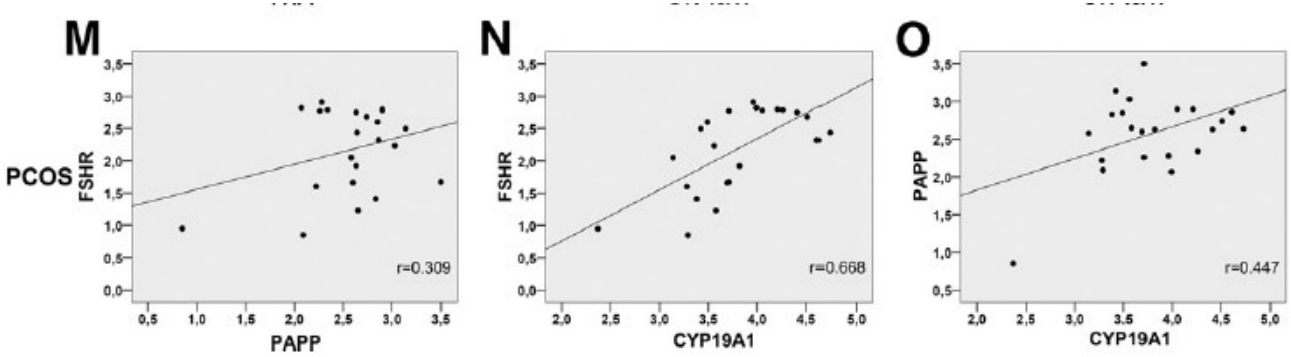
- Endometriosis:
  - Endometriosis-A (poor responders)→ FSHR expression correlates positively to Cyp19A1, but not with PAPP.
  - No correlation was observed between PAPP and Cyp19A1 (Fig. 2G–2I).



  - In endometriosis-B no correlation was observed between the three genes analyzed (Fig. 2J–2L) and a negative correlation was found between CYP19A1 expression and age (r =0.549, P<.05).



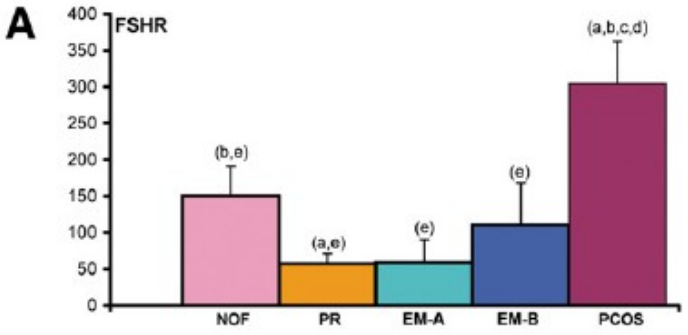
- Polycystic ovary syndrome:
  - The FSHR expression correlates positively with Cyp19A1 but not with PAPP.
  - However, PAPP and Cyp19A1 are positively correlated (Fig. 2M–2O).
  - With respect to clinical parameters we found a negative correlation between the expression of FSHR and E2 levels on the day of hCG administration (r=0.492, P<.05).



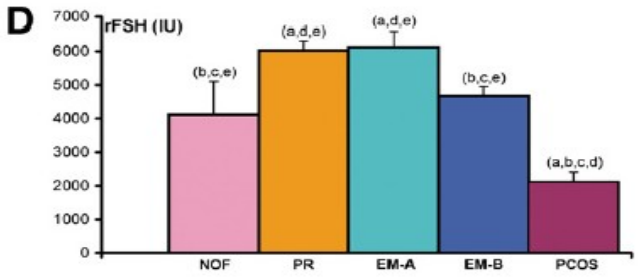
DISCUSSION

- We present data supporting significant differences in correlations of FSHR and the FSH-regulated genes CYP19A1 and PAPP among several diagnostic infertile groups of patients.
- Our data indicate that FSHR, PAPP, and Cyp19A1 gene expression is regulated in a well-coordinated fashion in women with no ovarian cause of infertility (NOF group), and that this coordination fails in patients with infertility of ovarian origin (poor responders, endometriosis, PCOS groups).

- We previously reported that expression levels of FSHR vary according to the origin of infertility



- FSHR expression levels are higher in PCOS compared to NOF (Fig. 1A) → Stimulation of patients with PCOS requires low doses of exogenous recombinant FSH.

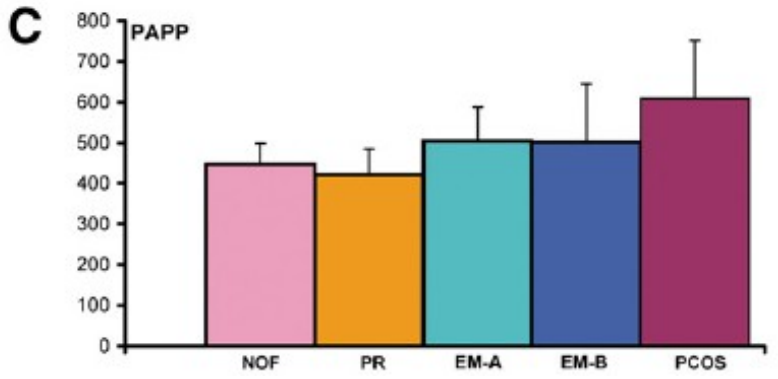


- poor responders and patients with endometriosis-A require higher doses of exogenous recombinant FSH or ovulation induction than NOF (Fig. 1D).

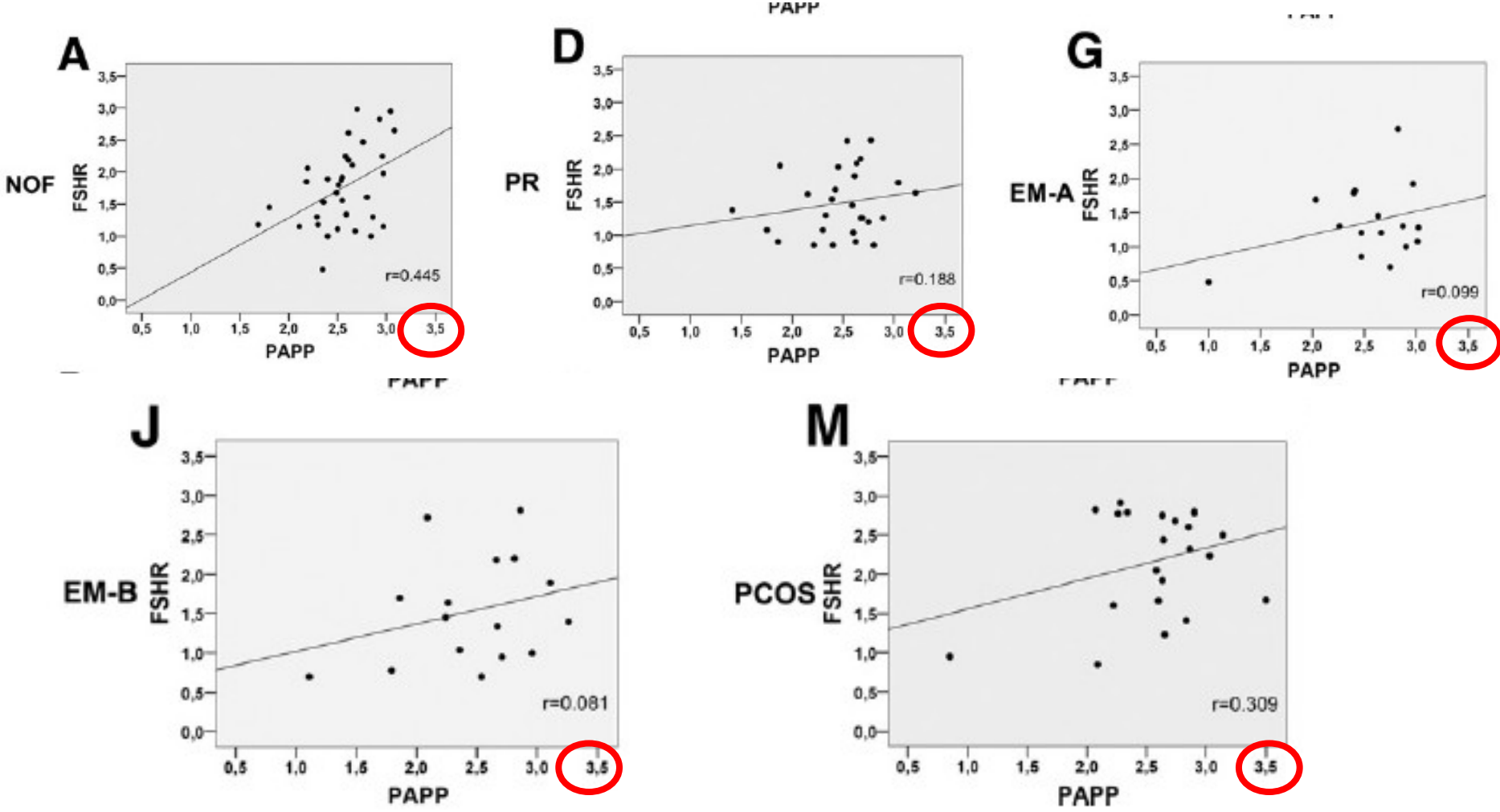
- FSHR levels are lower in poor responders and in patients with endometriosis-A → a similar mechanism for the poor response observed in both groups.

- Variations in the FSHR expression levels in GL cells could be related to an alteration of the regulatory circuit bone morphogenetic protein-15/Kit ligand
- This circuit is implicated in the inhibition of the FSH receptor expression and in the promotion of oocyte growth by a correct KL1:KL2 expression ratio
- Our group has recently published data supporting a positive correlation of the KL1:KL2 ratio and FSHR in patients with NOF and how this correlation is altered in poor responders and patients with PCOS

- Whereas in patients with NOF the expression levels of CYP19A1 and PAPP are positively related to FSHR expression, this does not happen in the other infertility groups studied.
- Mean PAPP expression remains similar among patients of all groups despite scattered expression levels of FSHR and variable amounts of exogenous FSH administered (Fig. 1C).



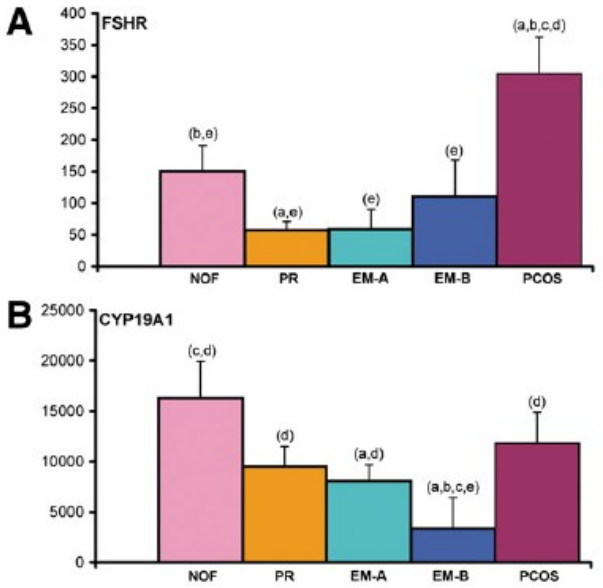
- This fact could be related to the existence of an apparent maximum of PAPP expression reached with relatively low levels of FSHR expression (Fig. 2A, 2D, 2G, 2J, and 2M).

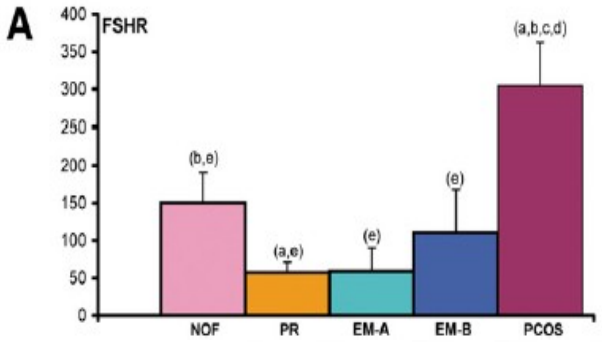


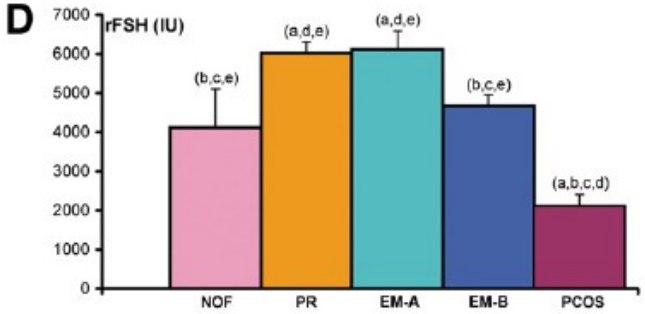
- The average CYP19A1 expression in GL cells of patients with PCOS is similar to patients with NOF, despite higher expression levels of FSHR in patients with PCOS (Fig. 1B).

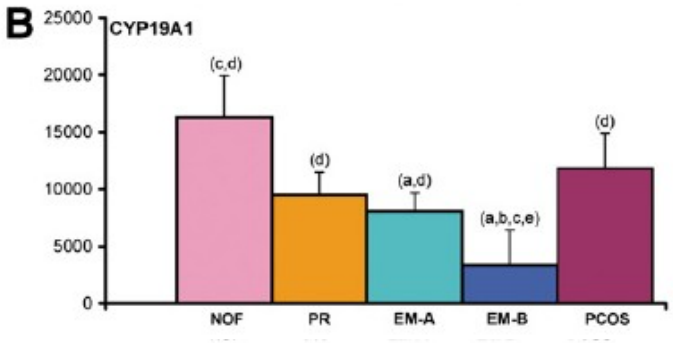
- Hypothesize: may be due to the much lower dose of exogenous FSH administered to patients with PCOS, which may have been insufficient to achieve maximal stimulation of the available receptors.

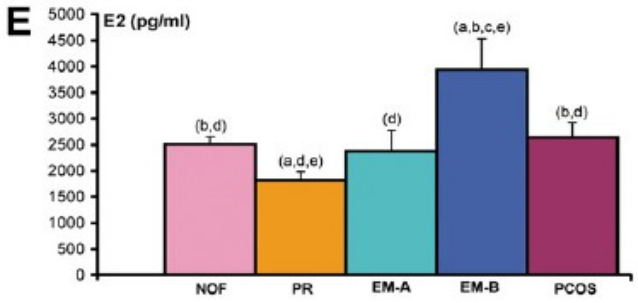
- Although patients with endometriosis-A and poor responders require similarly high doses of exogenous FSH and GL cells from both groups show a similar expression of FSHR and CYP19A1 (Fig. 1A and B), the level of E2 is higher and shows a much higher dispersion of values in patients with endometriosis-A.
- This last observation may reflect extraovarian E2 production in endometriotic implants











Patients with endometriosis-B and NOF have **similar** GC expression levels of FSHR and **require comparable gonadotropin doses**

expect a **similar expression level of CYP19A1** in the presence of normal signaling pathways

levels of CYP19A1-specific messenger RNA in endometriosis-B is approximately **five times lower** than in patients with NOF

a **failure in FSHR signaling** in this subgroup of patients with endometriosis

E2 levels are significantly higher than that in patients with NOF

the concept of E2 synthesis in endometriotic tissue

- The correct expression of FSHR-regulated genes depends on the amount of cellular receptor and on an equilibrated signaling pathway
- Our data support the concept that patients with endometriosis and poor responders not only have dysfunctional FSHR expression, but also an alteration of the postreceptor signaling pathways.

- The existence of a dysfunction of the FSHR-activated signaling pathways in these patients is corroborated by :
  - the correlation between FSHR expression and the expression of FSH-regulated genes CYP19A1 and PAPP observed in patients with NOF is missing in endometriosis and poor responders.
- In poor responders and patients with endometriosis-A the positive correlation between FSHR and CYP19A1 is similar to that in patients with NOF, but there is no CYP19A1/PAPP correlation as expected in a complete signaling pathway
- On the other hand, patients with endometriosis-B show a different pattern:
  - **both FSHR/CYP19A1 and CYP19A1/PAPP correlations are lost**

- In conclusion, we report evidence of
  - [1] a correlation between expression levels of FSHR in GL cells and various infertility causes
  - [2] possible alterations in signaling pathways activated through the FSHR in specific infertility states.

- Thanks for your attention!