Expression of *PGR*, *HBEGF*, *ITGAV*, *ITGB3* and *SPP1* genes in eutopic endometrium of infertile women with endometriosis during the implantation window: a pilot study

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ABSTRACT

Objective: Alterations in endometrial receptivity may be involved in the etiology of endometriosis-related infertility. The literature has suggested that patients with endometriosis present progesterin resistance, which could affect embryo implantation. We question the presence of alterations in the expression of the progesterone receptor gene (*PGR*) and the genes related to endometrium-embryo interaction regulated by progesterone. This pilot study compared the expression of *PGR*, *HBEGF*, *ITGAV*, *ITGB3*, and *SPP1* genes in eutopic endometrium during the implantation window (IW) in infertile women with endometriosis with that observed in the endometrium of fertile and infertile controls.

Methods: In this prospective case-control study, endometrial biopsies were performed during the IW in patients aged between 18 and 45 years old, with regular cycles and without endocrine/systemic dysfunctions, divided into endometriosis (END), infertile control (IC) and fertile control (FC) groups. Total RNA extraction, cDNA synthesis, and gene expression analysis by Real-Time PCR were performed. We assessed the size of the difference that our series was powered to detect.

Results: From the 687 patients who underwent diagnostic videolaparoscopy or tubal ligation at the University Hospital, 130 were eligible. Of these, 32 had endometrial samples collected, with 17 confirmed in the IW. Fifteen samples (5 END, 5 IC and 5 FC) were analyzed. There was no significant difference in the expression of any studied gene. Our sample size allowed us to identify or discard large differences (two standard deviations) among the groups.

Conclusion: Endometriosis doesn't cause large changes in the endometrial expression of *PGR*, *HBEGF*, *ITGAV*, *ITGB3* and *SPP1* during the IW.

Keywords: endometriosis, infertility, endometrium, embryo implantation

INTRODUCTION

Endometriosis is a disease characterized by the implantation and growth of ectopic endometrial tissue (glands and/or stroma) (Gupta *et al.*, 2006; Burney & Giudice, 2012), being frequently associated to infertility (Practice Committee of the American Society for Reproductive Medicine, 2012). However, the exact mechanisms involved in the etiology of infertility in patients with the disease have not yet been completely understood (Holoach & Lessey, 2010). In this sense,

evidences have suggested that impaired endometrial receptivity, resulting from molecular disorders in eutopic endometrium, may negatively impact fertility in women with the disease (Giudice & Kao, 2004; Wei *et al.*, 2009; Bulletti *et al.*, 2010).

In the receptive period, called implantation window, the human endometrium undergoes molecular, histological, and structural changes that promote embryo implantation and pregnancy development (Paulson, 2011; Sharma & Kumar, 2012; von Grothusen *et al.*, 2014). During the implantation window, there is an increased expression of various growth factors, cytokines and adhesion molecules involved in the embryo implantation process (Aghajanova *et al.*, 2008), so that inadequacies in the control of gene expression may result in aberrant endometrial growth and differentiation (Munro *et al.*, 2010).

Progesterone plays an important role in regulating the implantation window, by coordinating molecular changes that culminate with endometrial receptivity establishment (Young, 2013). Acting through specific nuclear receptors that function as ligand-activated transcription factors, this steroid starts a crucial gene cascade, whose products can have both autocrine and paracrine effects, and thus, regulate the expression of other genes essential for implantation (Young, 2013). Nevertheless, there is evidence that infertile women with endometriosis are partially resistant to progesterone (Burney *et al.*, 2007; Bulun, 2010), which may be due to an abnormal progesterone signaling as a result of aberrant expression of its receptors (Bedaiwy *et al.*, 2015). The human progesterone receptor is encoded by the *PGR* gene, whose function is to act as a transcriptional regulator of progesterone-responsive genes (Wu *et al.*, 2006). Among the molecules coordinated by progesterone that are crucial to implantation process, there is the heparin-binding epidermal growth factor (HB-EGF), whose secretion in stromal cells is induced by progesterone, and whose function is to increase $\alpha v \beta 3$ integrin expression in the adjacent epithelial cells (Sharkey & Smith, 2003). This integrin, in turn, is an adhesion molecule expressed on the apical surface of pinopodes during the implantation window that acts as a receptor of soluble ligands, such as osteopontin, and promotes signal transduction (Sharkey & Smith, 2003). Osteopontin is a glycoprotein of the extracellular matrix secreted into the lumen, which can mediate early endometrial-trophoblast interactions during implantation (Lessey, 2002; Jessmon *et al.*, 2009; Wei *et al.*, 2009). Thus, as progesterone acts directly and indirectly on the endometrium, defects in progesterone receptors

may trigger a series of alterations in the expression of molecules that are essential to the implantation process, such as those above mentioned.

In this context, the literature presents conflicting studies evaluating progesterone receptors (Attia *et al.*, 2000; Igarashi *et al.*, 2005; Shen *et al.*, 2015), $\alpha\beta 3$ integrin (Ordi *et al.*, 2003; Wei *et al.*, 2009; Casals *et al.*, 2012) and osteopontin (Burney *et al.*, 2007; Cho *et al.*, 2009; Wei *et al.*, 2009; Casals *et al.*, 2012) in the eutopic endometrium of women with endometriosis, especially with respect to disease-associated infertility. Moreover, to date, there is no study evaluating the expression of HB-EGF in these patients and no previous study simultaneously evaluated the expression of these five genes in infertile patients with endometriosis. Whereas the identification of molecular differences in the eutopic endometrium of women with endometriosis during implantation window is an important step to understand the pathogenesis of disease-related infertility, susceptible to support new therapeutic approaches, this study aimed to compare the expression of *PGR* (progesterone receptor), *HBEGF* (heparin-binding epidermal growth factor), *ITGAV* and *ITGB3* ($\alpha\beta 3$ integrin) and *SPP1* (osteopontin) genes in eutopic endometrium of infertile women with endometriosis, infertile controls and fertile controls, during the implantation window.

MATERIALS AND METHODS

Study design

A prospective case-control study was carried out in the University Hospital. The study was approved by the Research Ethics Committee of the University Hospital (grant 6383/2011).

Settings and duration

From December 2011 to December 2014, the patients who previously underwent diagnostic videolaparoscopy or tubal ligation at the University Hospital were evaluated according to the eligibility criteria, and those who were considered eligible were interviewed. An endometrial sample was collected from the patients who consented to participate.

Participants - Eligibility Criteria

The patients with regular cycles (every 24-38 days, 4.5 to 8 days of duration and flow up to 80ml per cycle) (Fraser *et al.*, 2007) for at least three months prior to the study, age between 18 and 45 years, body mass index (BMI) $\leq 30\text{kg/m}^2$, no polycystic ovary syndrome and no other etiologies of chronic anovulation, hydrosalpinx and chronic diseases such as *diabetes mellitus* or other endocrinopathies, cardiovascular disease, dyslipidemia, systemic lupus erythematosus and other rheumatologic diseases, HIV infection, any active infection, smoking, and use of hormonal medication, anti-inflammatory drugs during the three months preceding the beginning of the study were included. The endometriosis group consisted of patients with infertility exclusively associated to endometriosis diagnosed and classified by videolaparoscopy according to the American Society for Reproductive Medicine criteria (1997). The infertile control group consisted of patients with infertility attributable to male and/or tubal factors who had ruled out endometriosis and other pelvic diseases. The fertile control group was formed by sterilized patients, proven fertile (at least one living child), without possible associated endometrial factors.

The exclusion criteria for all groups were reports of menstrual cycle alterations and use of hormonal medication during the monitoring before sample collection,

and inability to attend at the scheduled date. All samples that were classified outside the implantation window after the endometrial dating were excluded.

Sample collection and processing

The patients had endometrial samples collected during the implantation window period (Wilcox *et al.*, 1999; Sharma & Kumar, 2012), according to the day of the menstrual cycle. For standardization, ovulation day was considered as the 14th day of a 28-day menstrual cycle and the implantation window was considered to be the one between the 20th and 24th days of the cycle. Endometrial biopsies were performed at the Human Reproduction Division, Department of Obstetrics and Gynecology, Ribeirao Preto School of Medicine (FMRP-USP), by an experienced physician using Pipelle catheter under sterile conditions.

Immediately after collection, the endometrial samples were divided into two parts: one placed in 10% formaldehyde for histological analysis, and the other placed in RNA-Later (Ambion Life technologies, Warrington, UK), maintained at 4°C for 24h and subsequently stored at -80°C until total RNA extraction.

Endometrial dating

All samples were classified according to the day of the menstrual cycle at the time of collection based on the menstrual history and confirmed by classical histological analysis with hematoxylin-eosin (HE) (Bedaiwy *et al.*, 2015), carried out according to the criteria of Noyes (Noyes *et al.*, 1975), by an experienced pathologist (F.M.C.) blinded to patients' clinical data and group-identifying information.

Gene expression

The integrity of extracted total RNA was evaluated in the Agilent Technologies 2100 Bioanalyzer™ apparatus (Agilent) according to the manufacturer's instructions. RNA samples with RNA Integrity Number (RIN) ≥ 7.0 were considered appropriate. The intact samples were quantified in the Qubit® fluorometer (Invitrogen).

One microgram of total RNA from each sample was reversely transcribed using the High Capacity cDNA Transcription Kit (Applied Biosystems Life Technologies) according to the manufacturer's instructions. Relative quantification of *PGR*, *HBEGF*, *ITGAV*, *ITGB3* and *SPP1* genes were analyzed using an ABI PRISM™ 7500 FAST Sequence Detection System (Applied Biosystems, Warrington, UK). The reactions were performed using the TaqMan Gene Expression Assays system (TaqMan® MGB probes, FAM™ dye-labeled), from Applied Biosystems. The *B2M* and *ACTB* genes were used as reference genes for the normalization of reactions. The primers and probes for *PGR* (Hs01556702_m1), *HBEGF* (Hs00181813_m1) *ITGAV* (Hs00233808_m1), *ITGB3* (Hs01001469_m1), *SPP1* (Hs00959010_m1), *ACTB* (Hs99999903_m1) and *B2M* (Hs00984230_m1) were obtained using the Assay-on-Demand™ Gene Expression Products (Applied Biosystems, Warrington, UK).

In order to test the efficiency of inventoried TaqMan® assays, and to identify the appropriate concentration of cDNA in which the efficiencies would be between 90 and 110%, we performed a real-time PCR efficiency curve test, with serial dilutions (1:2) using a cDNA pool of 3 samples from each group (9 samples in total). All assays had adequate efficiency and, based on this analysis, the 1:8 cDNA dilution was chosen. Quantitative Real-time PCR was performed in triplicate for each sample according to the following conditions: 5µL of TaqMan® Fast Advanced Master Mix (2x) (Applied Biosystems, Warrington, UK),

0.5µL of the TaqMan Gene Expression Assay, inventoried (20X) (Applied Biosystems, Warrington, UK), 4.5µL diluted cDNA in a final volume of 10µL reaction. The reaction conditions were 50°C for 2 minutes, 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

The relative quantification (RQ) of the analyzed genes was calculated for each sample, according to the efficiency of each assay by the $(1+E)^{-\Delta\Delta CT}$ method, where E is the efficiency obtained in the efficiency curve test. The *ACTB* and *B2M* (reference genes) and the calibrator (value obtained with the average of the CTs of the samples of the fertile control group) were used as standard-setting for the calculation.

Potential sources of bias

Selection bias was avoided by inviting all patients who underwent diagnostic videolaparoscopy or tubal ligation and fulfilled eligibility criteria to participate in the study. Between-group imbalances in covariates related to implantation and endometrial receptivity were avoided by adopting highly selective eligibility criteria for patient inclusion. The exclusion criteria were conditions potentially related to endometrial receptivity impairment or gene expression alteration. Reporting bias was avoided by presenting and analyzing all results obtained.

Study size

Considering the lack of data in the literature to estimate mean and standard deviation of *PGR*, *HBEGF*, *ITGAV*, *ITGB3* and *SPP1* genes simultaneously quantified, we designed a pilot study powered to detect or discard differences between the groups (Cohen, 1988). Thus, all eligible patients who agreed to participate and who donated appropriate samples were included in the study.

Statistical Methods

Statistical analysis was performed using the GraphPad Prism software (version 6.0, GraphPad Software, La Jolla, CA, USA). For comparisons between endometriosis, infertile control and fertile control groups, we applied the Kruskal-Wallis test. Significance was set as $p < 0.05$. We also determined the minimum difference (in SDs) that our analyzed sample had sufficient power to detect.

RESULTS

From December 2011 to December 2014, 687 patients who underwent diagnostic videolaparoscopy ($n=629$) or tubal ligation ($n=58$) at the University Hospital were evaluated according to the eligibility criteria. One-hundred thirty patients were eligible, 97 of them were interviewed and 33 were not found. Twenty-three of the interviewed patients did not agree to participate in the study and 32 of the 64 who wanted to participate were excluded (9 for menstrual cycle alterations, 14 for use of hormonal medication, 6 for starting Assisted Reproduction Treatment and 3 patients were unable to attend the sample collection at the scheduled date). Thus, 32 patients provided written informed consent and had endometrial samples collected. Among them, 11 were infertile women with endometriosis (4 stage I, 2 stage II, 2 stage III and 3 stage IV), 13 were infertile controls and 8 were fertile controls. Among the 32 samples collected, 17 samples had endometrial dating confirmed by histological evaluation, being 6 infertile women with endometriosis (2 stage I, 1 stage II, 1 stage III and 2 stage IV), 6 infertile controls and 5 fertile controls. Two patients had total RNA inadequate to perform the Real Time PCR. Thus, 15 samples had expression quantified, among them, 5 infertile women with endometriosis (2 stage I, 1 stage II, 1 stage III and 1 stage IV), 5 infertile controls and 5 fertile controls (Figure 1).

The endometriosis, infertile control and control fertile groups showed no significant differences concerning age and BMI (Table 1). According to Real Time PCR results, all genes had detectable expression in all samples. However, there was no significant difference in the expression levels of any of the studied genes when comparing endometriosis, infertile control and fertile control groups (Figure 2). The median and interquartile range of the gene expression levels are presented in Table 1. With a sample size of 5 women in each group, this study would have an 80% power to detect 2 standard-deviations difference in the expressions of *PGR*, *HBEGF*, *ITGAV*, *ITGB3* and *SPP1* genes among the groups (women with endometriosis, infertile controls and fertile controls).

DISCUSSION

Progesterone plays a fundamental role in endometrium preparation during the implantation window, coordinating several genes related to endometrial receptivity (Young, 2013), so that defects in its receptor can trigger a number of changes in the expression of molecules that are essential to the implantation process. Evidence has suggested that disorders in the eutopic endometrium are involved in impaired fertility in women with endometriosis (Giudice & Kao, 2004; Wei *et al.*, 2009; Bulletti *et al.*, 2010). In order to evaluate the role of endometrial receptivity in the etiopathogenesis of disease-related infertility, this study simultaneously compared the expression of progesterone receptor (*PGR*) coding genes and of molecules direct or indirectly regulated by progesterone, such as heparin-binding epidermal growth factor (*HBEGF*), $\alpha\beta3$ integrin (*ITGAV* and *ITGB3*) and osteopontin (*SPP1*) in eutopic endometrium of infertile women with endometriosis and infertile and fertile controls during the implantation window.

Although the literature reports on the occurrence of progesterone resistance (Osteen *et al.*, 2005; Burney *et al.*, 2007; Bulun, 2010) and changes in PR-A and PR-B receptors in women with endometriosis (Attia *et al.*, 2000; Igarashi *et al.*, 2005; Shen *et al.*, 2015), the preliminary findings of this study showed no large differences in the *PGR* gene expression among the groups. It is important to note that the sample size adopted in the present study only allowed evaluation of large differences among the groups. However, further studies with larger series may clarify the existence of moderate or small differences in the expression of this gene, so that the present findings could help in the respective sample size calculation. Another important point to consider is that the different selection criteria adopted in other studies may have contributed to the different findings, since other authors included fertile patients with endometriosis and did not exclude many of the possible factors that could influence the gene expression and/or endometrial receptivity.

Regarding $\alpha\beta3$ integrin, the findings corroborate the data of the only two studies that evaluated its levels in the eutopic endometrium of infertile patients with endometriosis I/II by immunohistochemistry and did not identify differences when compared to infertile and fertile controls (Ordi *et al.*, 2003; Casals *et al.*, 2012). Nevertheless, in both studies, the infertile control group consisted of patients with unexplained infertility, which could represent a sampling bias, since we cannot exclude a possible endometrial factor in these cases. Added to this, the testing power of the Ordi *et al.* (2003) study was low, while Casals *et al.* (2012) did not report this data, making it impossible to draw consistent conclusions about the role of this integrin in the disease-related infertility.

Figure 1. Study Flow Chart.

Table 1. Age, BMI and relative expression of *PGR*, *HBEGF*, *ITGAV*, *ITGB3* and *SPP1* genes by the (1+efficiency of assay)^{ΔΔCT} method in eutopic endometrium of infertile patients with endometriosis, fertile controls and infertile controls during de implantation window

Variable	Endometriosis (n=5)	Infertile Controls (n=5)	Fertile Controls (n=5)	p
Age	34.80 (29.80; 39.50)	33.50 (29.10; 38.50)	32.90 (27.85; 36.50)	0.63
BMI	21.90 (21.55; 28.85)	23.40 (20.35; 27.30)	27.20 (24.40; 29.26)	0.17
<i>PGR</i>	0.97 (0.73; 1.22)	1.02 (0.83; 1.26)	0.94 (0.76; 1.5)	0.87
<i>HBEGF</i>	0.63 (0.54; 1.46)	1.02 (0.50; 3.34)	1.14 (0.76; 1.27)	0.73
<i>ITGAV</i>	0.85 (0.81; 0.98)	1.03 (0.58; 1.20)	0.85 (0.81; 1.40)	0.61
<i>ITGB3</i>	0.29 (0.25; 0.96)	0.50 (0.45; 4.80)	0.83 (0.45; 3.10)	0.33
<i>SPP1</i>	1.03 (0.61; 1.73)	0.55 (0.46; 1.59)	1.03 (0.61; 1.73)	0.62

Note: data are presented as median (interquartile range). BMI: body mass index. *PGR*: progesterone receptor. *HBEGF*: heparin-binding epidermal growth factor. *ITGAV* and *ITGB3*: αvβ3 integrin. *SPP1*: osteopontin.

Figure 2. Relative quantification data for the *PGR* (A), *HBEGF* (B), *ITGAV* (C), *ITGB3* (D) and *SPP1* (E) genes by the $(1 + \text{efficiency of assay})^{-\Delta\Delta CT}$ method in the analysis of eutopic endometrium of infertile patients with endometriosis, fertile controls and infertile controls during the implantation window.

To date, there are no studies evaluating *HBEGF* expression in infertile women with endometriosis, once it is the first study to rule out differences in this gene among the studied groups. On the other hand, the literature presents contradictory data regarding osteopontin expression in these patients and the present findings confirm once more the data published by Casals *et al.* (2012), which did not report differences in the osteopontin protein levels among the groups. In contrast, Wei *et al.* (2009) found reduced osteopontin expression in the eutopic endometrium of women with endometriosis during the mid and late secretory phases. Another study by the same group showed lower levels of osteopontin RNA messenger (mRNA) in women with moderate to severe endometriosis during the early secretory phase (Burney *et al.*, 2007). On the other hand, Cho *et al.* (2009) showed that higher osteopontin mRNA was expressed in the eutopic endometrium and in the plasma of women with endometriosis when compared

to controls. However, the authors of these studies included fertile and infertile patients with endometriosis and during different periods of the cycle, precluding the comparison of their results with those found by Casals *et al.* (2012) and those presented here. In the present study, the strict eligibility criteria applied avoided other factors related to impaired endometrial receptivity and our control group included only infertile patients with male and/or tubal factor infertility, excluding hydrosalpinx. However, despite no large differences found, we cannot rule out the existence of moderate or small differences.

Recently, a pilot study was published, in which eutopic endometrium of infertile women with endometriosis and infertile controls were evaluated by a microarray diagnostic tool named "Endometrial Receptivity Array" (ERA). The authors have shown no difference between groups in the expression of the evaluated 238 genes related to receptivity (García-Velasco *et al.*, 2015). However, the study did not

present the clinical characteristics of the included patients, such as obesity, related diseases, endocrine diseases, use of medication and habits. Furthermore, within the five genes evaluated by the present study, only the *SPP1* gene was included among the genes evaluated in their microarray platform. Thus, the information about the simultaneous expression of *PGR*, *HBEGF*, *ITGAV* and *ITGB3* genes are unpublished and add new data about the molecular status of eutopic endometrium from these patients during the implantation window.

The main limitation of the study was its small sample, due to the restrictive eligibility criteria adopted, limiting its generalization. However, strict eligibility criteria, eliminating other factors possibly related to impaired endometrial receptivity and implantation are necessary in order to increase the internal validity. Also due to the small sample size it was not possible to stratify the endometriosis group into the stages of the disease.

Whereas the sample size and power of the present study enable us to rule out a large difference between the groups, our preliminary findings suggest that the eutopic endometrium of infertile women with endometriosis do not present a large difference in the expression of genes related to endometrial receptivity, when compared to women with male and/or tubal factor infertility and fertile women during the implantation window. However, we cannot rule out small or moderate differences between the groups. Our findings will be useful for sample size calculation for well-designed studies.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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