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Matrix Metalloproteinases 2 and 9 and E-Cadherin Expression in the Endometrium During the Implantation Window of Infertile Women Before In Vitro Fertilization Treatment

Vamberto O. A. Maia-Filho, MD, PhD[1,2], Andre M. Rocha, PhD[3], Fernando P. Ferreira, MD, PhD[1,2], Tatiana C. S. Bonetti, PhD[1], Paulo Serafini, MD, PhD[2,3,4], and Eduardo L. A. Motta, MD, PhD[1,2,3]

Abstract

Objective: To evaluate the expression of endometrial matrix metalloproteinases (MMPs) 2 and 9 and E-cadherin in peri-implantation phase of infertile women who have undergone in vitro fertilization (IVF) cycles. **Methods:** This prospective study included 51 patients who underwent endometrial biopsy during the receptive phase in a menstrual cycle prior to IVF treatment. The samples were evaluated by tissue microarray for immunohistochemical study. **Results:** The expression of MMP-2, MMP-9, and E-cadherin in the endometrium prior to IVF treatment was not associated with pregnancy. There was a decrease in E-cadherin immunodetection, the higher the age of the patients, a negative relationship between E-cadherin and MMP-2, and a positive association between MMP-9 and E-cadherin. **Conclusions:** The MMP-2, MMP-9, and E-cadherin are expressed in the endometrium of infertile patients during the receptive phase of the natural menstrual cycle. However, there is no correlation between the expression of these molecules and the clinical IVF outcomes.

Keywords

metalloproteinase 2, metalloproteinase 9, E-cadherin, endometrium, IVF

Introduction

In spite of the development of ovarian stimulation and embryo culture technology, assisted reproductive techniques (ARTs) have a success rate of around 30% to 50%. One of the main limiting factors of ART is the failure of embryo implantation, which is a complex and poorly understood process. Approximately 70% of morphologically normal embryos transferred to the uterus are reabsorbed before implantation, without showing any sign of trophoblast action.[1-3]

The endometrium is receptive to embryo adhesion and invasion only during a certain time in the menstrual cycle, deemed the implantation window. Although there is still no consensus on the exact time of embryo implantation, human clinical studies suggest the implantation window is limited to between days 20 and 24 in ideal ovulatory cycles of 28 days.[4-9] During this period, stromal cells differentiate into decidual cells first modulated by progesterone and then other cell types and molecules interact to allow the coordination of embryo apposition, attachment, and invasion of the endometrium.[10-12] Moreover, other cells and molecules,[13] together with the presence of pinopodes are involved and can be also used to better define the implantation window.[14]

Among proteins involved in the implantation and placentation process, matrix metalloproteinases (MMPs) play a critical role in the degradation of the extracellular matrix (ECM), a process that is fundamental to the initial invasion of the blastocyst. The MMPs are expressed from a gene family of zinc-dependent endopeptidases and act on the remodeling of the

[1] Gynecology Department of Paulista School of Medicine, Federal University of Sao Paulo (EPM-UNIFESP), Sao Paulo, Brazil
[2] Centre of Human Reproduction, Hospital e Maternidade Santa Joana, São Paulo, Brazil
[3] Huntington Reproductive Medicine, Av República do Líbano, São Paulo, Brazil
[4] Gynecology Discipline of School of Medicine, University of Sao Paulo (USP), Sao Paulo, Brazil

Corresponding Author:
Vamberto O. A. Maia-Filho, Rua Dr Eduardo Amaro, 152-Paraíso, Vila Mariana, São Paulo, CEP 04104-080, Brazil.
Email: vambertof@gmail.com

decidua[15-18] and myometrium during pregnancy.[19] Among many types of MMPs expressed by human placenta,[20] MMP-2 and MMP-9 seem to have an important role in promoting ECM degradation and thus determine the invasiveness of cytotrophoblast.[21-23]

Cadherins are a group of cell surface glycoproteins responsible for maintaining adhesion between epithelial cells using a calcium-dependent mechanism. Cadherins are responsible for the organization, maintenance, and morphogenesis of epithelial tissues, thus establishing cell adherence.[24-26] One of the cadherin group of molecules, E-cadherin, is expressed in a variety of tissues and takes on an important role during embryogenesis in rodents.[27] On the other hand, expression of E-cadherin has been identified in human trophoblast but its involvement in human embryo implantation is still unclear.[28]

A rodent study shows that after selective injections of E-cadherin in the uterus of a pregnant mouse, pregnancy loss ensues at the site of application, possibly due to involution from the invasive capacity of trophoblast. Also, uterine E-cadherin messenger RNA expression decreases after MMP-2 and MMP-9 injections, suggesting that E-cadherin may be involved in negatively regulating the process of implantation as a result of the action of MMPs.[29]

Studies in different cancer types also show MMPs and E-cadherin are related to metastasis potential, as the invasion through membranes and interstitial ECM requires the action of proteolytic enzymes. The MMPs have largely been related in both invasive abilities in tumor cell lines and overexpression in malignant tumors in vivo.[30] The MMPs are able to cleave E-cadherin at the cell surface,[31] and E-cadherin shedding and the resulting MMP upregulation[32] represent a mechanism by which both molecules play functional roles in metastasis modulation mechanism. Also, the loss of E-cadherin function is a causal factor in the promotion of invasion and metastasis.[33] High levels of soluble E-cadherin in serum[34] and urine[35] of patients with cancer and coexpression of MMPs and E-cadherin cell metastasis studies[36] support that statement.

Based on animal studies, and the role of MMPs and E-cadherin in modulation mechanism of cancer invasion, we hypothesized that MMP-2, MMP-9, and E-cadherin can be expressed in the endometrium during the implantation window and are involved in the implantation process in women. When women undergo ART, endometrial tissue is collected in a preceding menstrual cycle and is later evaluated following the outcomes of the pregnancy. Thus, the aim of this study was to investigate the expression of MMP-2, MMP-9, and E-cadherin in the endometrium of infertile patients during the implantation window before in vitro fertilization (IVF) treatment.

Materials and Methods

Patients and Biopsy

This study was conducted in the Human Reproduction Section of the Gynecology Department of the Federal University of São Paulo, São Paulo, Brazil, with the collaboration of the Reproduction Center of the Santa Joana Hospital and Maternity Unit and the Huntington Center of Reproductive Medicine, São Paulo, Brazil. This study was approved by The Ethics Committee of the Federal University of São Paulo, Sao Paulo, Brazil (number 1392/07), and a signed informed consent was obtained from all patients who agreed to take part. The study was conducted on endometrial tissue samples obtained in a menstrual cycle prior to IVF treatment.

Between 2008 and 2010, 51 women undergoing infertility investigation with a view to IVF treatment were prospectively enrolled. The inclusion criteria were to be between 21 and 38 years old; both ovaries to be present; basal serum levels of follicle-stimulating hormone (FSH) $\leq$ 15 IU/L and estradiol (E2) $\leq$ 60 pg/mL; normal endometrial cavity at ultrasound scan; and body mass index (BMI) < 30 kg/m^2. Patients were excluded whose endometrium was $\leq$ 6-mm thick in the secretory phase, as were those who had had uterine surgery, those whose endometrial biopsy samples were not substantial enough for analysis; whose endometrium presented infectious hyperplastic, malignant disease; or those who were other than in the receptive phase on histological evaluation.

In a natural menstrual cycle before IVF treatment, patients were monitored by ultrasound from cycle day 10 until the dominant follicle was seen to be 16-mm thick out. From this moment forward, serum luetinizing hormone (LH) levels were measured daily and the day of the LH surge was defined when a significant increase in the LH measurement was observed in 2 subsequent tests. The LH surge day was considered as day LH + 0 and endometrial biopsies were collected between days LH + 9 and LH + 11. All endometrial biopsies were collected from the posterior and lateral walls of the uterine fundus using a Pipelle catheter (CooperSurgical Inc, Trumbull, Connecticut) under sterile conditions.

In the next menstrual cycle of endometrial biopsies, patients were submitted to pituitary blockage and an ovarian stimulation cycle in accordance with routine clinical practice. Briefly, pituitary blockage was obtained using a gonadotrophin-releasing hormone antagonist, and ovarian stimulation was achieved with recombinant FSH. Recombinant FSH doses were adjusted according to each patient's profile and response to stimulation. For final oocyte maturation, recombinant human chorionic gonadotropin (hCG) was used (Ovidrel; Serono Laboratories) and oocyte recovery was carried out 35 to 37 hours afterward. Oocytes were fertilized by intracytoplasmic sperm injection (ICSI) and embryos were transferred on days 3 to 5 of culture per routine clinical practice.

Endometrial Processing

Endometrial biopsies were split into 2 portions: 3 mm^3 of tissue sample was fixed in 4% formaldehyde, embedded in paraffin, and histological sections were stained with hematoxylin and eosin for histological dating analysis. The remaining portion of the biopsies was fixed in 4% formaldehyde, embedded in paraffin, and used for immunohistochemistry analyses.

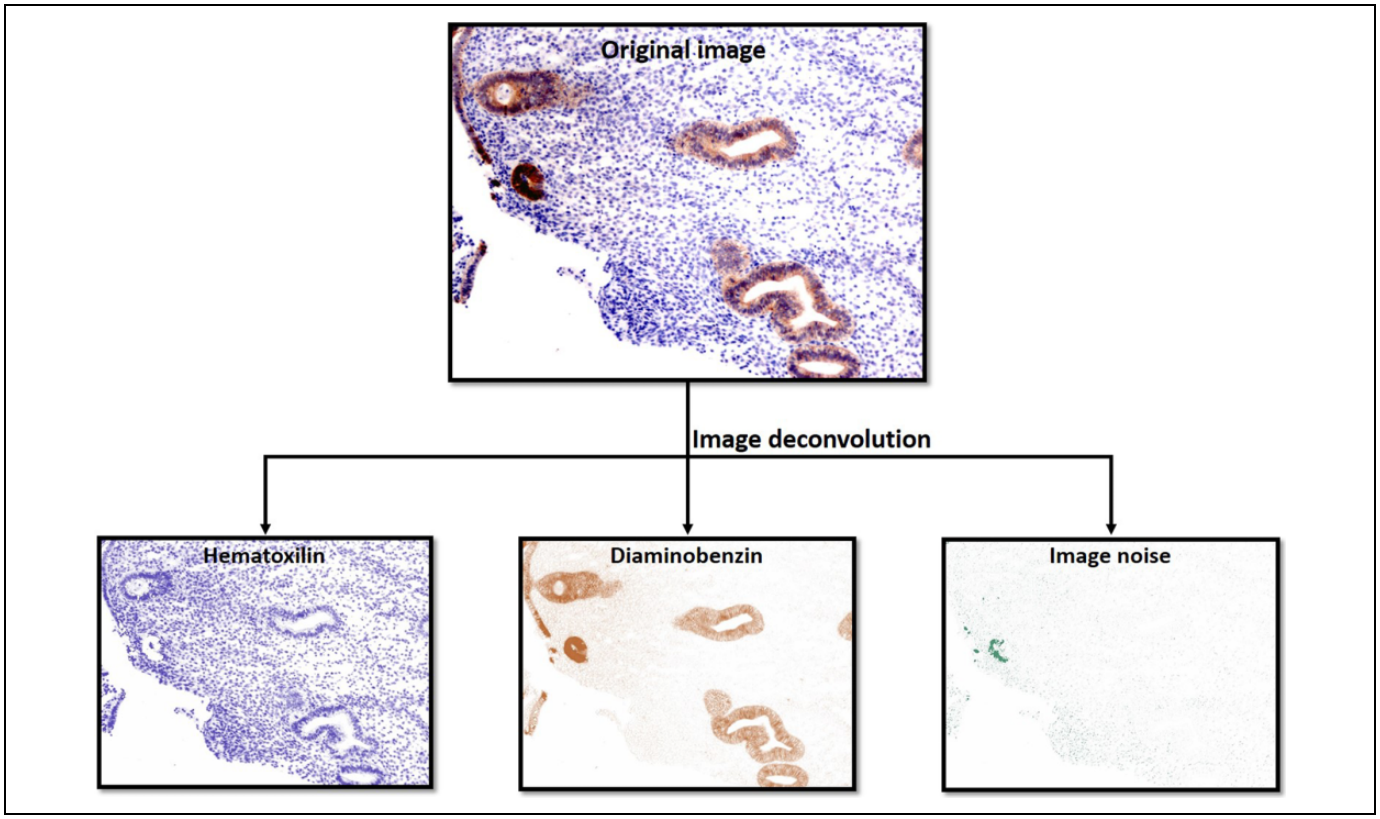

Figure 1. Representative figure of deconvolution of images quantified densitometrically using digital image analysis Image J software (National Institutes of Health, Bethesda, MD) and analyzed with a "plug-in" package developed by McMaster University (http://www.macbiophotonics.ca/ ImageJ).

Endometrial Histologic Dating

Histological dating analysis was performed by the same pathologist for all biopsies following the Noyes criteria.[37,38] The pathologist was blind as to the subject and menstrual cycle phase. The samples were dated as proliferative, prereceptive, receptive, or postreceptive as per their histological features. Only patients presenting receptive endometrium were used in this study.

Immunohistochemistry for MMP-2, MMP-9, and E-Cadherin

For the immunohistochemical study of MMP-2, MMP-9, and E-cadherin, 4-µm thick histological sections were used, and sections presenting less artifacts of each biopsy were chosen for the production of tissue microarray following protocols established previously.[39] Briefly, after sections were fixed, the slides were deparaffinized and rehydrated and rinsed in 10 mmol/L citrate buffer (pH 6, 95°C) for 20 minutes to retrieve antigen. Then, the slides were incubated for 1 hour at 37°C with polyclonal MMP-2 (*RB-1537-P*; Neomarkers, Thermo Fisher Scientific, Fremont, California), MMP9 (RB-1539-P; Neomarkers) antibody (1:300), and E-cadherin (*Mouse Anti-E-Cadherin Cat. N° 18-0223*; Zymed, Life Technologies, Grand Island, New York) antibody (1:1200) followed by overnight incubation at 4°C to 8°C. Secondary antibody (*Anti-mouse BA 200*; Vector, Burlingame, California) incubation was carried out at room temperature for 45 minutes. The revelations used diaminobenzidine diluted in Trizma and hydrogen peroxide in equal proportion. Slides were counterstained with Mayer hematoxila (Sigma-Aldrich Co, St Louis, Missouri). Positive and negative controls were used for each marker.

The images were captured using a microscope (Nikon Eclipse 80i, Nikon Instruments Inc, Melville, New York) coupled to a digital camera (Nikon DXM1200, Nikon Instruments Inc, Melville, New York) and images were captured at 100-fold increase in the program ACT-1 (Nikon ACT-1 Software 2.70 for DXM1200 and DXM1200F digital camera, Nikon Instruments Inc, Melville, New York). In order to improve the accuracy of signal quantification, the slides were quantified densitometrically using digital image analysis Image J software (National Institutes of Health, Bethesda, Maryland) and analyzed with a "plug-in" package developed by McMaster University (http://www.macbiophotonics.ca/ ImageJ).

The images were analyzed as follows: a deconvolution procedure specific for immunohistochemistry reactions using diaminobenzidine counterstaining with hematoxylin was developed, resulting in 3 images: (1) an image corresponding to the hematoxylin stain; (2) an image corresponding to the diaminobenzidine stain, and (3) an image corresponding to the image noise (Figure 1). Four squares were randomly selected for each slide and the intensity of the luminescence of the areas was measured.

Table 1. General Characteristics of Patients Undergoing ICSI Cycles According to Study Groups (Positive vs Negative β-hCG).

	Positive β-hCG (mean ± SD)	Negative β-hCG (mean ± SD)	*P*
N	33	18	
Age, years old	32.5 ± 4.7	34.1 ± 4.3	.208
FSH, mUI/mL	6.3 ± 1.8	6.8 ± 1.5	.266
E2, pg/mL	1355 (175-5944)	1267 (568-7862)	.828
P4, ng/mL	1.2 (0.2-22)	1.1 (0.2-12.7)	.662
LH, mUI/mL	3.9 ± 1.4	4.8 ± 2.1	.133
FSH total dose, UI	1902 ± 446	1931 ± 619	.870
Endometrium, mm	11.4 ± 1.9	11.2 ± 3.7	.797

Abbreviations: E2, estradiol; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; LH, luetinizing hormone; P4, progesterone; SD, standard deviation.

Table 2. Characteristics of ICSI Cycles According to Study Groups (Positive vs Negative β-hCG) Studied.

	Positive β-hCG (Mean, Range)	Negative β-hCG (Mean, Range)	*P*
Retrieved oocytes	15.2 ± 6.7	11.6 ± 7.2	.092
Oocytes MII	11 (2-28)	8 (4-24)	.142
ICSI	9 (0-24)	8 (2-24)	.798
Fertilized, %	7.0 (1-23)	6.5 (2-21)	.531
Cell numbers D^3	8 (6-10)	8 (6-9)	.434
ET average (8 cells)	2 (1-3)	2 (1-2)	.220

Abbreviations: ET, embryos transferred; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; MII, metaphase II.

Statistical Analysis

The statistical analysis was performed using SAS for Windows 9.2 (SAS Institute Inc, Cary, North Carolina) and Minitab Statistical Software 14 (Minitab Inc, State College, Pennsylvania). Samples were split into 2 groups per the clinical outcomes of the IVF cycles, as positive and negative pregnant patients, defined by β-hCG measurements 12 days after embryo transfer. The data variances and normality were analyzed by Bartlett and Shapiro-Wilk tests, respectively. Comparisons of the groups (positive and negative pregnancy) were evaluated by Student *t* or Mann-Whitney *U* tests as appropriate for normal and nonnormal data distribution. Pearson correlation analysis was used to evaluate whether the data were correlated. Data were described as mean ± standard deviation (SD) for normal data and mean and range for nonnormal data. $P < .05$ was considered statistically significant.

Results

The patients' infertility factors were defined as male factor (31%), unexplained infertility (23%), tubal factor (18%), multiple factors (16%), ovulatory factor (8%), and endometriosis (4%). The pregnancy rate of IVF cycles in patients included in the study was 64.7%; thus, 33 patients were allocated to group β-hCG positive and 18 patients to group β-hCG negative.

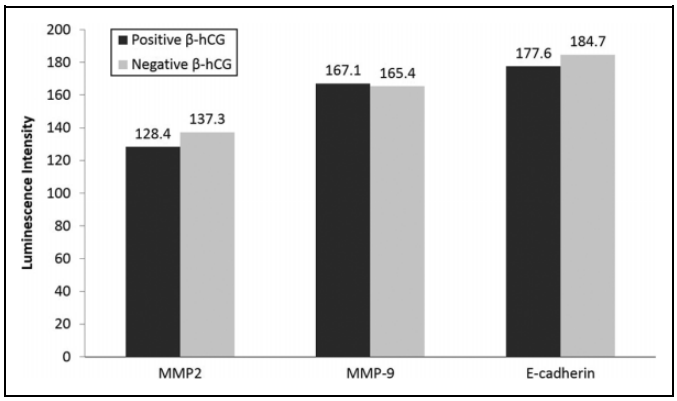

Figure 2. Detection of the intensity of the luminescence of matrix metalloproteinase (MMP) 2, MMP-9, and E-cadherin in the endometrium of patients in previous cycles of in vitro fertilization (IVF) per study group in the subsequent IVF cycle.

In order to evaluate the homogeneity of the groups, we compared age (years old), basal FSH and LH (day 2 of the cycle), E2 (pg/mL), and progesterone (ng/mL) on the days of the last ovulatory stimulus, the total dosage of gonadotropin used (UI), and the endometrial thickness at the end of the ovulatory stimulus (mm; Table 1). When comparing groups to the laboratory and clinical outcomes of IVF cycles, we observed that the groups were homogeneous as shown in Table 2.

Analysis of the intensity of the luminescence of endometrial biopsy on previous menstrual cycle of completion of IVF showed the expression of MMP-2, MMP-9, and E-cadherin was similar between the groups of positive β-hCG women compared to those of the negative β-hCG ones (Figure 2).

The Pearson correlation analysis showed a negative relationship between the age of the patients and the mean luminescence intensity of E-cadherin ($r = -.33$, $P = .02$) indicating that the detection of this protein in women was lower the higher their age. With regard to MMP-9, we found positive correlations between the mean intensity and the basal FSH levels ($r = .34$, $P = .02$). Also, MMP-2 versus E-cadherin ($r = .28$, $P = .049$) and MMP-9 versus E-cadherin ($r = .29$, $P = .03$) were directly correlated (Figure 3).

Discussion

The development of ovarian stimulation, IVF technology, and embryo culture has made a great contribution to the success of infertility treatments in the past 20 or so years. However, although these areas have been continuously developed, the endometrium was considered a less important factor in the process. However, attention has turned to the endometrium lately since it holds hundreds of molecules that play an important function in developing a pregnancy as the endometrium is an essential element of imbalance for improving success rates of ART in infertile women.[40]

Previous study in rodents showed that MMP-2, MMP-9, and E-cadherin act in a synergistic way on the implantation process. Although MMPs are expressed earlier and are essential for

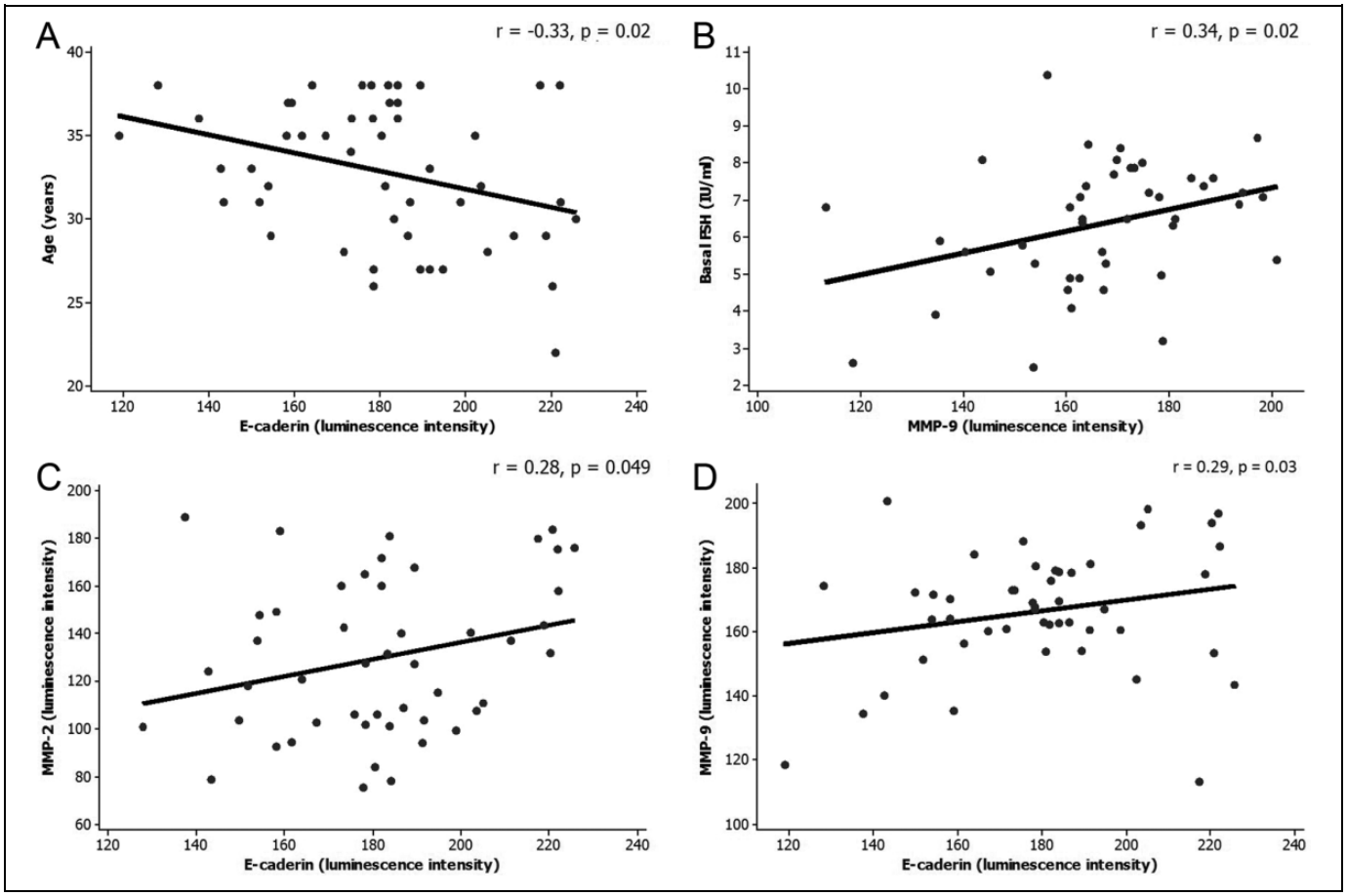

Figure 3. Graph representative of Pearson correlation analysis. A, Patients age (years) and mean detection of the intensity of the luminescence of E-cadherin. B, Basal follicle-stimulating hormone (FSH) levels (IU/mL) and mean detection of the intensity of the luminescence of matrix metalloproteinase (MMP) 9. C, Mean detection of the intensity of the luminescence of E-cadherin and MMP-2. D, Mean detection of the intensity of the luminescence of E-cadherin and MMP-9.

ECM degradation, thus supporting invasion, E-cadherin promotes epithelial cell adhesion.[29] Our study aimed to evaluate the expression of MMP-2, MMP-9, and E-cadherin in the endometrial biopsies of infertile women in the menstrual cycle prior to the IVF cycle. However, the expression of proteins according to pregnancy in the subsequent IVF cycle did not show significant differences.

In spite of difficulties in identifying the implantation window,[41] we based our study on the LH surge in order to collect the endometrial biopsy in the natural menstrual cycle prior to the IVF cycle so as to avoid endometrial trauma and bleeding in the current embryo transfer cycle or even the effects of ovarian stimulation. There is evidence, which strongly suggests that inducing local endometrial injury in the preceding cycle of ovarian stimulation improves pregnancy outcomes in women with unexplained recurrent implantation failures[42,43] while this is not observed when injuries are induced in current cycles of embryo transfer.[44] Hence, we consider that our practice is ethical and did not impair the implantation rates of IVF cycles. On the other hand, if the endometrial biopsy per se improved the pregnancy rates, it could feasibly adversely affect finding the expression of different molecules when we grouped patients in accordance with the pregnancy outcome.

A study evaluating mid-secretory endometrium of unexplained and endometriosis infertile patients showed a higher E-cadherin protein expression in endometriosis compared to healthy fertile controls. These outcomes suggested that the downregulation of E-cadherin during the implantation window is a potential mechanism for implantation that enables epithelial cells to dissociate and the blastocyst to invade.[45] On the other hand, another study showed that infertile patients with hydrosalpinx presented a lower expression of E-cadherin than fertile control patients and suggested that downregulation might be an explanation of the deficient receptivity of the endometrium in patients with hydrosalpinx.[46] Similarly, Marinakis and Nikolaou demonstrated the expression of endometrial E-cadherin was suppressed in women who had lower chances of getting pregnant.[47] These controversies show that the function of E-cadherin in the human endometrium is still unclear.

In the correlation analysis in our study, we observed an inverse association between patients' age and the expression

of endometrial E-cadherin. The small sample size in this study does not allow us to have any conclusion about that, as the correlation can be just a coincidence. On the other hand, this finding could suggest the downregulation of endometrial E-cadherin related to aging.

Animal[29] and in vitro[48] studies suggest that E-cadherin is involved in embryo implantation due to its decreasing expressions and activities of MMP-2 and MMP-9. But, inversely to those studies, we found that MMP-2 and MMP-9 were positively correlated with E-cadherin.

Cancer studies also show inverse relation between E-cadherin and MMPs. An in vitro study evaluating thyroid cancer cells showed E2 induces metastasis via downregulation of E-cadherin and upregulation of vimentin and MMP-9.[36] Other authors showed that malignant phenotype emerged in a breast cancer model, characterized by upregulation of MMP-9, among other molecules, and loss of E-cadherin.[49] A noninvasive human lung tumor cells study demonstrated that E-cadherin upregulates MMP-2 and MMP-9 expressions at the protein level.[32] Those studies let us to think the positive correlation found in our results can be a coincidence biased by small sample size. In the same time, we cannot ignore the fact that patients included in our study are infertile patients, and the infertility condition can be a factor influencing expression of those molecules.

We did not investigate the expression of molecules in uterine flushing fluid or other molecules involved in MMP-2 and MMP-9 pathways, which represent limitations of this study. On the other hand, our initial findings prompt us to develop further studies to continue investigating the differential expression of endometrial molecules in receptive endometrium and its relation to implantation success in assisted reproductive cycles.

Conclusion

Few research studies focus on the expression of protein from the human endometrium and most of them that do so include very few samples of endometrial biopsies. In conclusion, MMP-2, MMP-9, and E-cadherin are expressed in the endometrium of infertile patients during the receptive phase of the natural menstrual cycle preceding the IVF cycle. However, there is no correlation between the expression of molecules and clinical IVF outcomes. Moreover, in spite of there being a significant correlation between E-cadherin and a patient's age, and an inter-correlation between E-cadherin and MMP-2 and MMP-9, it is not clear what the mechanistic relation between them is. Although our outcomes confirm an interaction between these molecules, other articles report contradictory findings which therefore shows there is a need for further studies to clarify the mechanisms of the functions of such molecules and their association with embryo implantation.

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Declaration of Conflicting Interests

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