

High temperature requirement A1, transforming growth factor beta 1, phosphoSmad2 and Ki67 in eutopic and ectopic endometrium of women with endometriosis

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Abstract

Increasing evidence supports the hypothesis that TGFβ1 signalling may be mediated by high temperature requirement A1 (HtrA1) serine protease, acting on important regulatory mechanisms such as cell proliferation and mobility. Evidence is now accumulating to suggest that HtrA1 is involved in the development and progression of several pathologies. The aim of this study was to evaluate: i) if HtrA1 and TGFβ1 expressions differ in eutopic and ectopic endometrium in women with endometriosis; ii) if HtrA1 correlates to TGFβ1, pSmad and Ki67. This study was carried out including 10 women with ovarian endometriosis (cases) and 10 women with non endometriotic diseases (controls). Endometrial tissue underwent immunohistochemical H-score analysis for HtrA1, TGFβ1, pSmad and Ki67 molecules. Data evaluation was performed by a nonparametric Kruskal-Wallis test and Spearman correlation was applied to evaluate the relationship among the molecules investigated in the epithelial and in the stromal compartment. The HtrA1 was significantly decreased in ectopic and eutopic endometrium of women with endometriosis when compared with control endometrium in epithelial compartment. TGFβ1 was significantly increased in eutopic endometrium and decreased in ectopic

endometrium in epithelial and stromal compartment. In addition, Ki67 was significantly increased and an increase, but not significant, was detected for pSmad2 in eutopic and ectopic endometrium compared to control one. In summary, the significant direct correlation between TGFβ1 and pSmad2 as well as between HtrA1 and TGFβ1 and the very significant increase of Ki67 in stromal compartment of eutopic endometrium suggest a possible involvement of HtrA1 in the pathogenesis of endometriosis.

Introduction

Endometriosis is the most common gynaecological disorder,¹ characterized by endometrial-like tissue outside the uterus, most commonly in the pelvic cavity, including the ovaries, the utero-sacral ligaments and the rectovaginal septum.^{1,2} Endometriosis is strongly associated with infertility,^{3,4} chronic pelvic pain, dysmenorrhea, and deep dyspareunia with significant socio-economic impact around the world.⁵ The pathogenesis and physiopathology of endometriosis remains widely debated, but the disease is now understood as a chronic systemic, multifactorial disorder. There are factors that increase the risk for developing the disease, such as age, genetic and environmental factors, as well as the interactions among these factors.^{2,6} In particular, evidence shows that establishment of endometriosis can be facilitated by an endometrial dysfunction involving both eutopic and ectopic endometrium.⁷

Among the various theories proposed for the aetiology of this pathology, the implantation theory of the endometrial tissue, following menstrual back-flow is the most widely accepted one.⁸ Several studies have revealed that there are many fundamental differences between the eutopic endometrium from patients with and without endometriosis concerning the structure, proliferation, immune components, invasive properties, apoptosis, alterations in expression of specific genes and proteins, growth factors, steroid and cytokine production.⁹⁻¹⁵ In particular, TGF-βs are implicated in the gene expression, cell motility, proliferation, apoptosis, differentiation, immune responses and tumorigenesis.¹⁶ TGFβs are abundantly and differentially expressed in the endometrium and are secreted by endometrial cells and macrophages into the uterine fluid.¹⁶ Secretion of TGFβs into peritoneal fluid of women suffering from endometriosis suggests that they may be crucial in establishing and/or maintaining of endometriosis.¹⁷ Binding of TGFβ1 to its receptor leads to activation of transforming growth factor receptor I (TβRI), which then propagates the signal *via* the intracellular phosphorylation of

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receptor-regulated Smad proteins such as *e.g.* Smad2. The subsequent phosphorylated complex translocates into the nucleus where it functions as a transcription factor to regulate gene expression downstream of TGFβ signalling.¹⁸ Increasing evidence supports the hypothesis that TGFβ signalling may be mediated by high temperature requirement A1 (HtrA1) serine protease, acting on important regulatory mechanisms such as proliferation *in vivo*.¹⁹ HtrA1 plays a role in a variety of physiological processes, including protein degradation and cell signalling.²⁰ Evidence is now accumulating to suggest that HtrA1 is involved in the physiological development of many organs,²⁰⁻²³ as well as in development and progression of several pathologies, including neoplastic and degenerative diseases.²⁴⁻²⁶ In addition, Dentillo²⁷

and co-workers have identified HtrA1 as a candidate gene involved in endometriosis. They showed that HtrA1 transcripts increased in ectopic endometrial tissues. Interestingly, it has been demonstrated that mRNA abundance and protein level of HtrA1 significantly decrease with increasing grades of endometrial cancer.²⁸ However, to date, studies in human on the expression of HtrA1 in eutopic and ectopic endometrium in women affected by endometriosis are missing. Thus, the objective of the present study was to investigate the expression of HtrA1 in eutopic and ectopic endometrium of women with endometriosis compared to normal endometrium and to correlate HtrA1 with TGFβ1, with pSmad2 and with Ki67 expression.

Materials and Methods

Ethics statement

The experimental protocol was approved by the Ethics Committee of the Università Politecnica delle Marche for human subjects and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. Written informed consent was granted from all women prior to inclusion in this study and recorded on file.

Subjects

For the present study, we enrolled patients who had consecutively undergone hysterectomy and oophorectomy for severe ovarian endometriosis at the Clinic of Obstetrics and Gynecology, Università Politecnica delle Marche, Ancona, Italy, during the period January 2010 and December 2012 according to the 1996 Revised American Society for Reproductive Medicine Classification of Endometriosis.²⁹ Controls were obtained from women with regular menstrual cycles who underwent hysterectomy for benign diseases. Specimens were collected during proliferative phase, initially determined from the number of days since the last menstrual period, and confirmed by ultrasound³⁰ and by the histological criteria of Noyes *et al.*³¹ There was no known or diagnosed pathology present in the collected tissues. Subjects who had received steroid treatment during the past 6 months, and with pituitary, thyroid, or adrenal disorders, were excluded from the study.

During the study period we evaluated the following three groups: 10 eutopic endometria and 13 ectopic endometria from ovarian endometriosis and 10 control endometria. Three pathological subjects have got a bilateral ovarian involvement.

Morphological analysis

All the surgical samples underwent routine processing at the Section of Pathological Anatomy of Università Politecnica delle Marche for histologic diagnosis after tissue fixation overnight in 4% neutral buffered formalin. A pathologist (GG) reviewed the samples stained with haematoxylin and eosin to confirm the diagnosis and to select the more representative areas for immunohistochemistry. Histologically, ovarian endometriosis was defined by the presence of endometrial epithelium, endometrial glands or gland-like structures, endometrial stroma, and hemosiderin macrophages in the cyst lining.³² All endometrial samples from patients with endometriosis or from controls were devoid of any pathology at histological examination.

Immunohistochemistry

Paraffin sections were treated for HtrA1, TGFβ1, pSmad2 and Ki67 as we have previously described.^{26,33,34}

Briefly, paraffin sections were deparaffinised and rehydrated *via* xylene and a graded series of ethyl alcohol. Sections for pSmad2 and Ki67 were immersed in EDTA 1mM pH 8.0 and subjected to high temperature treatment for 15 min at 98°C. Sections for HtrA1 detection were treated with tween 0.3% in PBS for 25 min. at room temperature. Sections for TGFβ1 were immersed in 0.1M Citrate buffer pH 6 and subjected to high temperature treatment for 15 min at 98°C. To inhibit endogenous peroxidase activity, sections were incubated for 50 min with 3% hydrogen peroxide in deionized water. To block nonspecific background, the sections were incubated, 1hr at room temperature (RT), with normal goat serum (for HtrA1, TGFβ1 and pSmad2 antibodies) and normal horse serum (for ki67-MIB1 antibody). The sections were incubated overnight at 4°C with one of the primary antibodies listed in Table 1.

After washing in PBS, the sections were subsequently incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). The peroxidase ABC method (Vector Laboratories) was used. Sections were counterstained in Mayer's haematoxylin, dehydrated and mounted with

Eukitt solution (Kindler GmbH & Co., Freiburg, Germany). Negative controls were performed by omitting the first antibody or the secondary antibody. Further negative control were performed using an isotype control antibody (rabbit IgG: cat. ab27478, Abcam, Cambridge, UK).

Immunostaining analysis

Immunohistochemical evaluations were performed independently by four morphologists (M.D. and T.G. for HtrA1, pSmad2, TGFβ1; AZ and GG for Ki67). Staining scored positive when a brown colour was present in the nuclei for pSmad2 and Ki67 and in the cytoplasm for HtrA1 and TGFβ1. Percentages were determined by counting positive nuclei of at least 100 nuclei in multiple microscopic fields for each endometrial compartment (epithelia and stroma). Counting was performed on at least three different sections from each endometrial sample. In summary we counted 600 nuclei/cells (300 nuclei in the stroma and 300 nuclei in the epithelial structures) for each sample using an image analysis software (Lucia, ver. 4.6.). A semi-quantitative scoring system was used, which evaluated both staining intensity (0, no stain; 1+ weak stain; 2+ moderate stain; 3+ strong stain) and the percentage of stained cells/nuclei (0, <5%; 1, 5-25%; 2, 26-50%; 3, 51-75% and 4, >75%). We derived HSCORE value, summing the percentages of cells that stained at each intensity (i) and multiplying by the weighted intensity of the staining (0,1,2,3) for each specimen. For example: $HSCORE = \sum Pi (i)$ where i represents the intensity score and Pi the corresponding percentage of positive cells (Hscore range 0-300). The researchers independently reviewed all slides in blind. The level of concordance, expressed as the percentage of agreement between the observers was 89%. A concordant decision was taken for the remaining specimens.

Statistical analysis

Age is presented as means ± standard deviation (SD). The variables TGF-β1, HtrA1, pSmad2 and Ki67 were evaluated for normality of distribution using the Shapiro-Wilk test. As they were not normally distributed, all data were expressed as a median and interquartile

Table 1. Antibodies used in this study.

Antibodies	Specificity	Antibody dilution	Reference
HtrA1 (ab38610)	Rabbit polyclonal	1:20	Abcam plc, Cambridge, UK
TGF-β1	Rabbit polyclonal	1:30	Spring bioscience, Pleasanton, CA, USA
pSmad2	Rabbit polyclonal	1:50	Merk Millipore, Darmstadt, Germany
Ki67-MIB1	Mouse monoclonal	1:80	DakoCytomation, Glostrup, Denmark

range (IQR). The differences of expression levels among molecules considering the three groups (eutopic and ectopic endometrium from ovarian endometriosis and control endometrium) were evaluated using the non-parametric Kruskal-Wallis test. The study had about 80% power to detect a difference of 50 of HtrA1 intensity between controls and endometriosis, considering the following parameters: $\alpha = 0.05$ and k (number of groups) = 3. Spearman correlation was applied to evaluate the relationship among the molecules investigated in the epithelial and in the stromal compartment. P values < 0.05 were considered statistically significant. There were no missing data. All analyses were carried out using SAS/STAT statistical software.

Results

The mean age of patients was 41.3 ± 5.6 years for patients with ovarian endometriosis and 45.3 ± 2.7 years for controls ($P = 0.06$).

The results of TGF β 1, HtrA1, pSmad2 and Ki67 staining scores expressed as a median and IQR are summarized in Table 2. TGF β 1 was mainly present in endometrial epithelium while the stroma showed a weak staining for TGF β 1 in all specimens (Figure 1 a,b,c; Table 2). Its expression was weakly increased in eutopic endometrium, but it was clearly decreased in ectopic endometrium of patients with ovarian endometriosis compared to control one (Table 2). The ovarian endometriosis showed a weakly stained stroma (Figure 1c; Table 2). The differences in TGF β 1 expression among the different groups (control endometrium, eutopic and ectopic endometrium of patients with ovarian endometriosis) were statistically significant in either epithe-

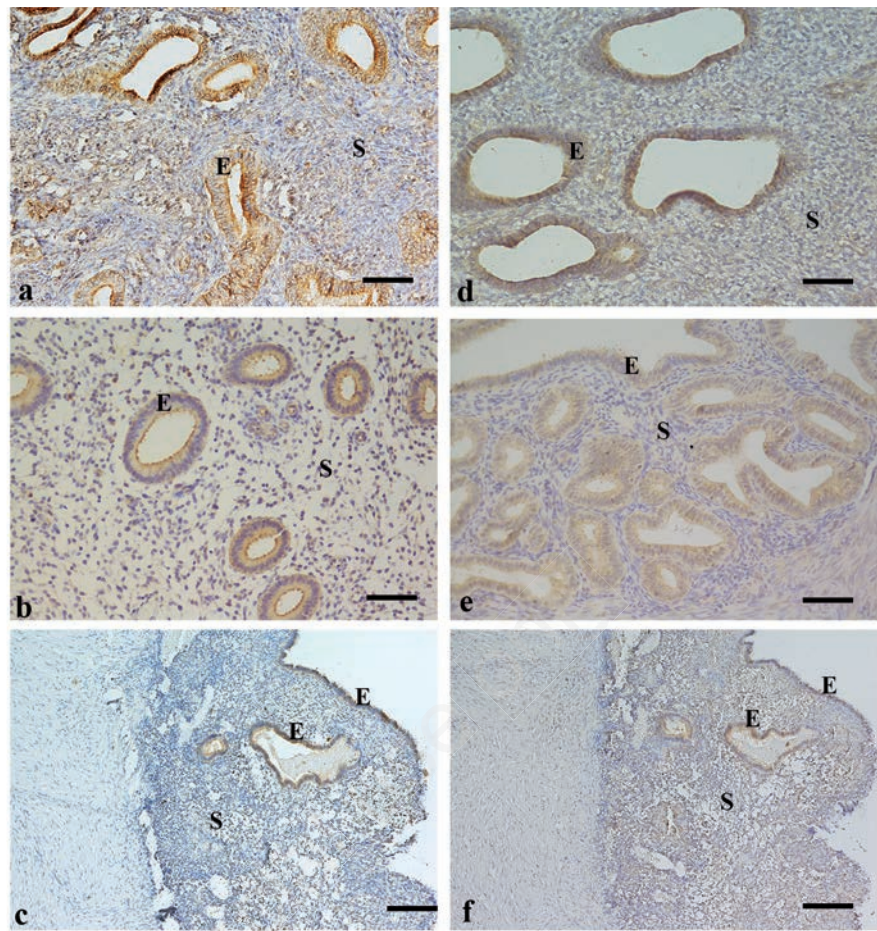


Figure 1. Expression of TGF β 1 and HtrA1 assessed by immunohistochemistry, in endometriosis and control samples. TGF β 1 (a,b,c) and HtrA1 (d,e,f) show a cytoplasmic staining, both in epithelial and stromal compartment, in control endometrium (a,d), in the eutopic (b,e) and ectopic (c,f) endometrium of patients with ovarian endometriosis. E, epithelium; S, stromal compartment. Scale bars: a,b,d,e) 50 μ m; c,f) 100 μ m.

Table 2. Immunohistochemical H-score.

Markers	Control endometrium (n=10)		Eutopic endometrium (n=10)		Ovarian ectopic endometrium (n=13)		Kruskal-Wallis test	
	Median	IQR	Median	IQR	Median	IQR	P	
Epithelium								
TGF β 1	134.5	50.5	153.3	53.5	86.5	79.5	0.03	
HtrA1	106.8	74.0	82.0	73.0	21.5	64.5	0.02	
pSmad2	78.3	19.5	86.0	20.0	82.5	24.5	0.80	
Ki67	14.2	4.5	35.2	157.5	16.5	30.0	0.09	
Stroma								
TGF β 1	20.8	32.0	39.8	32.5	10.0	12.5	0.02	
HtrA1	8.3	13.0	7.3	14.0	1.5	13.0	0.78	
pSmad2	43.8	34.5	72.8	29.5	67.5	34.0	0.19	
Ki67	13.5	12.0	212.2	226.5	24.0	6.5	0.002	

IQR, interquartile range.

lial ($P=0.03$) or stroma ($P=0.02$) compartments (Table 2). HtrA1 was present in the luminal and glandular epithelium, decreasing its expression from control endometrium to eutopic and ectopic endometrium of patients with ovarian endometriosis (Figure 1 d,e,f; Table 2). The differences in HtrA1 expression in the epithelial structures of the endometrium were statistically significant (Table 2) while endometrial stroma was very weakly stained for HtrA1 in all samples analysed and the differences among the three groups were not statistically significant (Table 2). pSmad2 showed a nuclear staining in all samples (Figure 2 a,b,c). No differences in pSmad2 expression were shown both in epithelium and in stromal cells comparing the three groups (Table 2). Ki67 was expressed in the nuclei of epithelial and stromal cells (Figure 2 d,e,f). Ki67 showed a non-significant increase expression in the luminal and glandular epithelium but a significant increase in the stroma of eutopic endometrium of patients with ovarian endometriosis (Table 2). In particular, the high increase of Ki67 in the stroma of eutopic endometrium was very significant comparing the three groups ($P=0.002$). The endometrium from controls exhibited a similar moderate staining for Ki67 in the epithelium and stroma (Figure 2 d,e,f). A statistically significant correlation was found in the stromal compartment between TGF β 1 and HtrA1 in ovarian endometriosis ($r=0.63$, $P=0.01$). pSmad2 and TGF β 1 exhibited a statistically significant inverse correlation in the epithelial compartment ($r=-0.71$, $P=0.02$) and a significant correlation in the stromal compartment ($r=0.76$, $P=0.01$) in eutopic endometrium. Not significant correlation was present between TGF β 1 and Ki67.

Discussion

This study describes the expression of HtrA1, TGF β 1, pSmad2 and Ki67 in eutopic and ectopic endometrium of patients with ovarian endometriosis and in control endometrium. Our data show that compared to control endometrium HtrA1 is significantly less expressed in the epithelium of eutopic endometrium and it shows a much lower expression in ectopic one. Because it is known that HtrA1 can directly or indirectly inhibit cell proliferation and mobility,²⁰ its down-regulation seems to be in accordance with the characteristics of endometriosis such as excessive proliferation and displacement of tissues outside their normal location. In addition, endometriosis shows close similarities with neoplastic processes represented by invasion of normal tissues and metastatic growth. It is

known that HtrA1 is reduced in tumours exhibiting an higher degree of malignancy,^{24,27,35} suggesting an involvement of this protein in invasive processes. Interestingly, the decrease of HtrA1 in endometriosis may be a mechanism involved in the displacement of endometrial tissue outside the uterus. The current study localized HtrA1 in cells of endometrium that produce TGF β 1, suggesting that HtrA1 may interact with TGF β 1 during endometrial remodelling processes. It has been suggested that HtrA proteins such as HtrA1 and HtrA3, can bind TGF β and inhibit TGF β signalling by their proteolytic activities,^{19,20,36} degrading TGF β or its receptors.¹⁹

Our study demonstrates that HtrA1 is significantly down-regulated and TGF β 1 is overexpressed in eutopic endometrium when compared to control one. In addition, both molecules are decreased in ectopic endometrium of

patients with ovarian endometriosis compared to other two groups suggesting that the mechanisms involved in endometriosis in eutopic and ectopic sites could be different. Interestingly, if HtrA1 proteolytic activity is involved in the regulation of TGF β 1 protein level, we can hypothesize that different tissue environment can influence the expression of HtrA1 in eutopic and ectopic endometrial location. In support of this hypothesis, it has been previously demonstrated that HtrA1 mRNA was up-regulated in the ectopic endometrium²⁷ leading to suppose an high expression of HtrA1 protein. On the contrary, our data show a decreased HtrA1 expression in ectopic endometrium suggesting possible post-transcriptional or post-translational modulations of this molecule in relationship to the different environment. Recently, new data generated on the possible embryological origin of

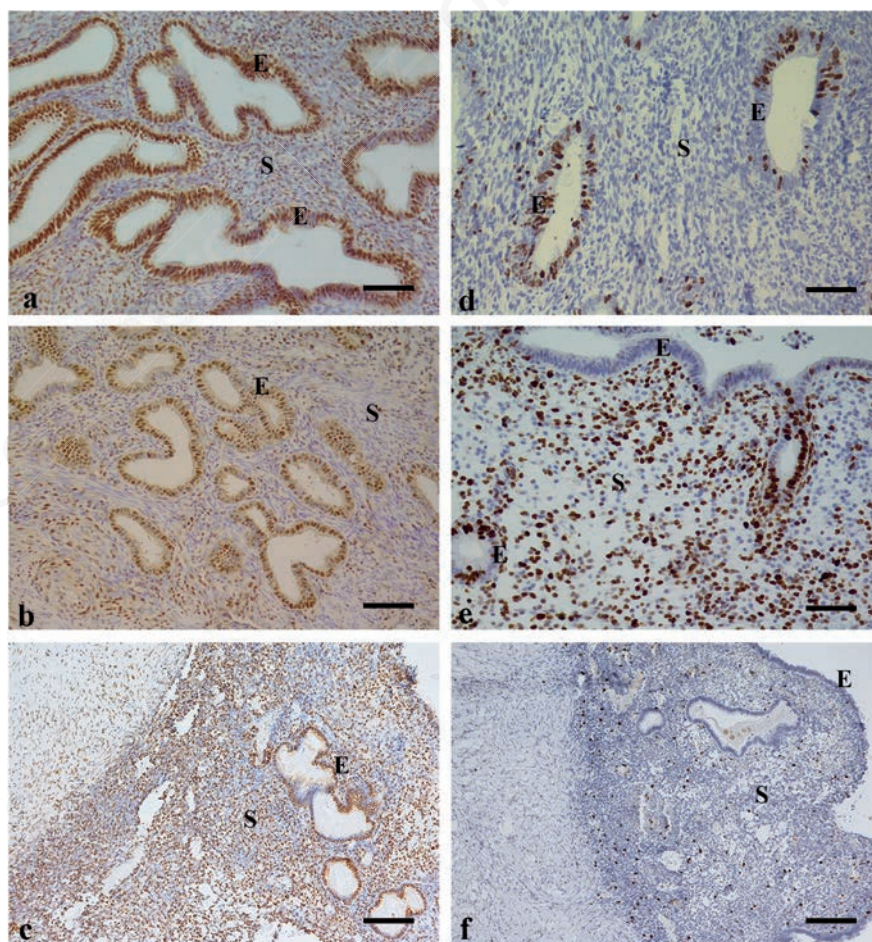


Figure 2. Expression of pSmad2 and Ki67 assessed by immunohistochemistry, in endometriosis and control samples. pSmad2 (a,b,c) shows a nuclear staining both in the epithelium and in the stromal cells of the endometrium in control (a), in eutopic (b) and in ectopic (c) endometrium of ovarian endometriosis. Ki67 (d,e,f) shows a nuclear staining in the epithelium as well as in the stromal cells of all samples. Ki67 is mainly negative in control samples (d) while it is highly expressed in the stroma of the eutopic endometrium (e). E, epithelium; S, stromal compartment. Scale bars: a,b,d,e) 50 μ m; c,f) 100 μ m.

endometriosis suggest that it is caused by molecular alterations of the embryological program for the correct development of uterus. These authors hypothesize that these molecular alterations may cause endometriosis under an opportune environment.^{37,38} Our data concerning the altered expression patterns of HtrA1 and TGF β in ectopic endometrium compared to the ectopic one can support this latter hypothesis. It is known that TGF β receptors propagate the signal downstream by Smads, in particular Smad2, which after its phosphorylation migrates in to the nucleus regulating gene expressions involved in many processes such as proliferation and cellular migration.^{39,40} No differences were observable in pSmad2 expression in the three groups analysed in epithelial as well as stromal compartment of endometrium. Interestingly, we showed a significantly direct correlation between pSmad2 and TGF β 1 expression levels in stromal cells of the eutopic endometrium and an inverse correlation between pSmad2 and TGF β 1 in the epithelium of the eutopic endometrium. In addition, no correlation was detectable between Ki67 and TGF β 1. Thus, we can hypothesize that other molecules, such as activin, a member of TGF β superfamily, may modulate the activation of Ki67 by pSmad2. In fact, although small members of TGF β superfamily play different biological activities, the signalling for all of them is essentially the same.⁴¹ A previous manuscript⁴² has analysed Ki67 expression in eutopic and ectopic endometrial epithelium considering women (cases and controls) in the age of 21-44, showing a decrease (from 38% to 1.2%) in proliferative rate in ectopic lesions. In addition, it has been demonstrated that Ki67 expression significantly decreases with age in normal endometrial tissues in women in the age 18-38.⁴³ We have considered women in the age 36-47 (with regular menstrual cycle) demonstrating a significant increase of Ki67 Hscore in the stroma of eutopic endometrium of women affected by endometriosis suggesting an important enhancement of the proliferative processes in this location.

In conclusion, the main result of this study concerns the eutopic endometrium, particularly the stromal compartment, of patients with ovarian endometriosis. The significant direct correlation between TGF β 1 and pSmad2 as well as between HtrA1 and TGF β 1 and the very significant increase of Ki67 in the stromal compartment of eutopic endometrium suggest a possible involvement of HtrA1 in the pathogenesis of endometriosis. The study of relationship among these molecules and other growth factors or cytokines could represent an attractive field of future *in vitro* and *in vivo* research in order to verify if HtrA1 could be considered a potential modulator or blocker of

cell proliferation in endometriosis. However, considering the small sample size, this finding need confirmation in larger studies.

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