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Article

Overexpression of ErbB-1 (EGFR) protein in eutopic endometrium of infertile women with severe ovarian endometriosis during the 'implantation window' of menstrual cycle

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Abstract

Strong association between endometriosis and infertility is of high clinical significance. High proliferative bias in eutopic endometrium during secretory phase is a hallmark of endometriosis, which may result in high occurrence of implantation failure and resultant infertility in endometriosis. ErbB family of proteins regulate the proliferation capacity in endometrium potentially causing endometrial hostility to implantation process in endometriosis. However, our knowledge regarding the involvement of ErbB family in human endometrium during the window of implantation (WOI) in endometriosis-associated infertility is thin. In the present study, the cellular profiles of immunopositive ErbBs-1 to -4 in endometrium of endometriosis-free, infertile women (Group 1; n = 11), and in eutopic endometrium of infertile women diagnosed with stage IV ovarian endometriosis (Group 2; n = 13) during mid-secretory phase were compared using standardized guidelines. Computer-aided standardized combinative analysis of immunoprecipitation in different compartments revealed an over-expression of ErbB-1 in the epithelial, stromal and vascular compartments along with marginally higher ErbB-3 expression ($P< 0.06$) in the vascular compartment and ErbB-4 expression ($P< 0.05$) in the glandular epithelium and stroma in endometrium during the WOI in women with primary infertility associated with stage IV ovarian endometriosis compared with disease-free endometrium from control infertile women. It appears that changes in ErbBs in the eutopic endometrium during WOI induce anomalous proliferative, inflammatory and angiogenic activities in it, which can antagonize endometrial preparation for embryo implantation in endometriosis. This knowledge appears usable in strategizing methods for treatment of endometriosis-associated infertility, as well as, preempting the oncogenic potential of endometriosis.

Keywords: endometriosis; endometrium; ErbB receptors; immunoprecipitation; implantation stage; infertility

Introduction

Endometrium occupies a unique place in the human body as it undergoes a cyclical pattern of cellular growth and differentiation to accommodate the arrival of fertilized embryo towards the establishment of pregnancy. According to the commonly accepted theory to explain the pathogenesis of endometriosis, the secretory phase of a non-fecund cycle ends with menstrual bleeding along with retrograde efflux of endometrial cells, some of which may adhere to organs within the peritoneal cavity [1]. Although such retrograde efflux of menstrual debris is a common event in women, it does not necessarily lead to development of endometriotic lesions [2]. In fact, one out of ten of women suffer from endometriosis, mostly while of reproductive age. Thus, it is possible that the primary defect exists in eutopic endometrium of women diagnosed with endometriosis as it bears significant differences with control endometrium from endometriosis-free women [3,4]. There exists a strong association between endometriosis and infertility; in infertile women, the prevalence of endometriosis may be as high as 50%, and at least one woman of three women with endometriosis is infertile [5,6]. It appears that the pathophysiological basis of endometriosis-associated infertility is multifactorial, and that inadequate endometrial preparation causing implantation defect is an important one [7]. Several groups have suggested that proliferative capacity of endometrium in women with endometriosis is higher than that compared with normal endometrium [8-13]. Such endometrial anomaly

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may be a putative cause of reportedly high occurrence of implantation failure in patients with endometriosis [14].

Epidermal growth factor receptor (EGFR) family mediates one important pathway in the regulation of proliferation in mammalian cells. This growth factor receptor family has four members: ErbB-1 (EGFR, HER1), ErbB-2 (HER2), ErbB-3 (HER3) and ErbB-4 (HER4). The ErbB family receptors are transmembrane glycoproteins with an extracellular ligand-binding domain, a transmembrane region and an intracellular domain displaying tyrosine kinase activity except in ErbB-3. ErbB-1 and ErbB-4 form either homo- or heterodimers, while ErbB-2 functions as a cofactor for the other receptors. ErbB-3 needs obligatory heterodimerization because of its lack of tyrosine kinase activity. Receptor dimerization is essential for activation of the intracellular tyrosine kinase domain of ErbBs and phosphorylation of C-terminal tail. Phosphotyrosine residues then activate, either directly or through adaptor proteins, downstream components of signaling pathways including Ras/MAPK, PLCγ1/PKC, PI(3)kinase/Akt, STAT and Par6-atypical PKC pathways [15-18].

A cyclical pattern of expression and localization of the ErbB family of receptor tyrosine kinases (RTKs) and their ligands in human endometrium has earlier been reported [19]. In endometriosis, mRNAs for ErbB-1 and ErbB-3 are upregulated in eutopic endometrium of women with endometriosis [20-22]. To our knowledge, there is a lack of knowledge regarding the association between endometriosis and expression of ErbB receptor family proteins in human endometrium during the window of implantation (WOI), i.e., days 20-24 of a typical 28 day menstrual cycle [23,24]. The accrued information, as summarized in Table 1, generally fails to provide any useful understanding in this regard, because the reported studies mostly failed to adhere to the WERF EPHect guidelines [25,26], and did not address the specific issue of cellular expression

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of ErbB family proteins in implantation stage endometrium [19-22]. Our previous studies have clearly pointed out that the physiology of secretory phase endometrium is significantly affected in ovarian endometriosis-associated infertility [4,13,22]. Thus, the aim of the present study was to immunolocalize the cellular profiles of ErbB family of proteins in endometrium of endometriosis-free, infertile women, and in eutopic endometrium of infertile women diagnosed with severe (i.e., stage IV) ovarian endometriosis during the WOI. Per the WERF EPHect guidelines, sampling and data mining of tissue samples were done from only one subtype of endometriosis (i.e., severe ovarian endometriosis, stage IV) patients along with clear annotations in the present study. Thus, tissue samples collected during the mid-secretory phase from consenting patients diagnosed with primary infertility either without endometriosis or with diagnosed stage IV ovarian endometriosis and showing implantation stage histological characteristics were used for immunohistochemical localization of ErbB receptor family proteins.

Table 1. Expression of ErbB family members in endometrium during endometriosis

Name of ErbB family of protein (*alias**)	Salient observations (References)
ErbB-1 (*EGFR*, *HER1*)	Epithelial as well as stromal compartment of human endometrium express EGF and ErbB-1 [19,27]. In endometriosis, EGFR (ErbB-1) mRNA is upregulated in endometriotic eutopic endometrium, especially during secretory period, compared with normal endometrium [20-22].
ErbB-2 (*HER2/neu*)	ErbB-2 showed high expression during the early secretory phase [19]. Endometrial mRNA for ErbB-2 was higher in endometriotic eutopic endometrium compared with normal endometrium [20].
ErbB-3 (*HER3*)	ErbB-3 showed high expression during the secretory phase [19,28]. Endometrial mRNA for ErbB-3 was higher in endometriotic eutopic endometrium compared with normal endometrium [20,22].
ErbB-4 (*HER4*)	ErbB-4 showed high expression during the secretory phase [19,28]. Endometrial mRNA for ErbB-4 was comparable between

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	endometriotic eutopic endometrium and normal endometrium [20].

*from GeneCards home page for human gene database [29].

Material and Methods

Patient selection and tissue processing

Patients enrolled in the Department of Obstetrics and Gynecology of the All India Institute of Medical Sciences-Delhi for surgical intervention for endometriosis and/or their evaluation at the Infertility Clinic voluntarily participated in the study after understanding its purpose and providing written consent, according to the standard protocol. The study approved by the Institutional Ethics Committee on the Use of Human Subjects (IECPG-546/21.10. 2020; RT-19/25.11.2020) was conducted according to the Declaration of Helsinki Amendment 2013. Infertile patients with primary infertility accompanied by stage IV ovarian endometriosis classified as the Patient group, or with no endometriosis classified as the Control group, were enrolled in the study as described elsewhere [4,13]. There were two groups: Group 1 (control group) comprised of seventeen (17) endometriosis-free patients and group 2 comprised of twenty (20) patients diagnosed with stage IV ovarian endometriosis. For both groups, patients showing evidence of polycystic ovarian syndrome according to Rotterdam criteria [30], male factor of infertility and unexplained infertility were excluded. Confirmation of ovarian endometriosis and exclusion of other types of endometriosis was achieved from reports of pelvic imaging based on ultrasound, MRI and/or diagnostic laparoscopy. Severity stages and sub-type of the disease condition were defined according to rASRM protocol at the time of surgical laparoscopy,

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and finally by histology as described elsewhere [4,22,31]. None of the patients in ovarian endometriosis group had prior clinical recording of the disease, and hence was not under any treatment for endometriosis. Exclusion criteria included the co-presence of any other endocrinological disorder, cancer and uterine conditions, such as fibroids (leiomyoma), adenomyosis, and tuberculosis, since these conditions might affect the results of the study as described elsewhere [22,32]. The patients who had taken contraceptives, GnRH analogues, aromatase inhibitors, danazol, dienogest or anti-tuberculosis therapy during the last 6 months and/or who had undergone any previous laparoscopic surgery were not included.

Table 2. Summary of patients' characteristics

Parameter	Group	
	1	2
Group description	Control	OE-IV
Fertility history	Infertile*	Infertile*
Duration of infertility (months)	21 ± 9.0	21.1 ± 8.7
Number		
Recruited	17	20
Selected**	11[a]	13[a]
Age in years[b]	29.1 ± 4.1	29.8 ± 4.7
BMI (kg/m^2)[b]	20.4 ± 3.2	21.9 ± 4.2
Length of menstrual cycle in days[b]	28.7 ± 1.3	28.8 ± 1.5
Cycle day of sample collection[b]	22.6 ± 2.3	21.8 ± 2.7

*despite frequent and unprotected coitus. **identified as mid-secretory phase endometrium typical of WOI seen between days 20 and 24 of a typical ovulatory cycle using standardized endometrial dating procedure [33,34]. [a]sample size was calculated using Stata 14.0 (StataCorp LLC, Texas, USA) and based on reported data [13] for power: 0.9 and α = 0.05 with attrition rate 20% yielding n = 11/each group. [b]for selected patients. Values are shown as means ± SDs.

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Endometrial samples during the mid-ovulatory period were obtained from upper uterine fundus and collected in cold phosphate-buffered saline (PBS, pH 7.4) using a Karman cannula no. 4. Samples were immediately washed with cold PBS, transported to the laboratory on ice and immediately fixed with freshly prepared cold neutral phosphate buffered 4% (w/v) paraformaldehyde, processed and embedded in paraffin for histological assessment of

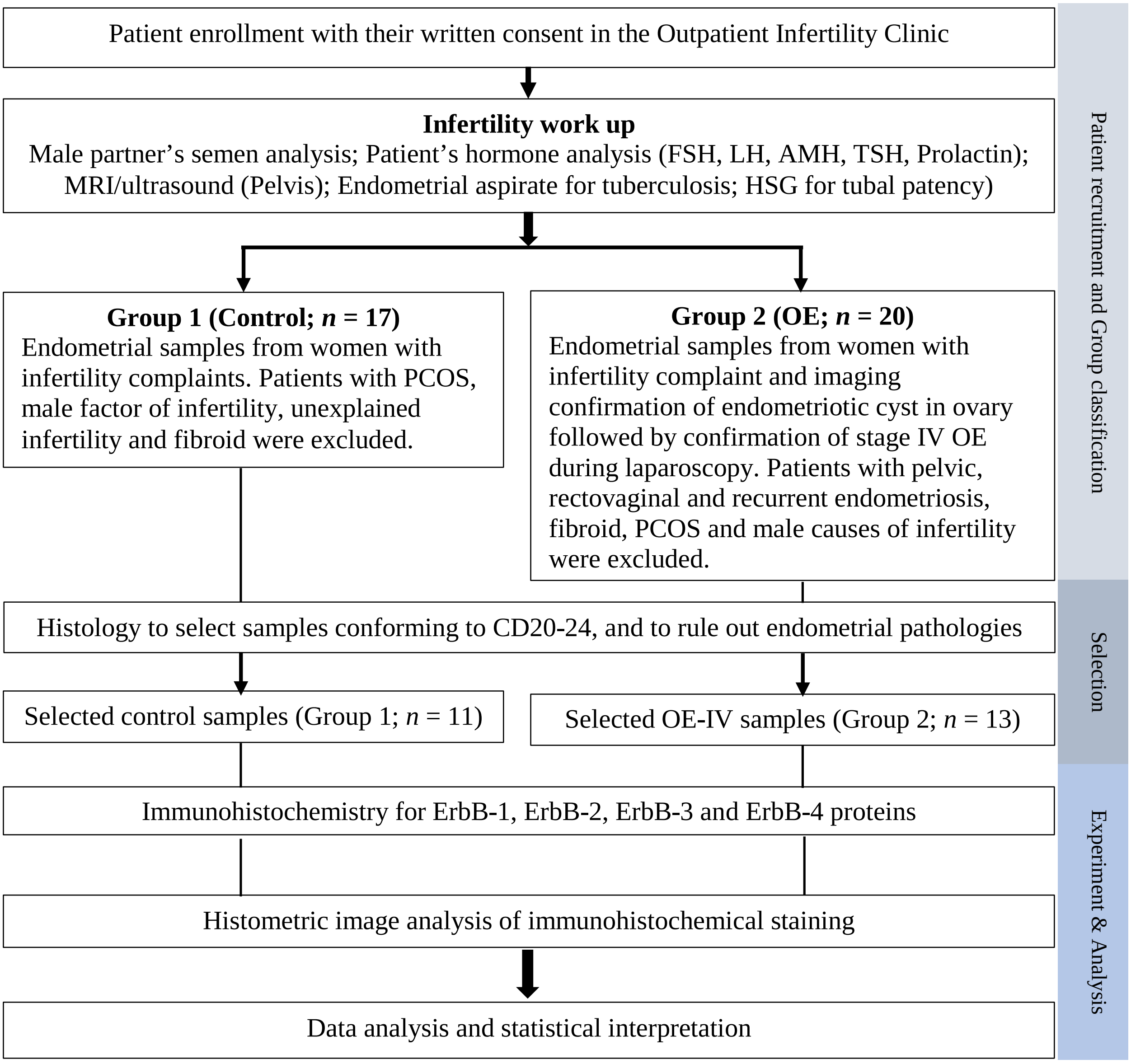

Figure 1.

A flowchart of overall experimental design used in the present study. AMH, Anti-Mullerian hormone; CD, cycle day; FSH, follicular stimulating hormone; HSG, hystero-salpingogram; LH, luteinizing hormone; OE, ovarian endometriosis; OE-IV, stage IV ovarian endometriosis; PCOS, polycystic ovarian syndrome; TSH, thyroid stimulating hormone.

endometrium. Histological assessment towards endometrial dating to identify implantation stage endometrium was performed using hematoxylin-eosin stained 5 µm paraffin sections according to the guidelines and previously optimized criteria of Noyes [33,34]. Based on endometrial dating independently done by three investigators, 24 samples for both groups were finally identified as mid-secretory phase endometrium (MSE), seen between cycle days 20 and 24 of typical menstrual cycle of 28 days; those samples were used for immunohistochemistry as described below. All chemicals were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). A summary of the experimental design is shown in the form of a flowchart in Figure 1. Table 2 provides the synopsis of the samples used. Supplementary Table 1 provides the major clinical data for the selected patients in both groups.

Immunohistochemistry

Rabbit monoclonal antibodies against three antigens, namely ErbB-1, ErbB-2, and ErbB-3 and rabbit polyclonal antibody against antigen ErbB-4 obtained from Abcam (Cambridge, MA, USA) were used for immunohistochemistry (IHC) using 5 µm paraffin sections collected on poly-l-lysine coated glass slides. Tissue sections were deparaffinized and subjected to heat retrieval in 0.1 M sodium citrate buffer (pH 6.1) for ErbB-1 and ErbB-4, and 0.5 M Tris-EDTA buffer (pH 9.0) for ErbB-2 and ErbB-3 using standard methods described elsewhere [35]. Briefly, endogenous peroxidase quenching was performed using 0.3% (v/v) freshly prepared hydrogen peroxide in phosphate buffer (pH 7.4) followed by blocking of non-specific binding using goat non-immune blocking serum (1:50) obtained from Vector Laboratory (Burlingame, CA, USA). Dilutions of stock primary antibodies for incubation were pre-calibrated based on 5-point titration and the information provided by the manufacturer. The final visualization was achieved using AEC-IHC kit (ab64260) obtained from Abcam (Cambridge, MA, USA) and Gill's hematoxylin obtained from Sigma-Aldrich (St. Louis, MO, USA). For IHC controls, sections were processed as above with the omission of either primary antibody or secondary antibody.

Image and data analysis

All images were viewed, documented and analyzed using a Leica DMRD microscope and a Leica computer-assisted image analysis system (QWin DC 200) obtained from Leica Microsystems (Wetzlar GmbH, Germany). Three trained observers independently performed combinative scoring for immunostaining in different compartments for all samples. Staining vectors were digitally set using positive controls for spectral reference, which was applied in default mode individually to every slide to ensure meaningful detection and quantification of positive cells for every sample. The observers were blinded for the patients' clinical data during the scoring procedures. For every parameter, the optimal score in each compartment was assessed using a pre-calibrated standardized five-scale scoring scales that transformed continuous quantitative data into ordinal data [0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%), 4 (>75%)] according to the previously detailed procedure [35,36]. All scores provided by the observers were entered into a database using Excel sheet and analyzed using weighted κ-statistics for assessment of inter-observer errors yielding a mean κw-score of 0.67, suggestive of good agreement beyond chance [37]. This approach of histometric analysis of immunohistochemical staining has been observed to be satisfactory and recommended as an acceptable routine procedure in pathological studies [38,39]. Statistical analyses between two groups were done using Mann-

Whitney *U*-test [40] using Graph Pad version 9 (GraphPad Software Inc., La Jolla, CA, USA) statistical packages.

Results

General

As seen in Table 2, 24 samples obtained from women during the mid-secretory phase of endometrial cycles from both groups were considered usable in the present study. These volunteers displayed similar physiological characteristics in terms of their reported duration of infertility, age at the time of tissue sample collection, and normal BMI and length of their menstrual cycles. Their tissue samples also displayed histology typical of 'implantation window'. In the following section, the profiles of immunopositive ErbB family proteins in selected endometrial samples from control (group 1) and stage IV ovarian endometriosis (group 2) groups are presented. A composite plate showing representative photomicrographs at low magnification for ErbBs immunostaining in MSE obtained from both groups is provided in the Supplementary Figure 1.

Immunohistochemical localization

ErbB-1: Figure 2 displays the representative photomicrographs of ErbB-1 immunostaining in different compartments in MSE obtained from both groups and the ordinal scores in respective compartments. In the control samples, ErbB-1 immunoprecipitation in basal and apical regions of luminal epithelial cells with sparse immunostaining in glandular epithelial cells and minimal membrane and cytoplasmic staining within stromal cells of subluminal zone and in stromal cells surrounding glands of zone III was seen (Fig. 2A, B). Eutopic endometrial samples obtained from endometriosis patients displayed higher ErbB-1 immunoprecipitation in

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cell cytoplasm, cell borders and nuclei of luminal epithelia in basal and apical regions, and nuclei of glandular epithelial cells, in stromal cells of subluminal compartment and in cells surrounding

ErbB-1

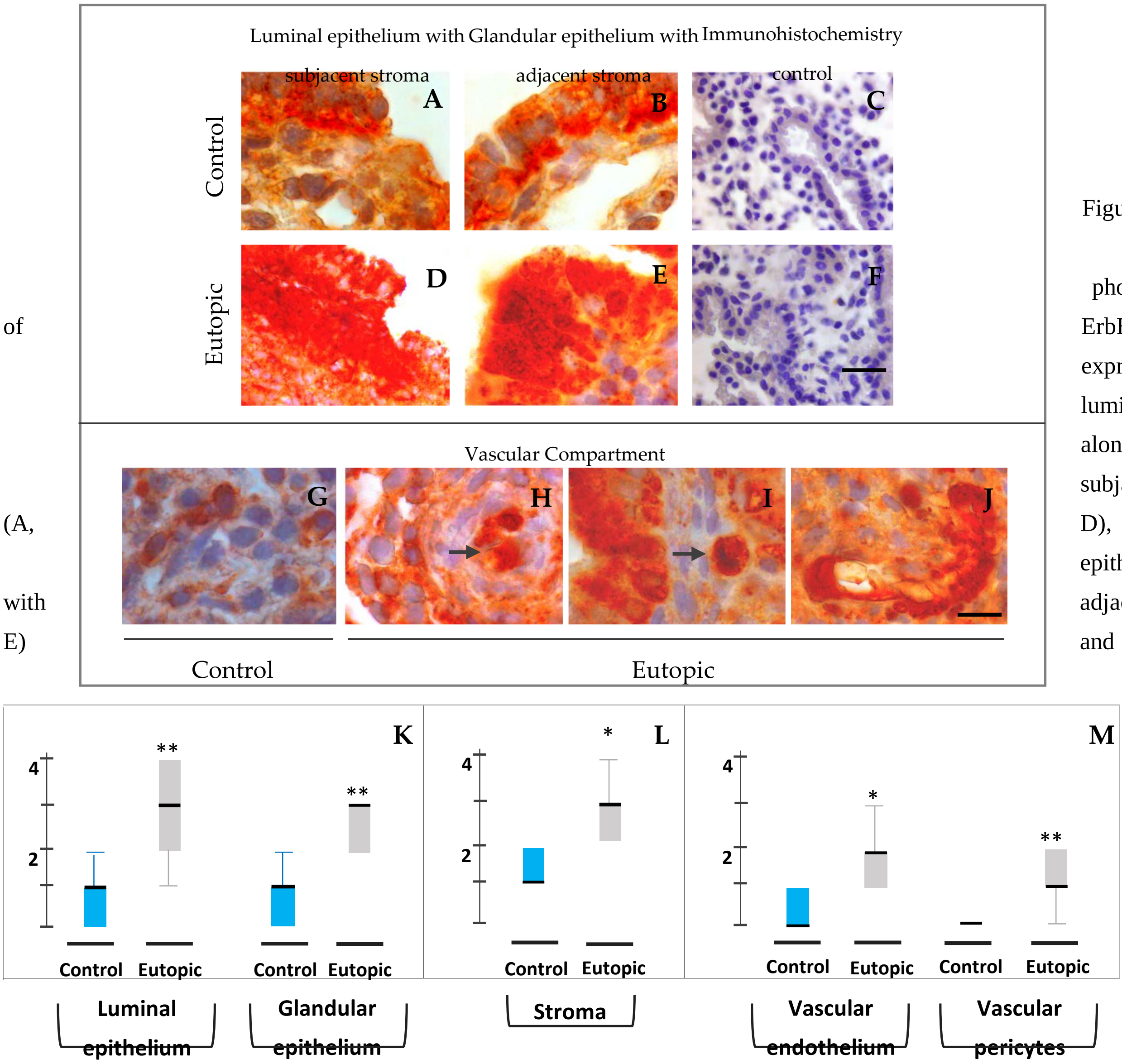


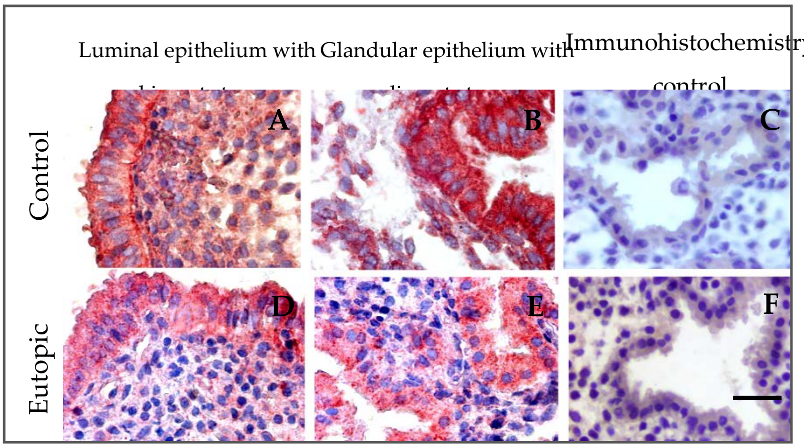
Figure 2. Representative photomicrographs of ErbB-1 (A-J) expressions in luminal epithelium along with subjacent stroma (A, D), glandular epithelium along with adjacent stroma (B, E) and in cells of the vascular compartment (G-J) in endometrial samples collected from endometriosis-free control women of group 1 (A-C, G) and stage IV ovarian endometriosis patients of group 2 (D-F, H-J). Semi-quantitative assessments of immunopositivity scores are shown in form of box plots of 10-90 percentile distribution of scores along with median values (K-M). Markedly higher ErbB-1 immunostaining is seen in the luminal and glandular epithelium and in stromal cells of endometrium obtained from group 2 subjects, i.e., infertile patients with stage IV ovarian endometriosis (D, E) as compared to control endometrium obtained (A, B) from endometriosis-free control women (group 1). High levels of cytoplasmic distribution besides membranous localization of ErbB-1 are seen, more predominantly in eutopic endometrial epithelium (D, E). Minimal immunopositivity in endothelial cells and pericytes of control endometrium (G) is seen, while endothelial cells and pericytes of eutopic endometrium obtained from patients with severe endometriosis show strong immunostaining for ErbB-1 (H-J). Intravascular (H) and extravascular (I) monocytes in eutopic endometrium show moderate to high immunoprecipitation (shown as *arrows*). This feature was generally absent in control endometrium obtained from disease-free

control patients. Marked cytoplasmic and nuclear distribution besides membranous localization is notable for ErbB-1 expression in eutopic endometrium of stage IV ovarian endometriosis infertile patients. Controls for immunohistochemistry staining were done by omitting the primary antibody (C) or the secondary antibody (F). *P< 0.02. **P< 0.01. Bar: 20 µm (A, B, D, E, G-J), 100 µm (C, F).

glands in zone III (Fig. 2D, E). Marked difference was observed in the ErbB-1 immunopositivity in the vascular compartment of eutopic endometrial samples from endometriosis patients compared with the control (Fig. 2G-J). Minimal ErbB-1 immunopositivity was detected in endothelial cells of spiral arterioles and surrounding pericytes in control endometrium (Fig. 2G), while significant immunopositivity was detected in the vascular endothelium and in surrounding pericytes of eutopic endometrium as well as in the blood borne cells lying within blood vessels and in the extracellular matrix surrounding glands of eutopic endometrium (Fig. 2H-J). These features were generally absent in control endometrium. The histometric scoring for ErbB-1 protein in epithelial, stromal and vascular compartments of endometrial samples revealed significantly higher immunoprecipitation in all four compartments (viz., luminal epithelium: P< 0.01; glandular epithelium: P< 0.01; stroma: P< 0.02; endothelium: P< 0.02, and vascular pericytes: P< 0.01) of functionalis of eutopic endometrium obtained from women with primary infertility and ovarian endometriosis (group 2) as compared to endometrium obtained from women with primary infertility endometriosis free control group (group 1) (Fig. 2K-M).

ErbB-2: Figure 3 displays the representative photomicrographs of ErbB-2 immunostaining in different compartments in MSE obtained from both groups (Fig. 3A-J) and the scores in in respective compartments. Immunohistochemical localization of ErbB-2 in endometrium showed basal and apical distribution of immunoprecipitates in luminal and glandular epithelium, often around nuclear regions along with its widespread presence in stromal cells particularly in the subluminal zone and around the glands. There were no marked

ErbB-2



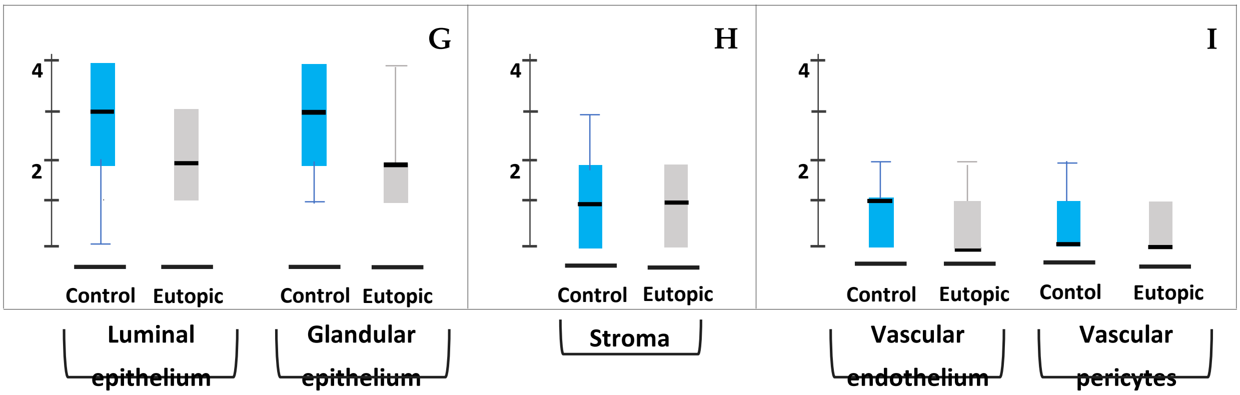


Figure 3. Representative photomicrographs of ErbB-2 (A-F) expressions in luminal epithelium along with subjacent stroma (A, D), glandular epithelium along with adjacent stroma (B, E) in endometrial samples collected from endometriosis-free control women of group 1 (A-C) and stage IV ovarian endometriosis patients of group 2 (D-F) and their semi-quantitative assessments shown in form of box plots of 10-90 percentile distribution of scores along with median values (G-I). No marked difference observed in any compartment between the two groups. Controls for immunohistochemistry staining were done by omitting the primary antibody (C) or the secondary antibody (F). Bar: 75 µm.

differences in samples between control women with primary infertility without endometriosis (group 1) and with stage IV ovarian endometriosis (group 2). No difference was seen in semi-quantitative scoring of immunopositivity detected for ErbB-2 protein in epithelial, stromal and vascular compartments of endometrial samples obtained from eutopic endometrium of women

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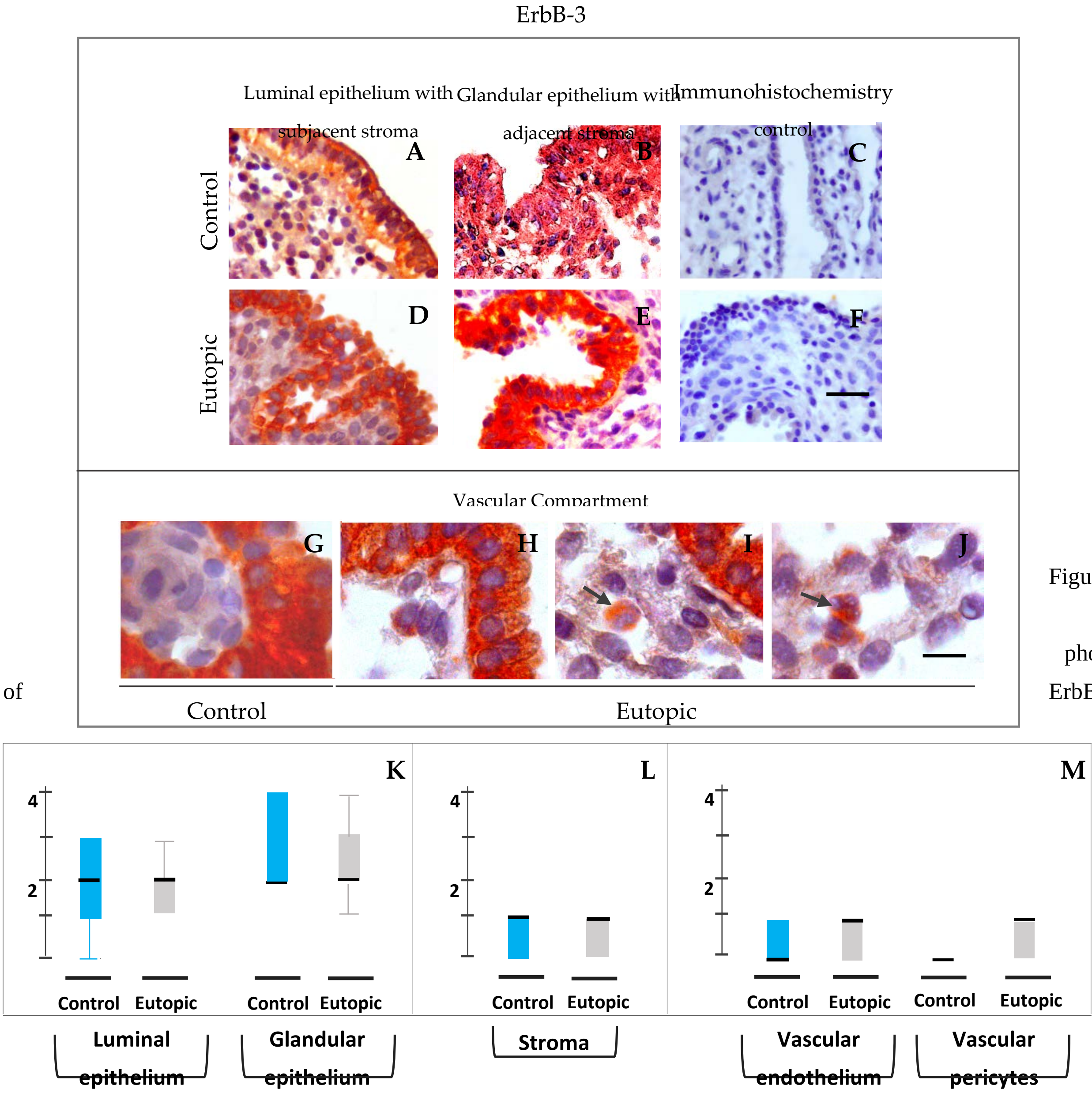

Figure 4. Representative photomicrographs of ErbB-3 (A-J) expressions in luminal epithelium along with subjacent stroma (A, D), glandular epithelium along with adjacent stroma (B, E) and in cells of the vascular compartment (G-J) in endometrial samples collected from endometriosis-free control women of group 1 (A-C, G) and stage IV ovarian endometriosis patients of group 2 (D-F, H-J). Semi-quantitative assessments of immunopositivity scores are shown in form of box plots of 10-90 percentile distribution of scores along with median values (K-M). Despite apparent higher expression in vascular compartment of eutopic endometrium from ovarian endometriosis group, it was statistically not significant. Intravascular monocytes in eutopic endometrium showed moderate to high immunoprecipitation (shown as *arrows*). This feature was generally absent in control endometrium obtained from disease-free control patients. Marked cytoplasmic and nuclear distribution besides membranous localization is notable for ErbB-3 immunoexpression in eutopic endometrium of stage IV ovarian endometriosis infertile patients. Controls for immunohistochemistry staining were done by omitting the primary antibody (C) or the secondary antibody (F). Bar: 20 µm (G-J), 75 mm (A, B, D, E), 100 µm (C, F).

with primary infertility as compared to that of obtained from control endometrial samples from endometriosis-free women with primary infertility (Fig. 3G-I).

ErbB-3*:* Figure 4 displays the representative photomicrographs of ErbB-3 immunostaining in different compartments in MSE obtained from both groups and the scores in the respective compartments. Figure 4A-J shows representative photomicrography of immunopositivity detected for ErbB-3 protein in epithelial, stromal and vascular compartments of control and eutopic endometria of women with primary infertility and without or with severe ovarian endometriosis. In control endometrium, marked ErbB-3 immunostaining was detected in the basal and apical regions of epithelial cells (Fig. 4A, B) along with minimal ErbB-3 immunopositive staining in vascular endothelial cells and pericytes (Fig. 4G). A similar profile of immunopositivity for ErbB-3 was detected in eutopic endometria in epithelial and stromal compartments (Fig. 4D, E). Although a marginally higher ($P< 0.06$) expression of ErbB-3 was seen in the vascular cells of eutopic endometrium from severe ovarian endometriosis, it was statistically not different from control values (Fig. 4M).

ErbB-4: Figure 5A-F documents the representative photomicrography of ErbB-4 immunopositivity in the epithelial and the stromal compartments of endometriosis-free control endometrium (Fig. 4A, B) and eutopic endometrium from ovarian endometriosis patients (Fig. 5D, E). Semi-quantitative scores of immunopositivity detected for ErbB-4 protein in endometrial samples (Fig. 5G-I) obtained from control women (group 1) and patients with stage IV ovarian endometriosis (group 2) revealed only a marginally ($P< 0.05$**)** higher trend of ErbB-4 immunopositivity in the glandular epithelium and stromal components of eutopic endometrium as compared to control endometrium (Fig. 5G, H).

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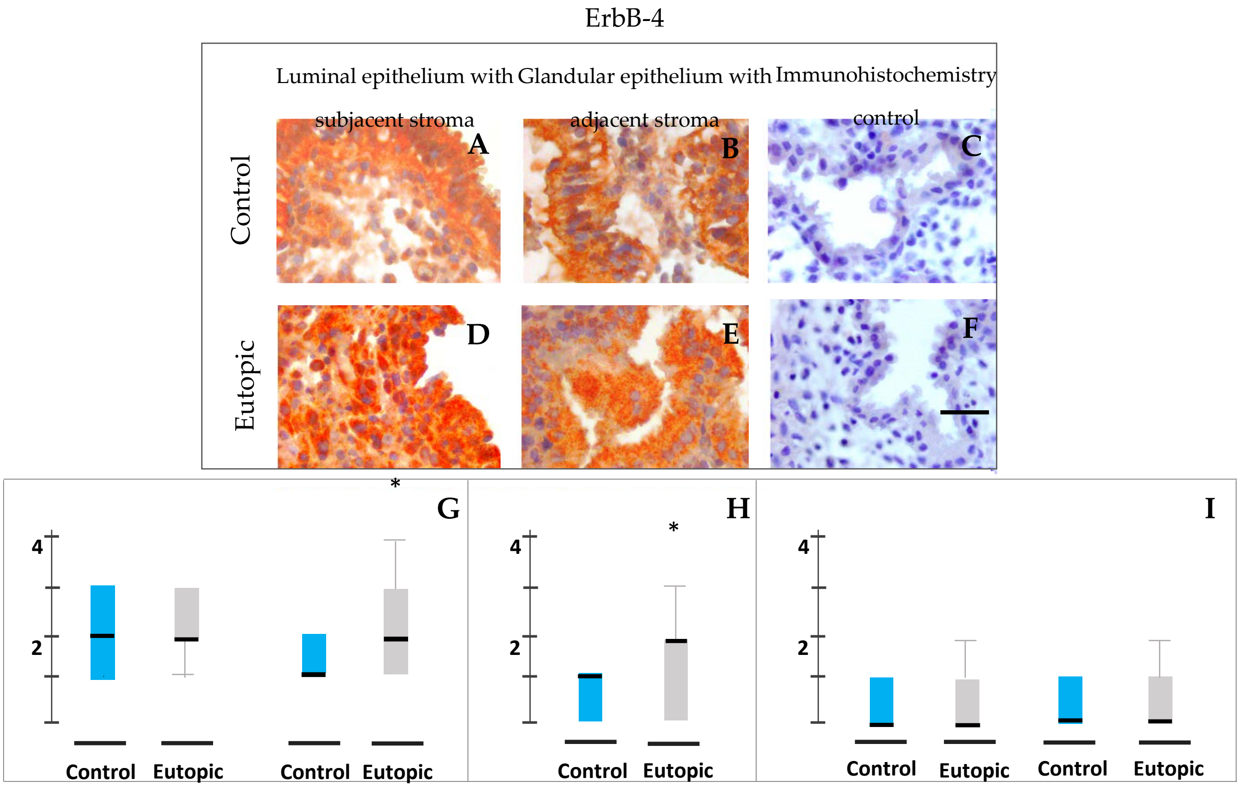

Figure 5. Representative photomicrographs of ErbB-4 (A-F) expressions in luminal epithelium along with subjacent stroma (A, D), and glandular epithelium (B, E) in endometrial samples collected from endometriosis-free control women of group 1 (A-C) and stage IV ovarian endometriosis patients of group 2 (D-F) and their semi-quantitative assessments shown in form of box plots of 10-90 percentile distribution of scores along with median values (G-I). High levels of cytoplasmic distribution besides membranous localization of ErbB-4 are seen, more predominantly in eutopic endometrial epithelium (D, E). Marginally higher trend of ErbB-4 immunopositivity is seen in the glandular epithelium and stromal components of eutopic endometrium as compared to control endometrium (G, H). Controls for immunohistochemistry staining were done by omitting the primary antibody (C) or the secondary antibody (F). *P< 0.05. Bar: 75 μm (A, B, D, E), 100 μm (C, F).

Discussion

This is the first report of differential expressions of ErbB family of tyrosine kinase receptors in mid-secretory phase endometrium (MSE) obtained from endometriosis-free women diagnosed with primary infertility versus patients with primary infertility and severe ovarian endometriosis. We observed an overexpression of ErbB-1 (EGFR/HER1) in the epithelial, stromal and vascular compartments along with marginally higher ErbB-3 expressions in the vascular compartment and ErbB-4 expression in the glandular epithelium and stroma in endometrium *functionalis* during the ‘window of implantation’ of women with

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severe (stage IV) ovarian endometriosis compared with control endometrium. The results obtained in the present study appear clinically useful as it revealed the specific issue of association between endometrial expression of ErbBs during implantation stage and infertility associated with severe ovarian endometriosis per the rASRM guidelines.

ErbB-1 (EGFR) suggestively plays an integral role in establishing the cellular framework necessary for a successful pregnancy [41]. In a normal ovulatory cycle, ErbB-1 controls proliferative events, while ErbB-2, ErbB-3 and ErbB-4 influence the secretory maturation of endometrium [19,28,42-44]. There are previous reports indicating that the relative expressions of ErbBs family may markedly vary in eutopic endometrium with severe endometriosis from control endometrium obtained from endometriosis-free infertile patients [20-22]. It is generally known that ErbB-1 (EGFR) plays important roles in cell proliferation and that secretory phase endometrium from women with endometriosis display a proliferative molecular profile with an enrichment of genes involved in cell cycle regulation [45-47]. Additionally, it has been reported that ErbB-1/EGFR signaling may result in aberrant cAMP-induced *in vitro* decidualization of stromal cells in women with endometriosis *via* cooperation between EGFR and protein kinase A signaling in the regulation of PI3K/AKT/ mTOR) [48-50]. EGFR signaling pathways leading to altered *in vitro* responses to steroid hormones by endometrial stromal cells of endometriosis differ than that in normal endometrial cells [51]. Interestingly, in a mouse model of endometriosis, ErbB-1/EGFR mediated ERK1 and activator protein 1 signaling for the transcriptional activation of MMP-7 in epithelial cells was observed, and the treatment with an EGFR inhibitor led to the regression of endometriotic lesions along with decreased MMP-7 activities [52]. Taken together, it appears that overexpression of ErbBs proteins, especially ErbB-1 in epithelial, stromal and vascular compartments in the implantation stage endometrium obtained from patients with severe

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endometriosis may cause endometrial hostility and failure of embryo implantation due to hyper-proliferative status in eutopic endometrium during severe ovarian endometriosis [8-13].

Additionally, the observation in the present study that both ErbB-1 and ErbB-3 were over-expressed in vascular compartment of eutopic endometrium of women with severe stage endometriosis appears a matter of interest. Peripheral blood monocytes express ErbB-1, ErbB-2 and ErbB-3 on their cell surfaces [53,54]. An inhibitory effect of ErbB-3 on the proinflammatory activation of $CD14^{low}CD16^{+}$ monocytes that show marked adherence to endothelial cells was earlier reported [54]. ErbB-1 signaling is known to regulate macrophage function *via* EGFR signaling activated NF-κB and MAPK1/3 pathways to induce cytokine production and macrophage activation [55]. Thus, over-expressed ErbBs may cause observed 'hyperinflammatory bias' during the implantation window in eutopic endometrium with severe ovarian endometriosis [13].

It is notable in this connection that co-expression of EGF and EGFR in the secretory phase of normal menstrual cycle coupled with co-expressions of VEGF, FGF and their respective receptors coincides with the timing of the development of sub-epithelial capillary plexus [43,56]. Thus, higher expression of ErbB-1 in the endothelium and pericytes may result in angiogenic phenotypes in eutopic endometrium during severe ovarian endometriosis [57-59]. Furthermore, we have noted an apparent similarity of ErbB-1 expression between a WHO grade IV EGFR amplified glioblastoma sample and eutopic endometrium obtained from patients with severe endometriosis (Fig. 6). Such expression pattern of ErbB-1 induces higher proliferative capacity,

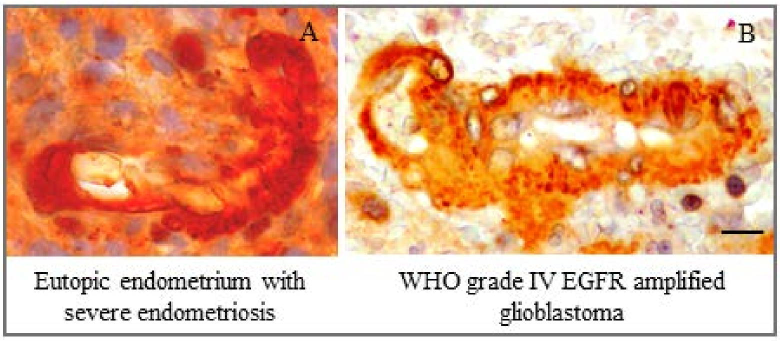

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Figure 6. Marked similarity of ErbB-1 expression in vascular compartment between (A) eutopic endometrium obtained on cycle day 24 from a woman with severe ovarian endometriosis-associated infertility and (B) a WHO grade IV EGFR amplified glioblastoma sample. Bar: 10 µm.

increased vessel density, cellular atypias, high mitotic activity, and distinctive infiltrative phenotype in both types of tissues, and these changes may bring forth their oncogenic potential [57,60-65]. The observed cytoplasmic and nuclear distribution of ErbBs, especially for ErbB-1 and ErbB-3, besides their membranous localization in the eutopic endometrium during endometriosis may trigger pathogenic potential of eutopic endometrium [66-70]. Collectively, it appears from the results of the present study that there was an overexpression of ErbB-1 in endometrial epithelium, stromal and vascular cells during implantation phase, which might explain how endometrial preparation for embryo implantation could be ruffled due to anomalous proliferative, inflammatory and angiogenic activities in the target tissues in severe ovarian endometriosis resulting in associated infertility'.

There were a few limitations in the present study. Firstly, we could recruit only limited number of subjects due to stringent application of WERF EPHect guidelines [25,26], and that of histological criteria for identifying mid-secretory phase having features of window of implantation as previously defined [33,34]. Additional ultrasound investigation of follicular rupture, which would provide solid support to the data of histological dating in individual patients, could not be done in the outpatients set up of the present study. Despite these limitations, we believe that the results of the present study were indeed useful due to stringent administration of EPHect model and endometrial dating model for tissue selection. Secondly, there was no design to undertake any functional studies towards understanding the specific roles ErbB-1 in epithelial, stromal and vascular cell types during severe stage ovarian endometriosis. Further investigations to interrogate the roles of ErbB-1 on these cell types and that of ErbB-1 and ErbB-3 in monocytic cells in eutopic

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endometrium in the disease state are necessary to address these limitations. In the present study, the issue of endometrioma-related reduction in ovarian reserve (ERROR) was not explored [71,72]. Thus, the question whether the overexpression of ErbB-1 in endometrium of infertile patients with endometriosis could be a consequence of altered endocrine milieu, particularly estrogen action, which is known to influence the regulation of ErbBs could not be addressed [73-75]. This could identify the association, if at all, between these two factors, namely estrogen receptors and ErbB-1 in endometrium of infertile patients during endometriosis. In this connection, it is notable that Miturski et al. failed to obtain any correlation between ErbB-1 and estrogen receptor expressions in endometrial carcinomas [76]. However, a higher level of tissue estrogen in eutopic endometrium of ovarian endometriosis compared to non-endometriosis group of infertile patients in secretory phase of menstrual cycle was observed in our previous study [4]. Furthermore, we have also proposed an association between estrogen and progesterone receptor subtypes in eutopic endometrium of infertile women with ovarian endometriosis based on our reported data that may lead to increased cell proliferation, cell migration, decidual incompetence, and inflammatory responses leading to failure of embryo implantation [4,10,77]. We now report of an added factor in this scenario, that is increased cellular (membrane and cytoplasmic) expressions of ErbB-1 in glandular, stromal and inflammatory cells of eutopic endometrium. Further study to link stage IV ovarian endometriosis with expression of the ErbB family of proteins and associated molecular pathways, as well as, to unravel the functional association between stage IV ovarian endometriosis and ErbB family expression in endometriosis-associated infertility will strengthen our understanding and yield improved mode of treatment and management of this disease. Lastly, parallel investigations on an additional control group of normal women with proven fertility donating endometrial samples during mid-secretory phase receptivity would yield higher order of knowledge; however, it was not possible in the outpatient hospital set up of the present study. Future studies using alternative experimental models, e.g., primary cell culture and cell lines may help in filling up some of the hiatus in this knowledge domain.

In conclusion, a preferential and accentuated expression of ErbB-1 in all compartments of endometrium *functionalis* during the critical 'window of implantation' in women with severe ovarian endometriosis and infertility appears novel and intriguing. This knowledge can be of help in strategizing methods for treatment of patients with endometriosis and infertility, as well as, preempting the oncogenic potential of endometriosis.

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