ENDOMETRIOSIS PERITONEAL FLUID FACTORS INVOLVED IN
THE ALTERATION OF DECIDUALIZATION PROCESS

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ABSTRACT
Endometriosis is accompanied by local and systemic proinflammatory dysregulation, which motivated us to screen for specific
marker molecules of the endometriotic peritoneal fluid. Endometriosis affects up to 10 % of the women of reproductive age and
is frequently associated with infertility. In the present study we analyzed the influence of endometriotic peritoneal fluid on the
decidualization process, by measuring the Prolactin secretion. Since we found that endometriotic peritoneal fluid inhibits human
endometrial stromal cells decidualization, we applied two-dimensional polyacrylamide gel electrophoresis in order to compare
the proteomic profiles of endometriotic vs. non-endometriotic peritoneal fluids and to identify the most significant protein
spots associated with the endometriosis condition. We used 2D DIGE densitometry analysis followed by bioinformatics gene
ontology enrichment of the putative proteins corresponding to the identified protein gel spots. Statistically significant pathway
identification analysis was performed to pinpoint the most likely key pathways that the putative proteins would be involved in.
The obtained results indicated the Toll-like receptor pathway, NFκB cascade pathway, and Cell surface receptor linked signal
transduction pathways, as most significantly contributing to the pathogenesis of endometriosis.


Keywords: endometriosis, decidualization, two-dimensional electrophoresis, factor analysis, gene ontology networks
enrichment analysis

Abbreviations: 8-bromo-cAMP: 8-bromo-cyclic adenosine monophosphate; 2D-PAGE: two-dimensional gel
electrophoresis; ePF: endometriosis peritoneal fluid; FBS–DMEM: fetal bovine serum–Dulbecco’s modified Eagle’s medium;
GO: gene ontology; HECSs: human endometrial stromal cells; ID: spot match; MW: molecular weight; nPF: non-endometriosis peritoneal fluid; PF: peritoneal fluid; pl: isoelectric point; PRL: prolactin; SDS-PAGE: sodium dodecyl
sulphate polyacrylamide gel electrophoresis

Introduction
Endometriosis is a common, benign and estrogen-dependent gynecological disorder, which is chronic and is associated
with pelvic pain and infertility. It is defined as the presence of endometrial glands and stroma outside the uterus, mainly on
the pelvic peritoneum, on the ovaries, and in the rectovaginal septum (4). The condition affects up to 10 % of the women of
reproductive age (11). Although endometriosis and normal eutopic endometrium might be similar in histology, many
differences in gene and protein level expression, steroid pathway, cytokine signaling, have been found (17). So far, the
precise etiology of endometriosis remains unknown, with the most widely accepted pathogenesis theory being the Sampson’s
one (15, 16). According to this theory, the disorder might originate from retrograde menstruation of viable endometrial
cells through the fallopian tubes into the peritoneal cavity where they implant on the peritoneal surface or pelvic organs.

Evidence from numerous investigations indicates that endometriosis develops as a result of specific molecular
characteristics of the peritoneal environment and in particular, of the peritoneal fluid (PF) (5, 8, 9, 14). Since peritoneal
fluid reaches the site of human oocyte fertilization, it could contribute to the outcome of this process (2, 7). Hence, changes
in the characteristics of the peritoneal fluid might affect the natural conception.

The mammalian decidua is a highly specialized structure and develops after trophoblast invasion triggered differentiation
of the endometrial stromal cells, having the major function to guarantee the best conditions for embryo implantation and
placentation (10).

In the present study we investigated whether endometriosis peritoneal fluid might violate the decidualization of normal
endometrial stromal cells in an in vitro model, and sought target factors which play a role in it.

Materials and Methods
All biological materials (PFs, tissues) were collected after receipt of written informed consent from the patient, according
to the requirements of the Ethics committees of the Second Municipal Hospital for Obst/Gyn “Sheinovo” PLS, Sofia,
Bulgaria, and that of Ob/Gyn Hospital “Dr. Shterev”, Sofia, Bulgaria.
Peritoneal fluid collection
Peritoneal fluid samples were collected from women with endometriosis and control subjects who underwent surgical laparoscopy for infertility or tubal sterilization. Briefly, all visible PFs were aspirated from both cul-de-sac and vesico-uterine fold at the beginning of laparoscopy and the samples were mixed before centrifugation at 600×g for 10 min; the supernatant was collected and stored at -80 °C until analyzed.

Human endometrial stromal cells (HESCs) – isolation procedure, cell culture conditions and in vitro decidualization
Five sample sets of human endometrial stromal cells were collected from healthy women aged 27–32 who had undergone hysterectomy. Each sample was kept in sterile phosphate-buffered saline (PBS, pH = 7.4) supplemented with 0.1 % bovine serum albumin (BSA, Austria). In the laboratory, HESCs were isolated from endometriotic tissues by enzymatic digestion (0.5 % collagenase) and the dispersed cells were nylon mesh filtered, washed, and seeded in culture dishes. The non-adherent cells were washed out after 24 h of incubation at 37°C, as the culture medium was refreshed every other day.

Decidualization of HESCs. The cells on third passage were plated in 24-well plates (Orange Scientific, Belgium) at a concentration of 5×10⁴ cells/cm². When the cells reached 80 % confluence, induced decidualization was initiated with 17-β-estradiol (10⁻⁸ mol/l), medroxyprogesterone (10⁻⁶ mol/l) and 8-bromo-cyclic adenosine monophosphate (8-bromo-cAMP) (0.5 mmol/L) in 2 % charcoal-stripped Fetal Bovine Serum–Dulbecco’s modified Eagle’s medium (2 % FBS–DMEM); and it was maintained for 10 days, with culture medium being collected every 48 h. Successful decidualization was validated by monitoring the prolactin (PRL) secretion and endometrial cell morphology. PRL concentrations were measured with the Access Prolactin kit and Access Immunoassay System (Beckman Coulter, CA). The test sensitivity is 0.25 ng/ml and the precision has a coefficient of variation (CV) of <10 %. The decidualized cells in the test group were detected in our endometrial stromal cell cultures, with demonstrated multi-layering, and increased vacuolization, equilibrated and subjected to 2nd dimension separation by SDS-PAGE, where the proteins were transferred to a 12 % polyacrylamide gel in a standard dual cooled vertical unit (SE 600 Ruby; GE Healthcare), without the use of stacking gel. Analytical gels were silver-stained with ProteoSilverPlus Stain Kit (Sigma) and digitalized at 300 dpi resolution (Image Scanner III, GE Healthcare) with the specialized gel-scanning and processing software LabScan (GE Healthcare).

Analysis of protein expression
Spots differential analysis of the protein gel was done with ImageMaster 2D Platinum 7.0 (GE, Healthcare) software. Gels were compared for differences in protein expression based on the normalized percent volume of the spots, (%Vo) = (spot volume/volume of all spots in the gel)×100. The MW and pI of each spot in the clusters were approximated based on MW marker and isoelectric focusing strip pl values.

Bioinformatics statistical analysis
The variations in protein expression among the gels were evaluated through gel matching. The analytical methods used included scatter plots, descriptive statistics, histograms, and factor analysis.

Database search
The putative protein targets corresponding to the individual protein spots (defined by their pI, MW, and %Vo) on the gel were identified with the ExPASy tool Tagident. The putative protein IDs were filtered and the Gene Ontology (GO) network was enriched, using the Functional Annotation Tool of the DAVID system.

Results and Discussion
Using medroxyprogesterone (10⁻⁶ mol/L) in combination with 17-β-estradiol (10⁻⁸ mol/L) and 8-bromo-cAMP (0.5 mmol/L), we were able to induce decidualization of human endometrial stromal cells in vitro. Ten days after the initiation of the decidualization process, morphological changes were easily detected in our endometrial stromal cell cultures, with demonstrated multi-layering, and increased vacuolization, mimicking the in vivo decidual transformation (Fig. 1).

The functional modulation of the decidualized stromal cells was characterized by their PRL secretion. To trace the influence of ePF on the decidualization process, ePF and control nPF were used in three different concentrations of hormone-supplemented media: 1 %, 5 % and 25 %. As shown in Fig. 2, there was a decrease in the secretion of prolactin (12.4 ± 0.0412) during treatment with ePF as compared to either treatment with nPF or any of the other two control groups – endometrial stromal cells control (treated only with 2 % DMEM), or decidual cells control (treated with steroid hormones and cAMP in 2 % DMEM).
Fig. 1. Morphological changes of HESCs during the decidualization process for 10 days. There were clear changes in the cell shape and volume (A–D), while there were no changes in the control group (E–H).

Fig. 2. Secreted prolactin (PRL) during the decidualization process and influence of different concentrations of PFs. Data are mean values ± standard error.

These results clearly demonstrate that the decidualization process in HESCs was inhibited in the presence of ePF. On the other hand, the level of secreted PRL was highest when ePF was added at 25% concentration. This fact could explain the presence of a large amount of growth factors, normally existing in the peritoneal environment.

These findings motivated us to seek factors in the ePF that could impede normal decidualization, resulting in infertility of patients with endometriosis. For that purpose, a 2D-PAGE approach was chosen to identify the specific proteins that could differentiate the 2D gels of PFs of endometriotic women from those of non-endometriotic ones. Therefore, the protein profiles of PFs from both groups of patients were compared (Fig. 3).

The mean number of PF protein spots per gel was approximately equal (357 ± 30). No protein spot was consistently (≥90% of the cases) present in PF of women with endometriosis and absent in PF of control subjects. Similarly, no protein spot was uniquely present in PF of control subjects. Totally, 74 protein spots were consistently presented in PF of women with and without endometriosis. A scatter plot for matched spots (protein spot match IDs) was drawn with ImageMaster Platinum to analyze for disparities in stain intensity of the spots on the two gels (groups). The linear dependence relationship between the spot values on the endometriosis gel vs. those on the reference non-endometriosis gel was assessed using a best fit-line through the data points, with a correlation coefficient of 0.58 (Fig. 4).

The population of endometriosis-specific sample spots was distinguished using factor analysis (FA) (Fig. 5) applied on the densitometry and morphological parameters of the detected spots. The factor projection plot displays the projection of each protein spot match ID (cross) and each gel (blue vector) on the two factorial axes, showing the relationships between endometriosis and non-endometriosis gel spot populations in terms of associations with specific spot patterns. The factors are ranked in order of importance, the first ones generally being the best for characterizing the gels and matches that behave similarly. The most significant matches are displayed on the projection plot; the further away a spot is from the origin, the more likely it is for this spot to be important in terms of characterizing the
The endometriosis-specific protein expression profile was determined by the matched spot IDs: 29, 36, 70, and 56; while the matched spot IDs determining non-endometriosis were: 7, 6, 66, and 65. The coordinates of each of the gels were projected on the axes and are listed in the factor projection table (Table 1), with the first axis generally correlating with the protein abundance, and the second axis related to the ratio between the mean spot values in the population of each gels. The protein spot matches at the top of the table are also those with the highest relative volumes (Table 1).

TABLE 1

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TABLE 2

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<tr>
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<tr>
<td>GOTERM FAT</td>
<td>I-kappa B kinase / NF-xB cascade</td>
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The GO functionally most enriched pathway was the Adherens junction (http://david.abcc.ncifcrf.gov), with the genes: EGFR, FGFR1, MAPKKK7, and PTPN6. From all enriched GO pathway databases, 3303 terms were searched, and only 36 terms passed the filter, with a similarity score (Kappa) between 0.3 and 1 (Table 2).

The GO terms “membrane receptor”, “Toll-like receptor pathway” and “NFκB pathway” were significantly enriched in the set union of the FA-derived putative protein targets. The most putative pathway found by probabilistic GO enrichment of the union of all eventual proteins that could be regarded as potential eventual targets, using the protein spots information, was already pointed out as very important in endometriosis pathogenesis: Ponce et al. (13) demonstrated that the Nuclear factor kB pathway and interleukin-6 are affected in eutopic endometrium of women with endometriosis. It is not surprising that IL-6 was found to be affected in eutopic endometrium after NFkB pathway activation, especially since we found the Toll-like receptor pathway to be enriched, and it is known to be an upstream NFkB activator.

Conclusions
We demonstrated that the decidualization process of HESCs inhibits their PRL secretion in the presence of ePF, suggesting that ePF could possibly impede the normal decidualization, eventually resulting in infertility in endometriosis patients. An integrated 2D-PAGE semi-quantitative densitometry, factor analysis of protein spot matches, and Gene Ontology network enrichment in corresponding putative protein targets, allowed for retrieval of series of differentially regulated pathways in ePF that might have an impact on the development and establishment of endometriosis or might be a subject of differential regulation due to endometriosis itself. In addition, the aberrant integrin expression (1), involved in adhesion interactions may trigger the cell surface receptor-linked signal transduction pathways, which we found to be enriched, for subsequent cell proliferation, differentiation and invasion that are necessary for disease progression.

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