# Endometrial expression and in vitro modulation of the iron transporter divalent metal transporter-1: implications for endometriosis

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**Objective:** To evaluate divalent metal transporter-1 (DMT1) expression in healthy women's and endometriosis patients' endometrium and to analyze DMT1 and ferritin light chain (Fn-L) expression modulation by iron overload and IL-1$\beta$ in endometrial stromal cells (ESCs).
**Design:** Observational and experimental study.
**Setting:** University hospital research laboratory.
**Patient(s):** Thirty-one healthy women and 24 endometriosis patients.
**Intervention(s):** Menstrual, proliferative, and secretory endometrial biopsies. Isolated ESCs from seven endometrial biopsies incubated with IL-1$\beta$ or $FeSO_4$ overload for 24 hours.
**Main Outcome Measure(s):** Divalent metal transporter-1 endometrial protein expression assessed by immunohistochemistry and Western blot. Divalent metal transporter-1 and Fn-L proteins expression in stimulated ESCs evaluated by Western blot.
**Result(s):** Divalent metal transporter-1 is expressed throughout the menstrual cycle in human endometrium. Four endometrial DMT1 variants were identified accordingly to their molecular weight: DMT-80, -65, -55, and -50. Endometrial expression of DMT-80 and -55 is higher in endometriosis patients than in healthy women. In ESCs, iron overload induces an overexpression of DMT-80, DMT-50, and Fn-L, whereas IL-1$\beta$ increases DMT-80 and -50 expressions and decreases Fn-L expression.
**Conclusion(s):** Divalent metal transporter-1 overexpression in endometriosis patients' endometrium can increase iron influx to endometrial cells, inducing oxidative stress–mediated proinflammatory signaling. In turn, endometriosis-related conditions, as iron overload and inflammation (IL-1$\beta$), enhance endometriosis patients endometrial DMT1 expression, creating a vicious circle on DMT-1–modulated pathways. (Fertil Steril® 2016;106:393–401. ©2016 by American Society for Reproductive Medicine.)
**Key Words:** DMT1, endometriosis, interleukin-1$\beta$, iron

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The peritoneal environment of endometriosis patients has been described as highly oxidative and proinflammatory, possibly owing to the abundant menstrual reflux of cell debris and blood observed in these patients, as well as pelvic endometriotic lesions bleeding themselves (1–4). Erythrocytes lysis releases pro-oxidant factors like hemoglobin and its by-products, heme and iron, being the source of iron overload in the peritoneal cavity of women with endometriosis (5–8). Iron overload has been postulated as an important factor in the etiology and pathophysiology of peritoneal endometriosis, affecting macrophage scavenging function, inducing oxidative stress, nuclear factor-kappa B (NF-$\kappa$B) activation and its consequent inflammatory response, and promotion of ectopic endometrial cells survival, adhesion, invasion, proliferation, and angiogenesis (9–14).

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Iron homeostasis is influenced by intracellular iron level and external stimuli like hypoxia and inflammation (15). In blood, non–hemoglobin-captive iron is normally in oxidized state (+3), bound to transferrin. Iron must be reduced to the +2 state before transport into the cytoplasm by the transmembrane protein divalent metal transporter-1 (DMT1) (16). Alternative splicing of exons at the 5′ and 3′ ends of DMT1 messenger RNA (mRNA) produces four distinct DMT1 mRNAs, depending on which exon (1A or 1B) is transcribed and whether a conserved iron-responsive element (IRE) is present or not (+IRE or −IRE, respectively) (17). The four proteins have the same iron transport capacity and differ mainly in their intracellular location (18, 19). Excessive intracellular iron is stored in ferritin, a multimeric protein conformed by 24 subunits of either heavy or light chains, ferritin light chain (Fn-L) being the structure where nucleation sites for iron crystal formation are present (20). In a cell-specific response, inflammation is a stimulus for the regulation of DMT1 and Fn-L protein synthesis (21–29).

Inherent imbalances in iron endometrial cells traffic, caused by altered DMT1 basal and/or modulated expression, as well as variations in the iron storage protein Fn-L, may facilitate endometriosis development (12). Thus, the objective of this study was to evaluate DMT1 expression in eutopic endometrium from healthy women and endometriosis patients, and to assess the in vitro effects of two endometriosis-related conditions, namely iron overload and a proinflammatory environment (interleukin-1$\beta$, IL-1$\beta$), over DMT1 and Fn-L expression in healthy endometrial stromal cells.

## MATERIALS AND METHODS

### Endometrial Biopsies

The ethical review boards of the University of Chile, San Borja-Arriarán Clinical Hospital and Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) approved the use of human tissue for this study. Informed consent was obtained from all patients donating endometrial tissue for research purposes. Endometrial biopsies were obtained by using a Pipelle de Cornier biopsy curette (Laboratoire CCD) from 24 healthy women and 24 endometriosis patients, not receiving hormonal therapy, with regular menstrual cycles and undergoing laparoscopic surgery for tubal sterilization (excluding endometriosis) or endometriosis treatments. Twelve patients had endometriosis grade 1–2, and 12 patients had endometriosis grade 3–4, according to the revised American Society for Reproductive Medicine classification (30). Menstrual endometrial samples were obtained on day 1–4 of the menstrual cycle from eight healthy women and eight endometriosis patients. Proliferative endometrium was taken from eight healthy women and eight endometriosis patients on day 5–13 of the menstrual cycle. Secretory endometrium was obtained from eight healthy women and eight endometriosis patients on day 18–30 of the menstrual cycle. Samples were immediately placed on ice, transported, and cleaned of blood excess and clots with sterile phosphate-buffered saline (PBS) (Gibco). A portion of each sample was fixed in 4% buffered formaldehyde and embedded in paraffin for dating and immunohistochemical staining. Another endometrial portion was stored at −80°C until use for protein extraction techniques. The endometrial phase of the biopsies was histologically confirmed according to standard criteria (31). Additionally, for cell culture experiments, endometrial biopsies were obtained during the proliferative phase (days 5–13) of the menstrual cycle from a total of seven healthy women undergoing laparoscopic tubal sterilization and absent endometriotic lesions. In healthy women, adenomyosis was clinically excluded because they had no adenomyosis symptoms, their transvaginal ultrasound scans were normal with no ultrasonographic signs of adenomyosis, and results of laparoscopic inspection and instrumental palpation of the uterus were normal.

### Immunohistochemistry

Fixed tissue samples were embedded in paraffin, and 5-$\mu$m-thick sections were cut. Slides were dewaxed in xylol and rehydrated in ethanol solutions. Antigen exposure was achieved submerging slides in sodium citrate 0.01 M, pH 6.0 (Sigma), and heating at 120°C with pressure in a Retriever 2100 device (Prestige Medical). Endogenous peroxidase activity was quenched by incubation with 3% (vol/vol) $H_2O_2$ in methanol (Merck) for 30 minutes. The tissue sections were then blocked with 1% (wt/vol) bovine serum albumin (BSA) (Sigma) in PBS at room temperature for 2 hours, and then incubated overnight at 4°C in a humidified chamber with pan-DMT1 rabbit polyclonal primary antibody, which recognizes a common region to four DMT1 isoforms (provided by M. T. Núñez) (32), diluted 1:300. Normal rabbit IgG was used for negative control instead of the primary antibody. Next, slides were washed with PBS, and detection of the primary antibody was achieved by using the Histostain-SP Broad Spectrum kit (Invitrogen). Counter-stain was done using hematoxylin (Sigma) according to standard procedures. Images were visualized on an Olympus BX-51 microscope and captured with Image-Pro Plus v.6.2 software (Media Cybernetics). Divalent metal transporter-1 immunohistochemistry was carried out in two different slides from each endometrial sample.

### Cell Culture and Stimulation

Endometrial samples for in vitro experiments were transported to the laboratory in PBS on ice. Isolation of endometrial stromal cells (ESCs) was performed as described previously (14). For stimulation experiments, cells from second passage were plated in 100-mm Petri dishes (Orange Scientific) until they reach 80% confluence. Then the medium was discarded and replaced for 18–24 hours by FBS-free medium supplemented with 0.1% (wt/vol) BSA. The next day, plates were washed with PBS, FBS-free medium was replaced, and ESCs were stimulated with $FeSO_4 \cdot 7H_2O$ (Sigma) at 50 $\mu$M (17.5 $\mu$L from fresh 20 mM stock solution in 7 mL FBS-free medium) or IL-1$\beta$ (Sigma) at 45 pg/mL (6 $\mu$L from fresh 3 nM stock solution in 7 mL FBS-free medium) for 24 hours. Control condition corresponds to ESCs