Invasion of human deep nodular endometriotic lesions is associated with collective cell migration and nerve development

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Objective: To study the mechanisms of invasion and innervation of deep endometriosis in women.
Design: Morphologic and immunohistochemical analysis of human endometriotic lesions.
Setting: Academic research unit.
Patient(s): Seventeen biopsy samples of deep endometriotic lesions were collected from patients undergoing surgery for deep endometriosis.
Intervention(s): The endometriotic samples were divided into two parts: the front (the most invasive area of lesions, approaching rectal infiltration) and center (the area close to the posterior part of the cervix).
Main outcome(s): To elucidate: gland morphology, proliferation, and expression of adhesion molecules (β-catenin, E-cadherin, and N-cadherin) to determine the possible role of collective cell migration (CCM) in the invasion process; and nerve growth factor (NGF) and nerve fiber density (NFD) values to shed further light on the mechanism of innervation.
Results: Glands from the front showed significantly reduced thickness, but significantly higher proliferation. β-Catenin expression was similar between the lesion center and front. E-cadherin levels were significantly lower and N-cadherin levels significantly higher in glands located at the front of the lesions. Expression of matrix metalloproteinase-9 was significantly higher in glands and stromal cells located at the invasion front. NFD and NGF expression were also significantly higher at the lesion front.
Conclusion: Although some data in the literature point to features of epithelial to mesenchymal transition in human deep nodular endometriosis, our study suggests that gland invasion in these lesions is dominated by CCM. Innervation of deep nodular endometriotic lesions may be a consequence of nerve recruitment from surrounding organs. (Fertil Steril® 2018;110:1318–27. ©2018 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Deep nodular endometriosis, collective cell migration, innervation, invasion

Endometriosis is one of the most commonly encountered gynecological diseases, affecting 7%–10% of women of reproductive age (1, 2). Three different forms of pelvic endometriosis have been identified: namely, peritoneal, ovarian, and deep endometriotic nodules of the rectovaginal septum, each probably with its own pathogenesis (3, 4). This idea was first proposed by Donnez and Nisolle more than 20 years ago and is still supported by abundant clinical and experimental data, such as the different patterns of homebox A genes observed between peritoneal and rectovaginal lesions (5). Moreover, very recently, Anglesio et al identified some specific driver mutations in deep nodular endometriotic lesions (6). These mutations are intrinsic to this form of the disease, reinforcing the idea that deep nodular endometriosis is a specific disorder with a pathogenesis different from that of peritoneal and ovarian endometriosis.

Deep endometriosis has been reported to be the most innervated and

Received March 30, 2018; revised July 26, 2018; accepted August 3, 2018.
J.G.-S. has nothing to disclose. M.-M.D. has nothing to disclose. J.-L.S. has nothing to disclose. J.D. has nothing to disclose. O.D. has nothing to disclose.
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Fertility and Sterility® Vol. 110, No. 7, December 2018 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2018.08.016

painful type of endometriosis (7, 8), indicating possible involvement of nerve development in pain experienced by patients. Most of these lesions originate from the posterior part of the cervix (types II and III) and secondarily infiltrate the anterior wall of the rectum and other organs (type III) (9, 10). Some authors have highlighted the significance of smooth muscle hyperplasia and fibrosis in this process (4), whereas others stress the importance of the fibrotic component, in which endometrial stroma and epithelium can be identified (11).

Epithelial to mesenchymal transition (EMT) is often considered to be one of the crucial events in cancer invasion. During this process, stationary polarized epithelial cells lose their cell–cell adhesions and convert to highly motile mesenchymal cells, boosting their migratory and invasive capacities. Expression of EMT-inducing transcription factors, such as ZEB-1 or Snail-1, was reported to be significantly higher in deep endometriotic lesions than in eutopic endometrium (12), which may suggest possible involvement of this biological process in the pathogenesis of deep endometriosis (12).

On the other hand, Matsuzaki et al proposed that deep nodular endometriosis may be regulated by the opposite process, namely mesenchymal to epithelial transition (MET) (13). Indeed, these authors found significantly higher expression of the adhesion marker E-cadherin and other epithelial markers in deep endometriotic lesions compared to eutopic endometrium (13). This may indicate that a more epithelial cell–like phenotype could facilitate invasion in this disease (13, 14).

In 2013, we developed an in vivo baboon (*Papio anubis*) model, recreating deep nodular endometriosis and allowing us to investigate its pathogenic evolution and invasion process (15–17). Results obtained from this animal study pointed to the involvement of collective cell migration (CCM) and nerve development in the invasiveness of deep endometriosis (15–17). CCM occurs when groups of cells that retain their cell–cell junctions move together to invade other tissues. This process is stratified by a combination of different parameters, including multicellular morphology, degree of cell–cell adhesion, cell proliferation, and degradation of the surrounding extracellular matrix (ECM) (18, 19). In this model, lesion innervation increased significantly over time (15, 20), and the presence of nerve fibers was associated with pain experienced by patients. However, the origin of these nerve fibers has not yet been elucidated, and the mechanism of lesion innervation remains unknown.

We hypothesize that CCM could well be implicated in the invasion process of deep nodular endometriotic lesions observed in women. Moreover, innervation of these lesions may be a consequence of nerve recruitment from surrounding tissues.

To investigate our hypothesis, we used human biopsy samples of deep nodular endometriosis to study the following: [1] gland morphology, proliferation, and expression of adhesion molecules (β-catenin, E-cadherin, and N-cadherin) to determine the possible role of CCM; and [2] nerve growth factor (NGF) and nerve fiber density (NFD) values to shed further light on the mechanism of innervation.

MATERIALS AND METHODS

Patients and Histology

Tissue samples (mean volume: 7.85 $\pm$ 5.62 cm^3) were collected from 17 patients (one biopsy per patient) not undergoing any hormonal treatment (mean age: 33.22 $\pm$ 6.37 years) and presenting with deep nodular type III endometriosis. In all cases, bowel infiltration was confirmed by barium enema. Surgery was performed by the shaving technique, as reported by Donnez and Roman (21). Use of human tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain (2016/03MAI/202). In invasive lesions in the baboon model, a morphological distinction was made between the center (glands and stroma remaining in the grafting site) and front (glands and/or stroma invading adjacent organs) of lesions (15–17). To extrapolate this concept of center and front to the present study, all human lesions were divided into two sites, depending on anatomical location: namely, front (the most invasive area of lesions approaching rectal infiltration) or center (the area situated close to the posterior part of the cervix) (Supplemental Figure 1). All biopsy samples were fixed for 24 hours in 4% formaldehyde, embedded in paraffin, and serially sectioned (5 μm) for histological confirmation of endometriosis (presence of glands and stroma) and immunohistochemical analysis. Every fifth slide was stained with hematoxylin and eosin to identify the section with the largest surface area of endometriotic glands and stroma. Consecutive serial sections were used for this study.

Morphological Analysis

Gland thickness was calculated by measuring the distance between the external (basal) and internal (apical) sites of glands. To avoid confounding artifacts due to sectioning, we excluded glands without a lumen and areas with multilayer cells.

Immunohistochemical Analysis

Proliferation was assessed by Ki67 immunohistochemical staining and counting of Ki67-positive nuclei per gland (proliferation index) in histological sections. Briefly, deparaffinization was achieved with X-Solv paraffin-clearing solvent (Yvsolab, Turnhout, Belgium), followed by permeabilization with Tris-buffered saline solution/Triton 0.1%. Endogenous peroxidase activity quenching, heat epitope retrieval, and blocking of nonspecific staining (30 minutes) were then carried out. Thereafter, Ki67 monoclonal antibody (1:50; Dako M7240, Glostrup, Denmark) was incubated overnight at 4°C, before incubation with secondary antibody (1:2; B17 Dako K4001, EnVision anti-mouse, Carpinteria, CA). Labeling was done with use of the DAB substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Human bowel mucosa was used as a positive control for Ki67, showing strong nuclear staining in proliferative goblet cells of the intestinal epithelium. Negative controls were processed by omitting the primary antibody, and no staining was detected.

Concerning the presence of cell–cell attachment in glands, immunostaining of the cell–cell adhesion markers: β-catenin (1:5000; Beckton Dickinson BDB610154, San José, CA), E-cadherin (1:100; Dako M3612, Glostrup, Denmark), and N-cadherin (1:400; Abcam ab98952, Cambridge, United Kingdom) also involved deparaffinization, incubation with blocking solution (30 minutes), antibody incubation, and labeling with the DAB substrate kit (Vector Laboratories, Burlingame, CA), as above. Levels of E-cadherin, β-catenin, and N-cadherin were determined by a score obtained using a freehand selection tool to measure diaminobenzidine (DAB)–positive pixels and surface area of glands, excluding the lumen. Eutopic human endometrium served as a positive control for E-cadherin and β-catenin, showing strong expression in the membrane of epithelial cells, but no staining in the absence of the primary antibody. Human liver was used as a positive control for N-cadherin, with strong staining in the membrane of hepatocytes. Negative controls were conducted without the primary antibody, and no labeling was found.

Immunostaining for matrix metalloproteinase-9 (MMP-9) was performed as reported above, using mouse anti-human MMP-9 (1:300 Calbiochem/Merck Millipore IM37, Japan) as the primary antibody. Human inflammatory tonsil tissue was used as a positive control, showing strong labeling in immune cells (lymphocytes). Negative controls were processed by omitting the primary antibody, and no staining was detected. Expression of this protease was evaluated by determining the ratio between stained (DAB-labeled) and total area in glands and stroma in each sample, using the Tissue IA system (Leica Biosystems, Dublin, Ireland).

To evaluate the presence of nerves and the innervation process, protein gene product (PGP) 9.5 was selected because it is one of the broadest markers of nerve fibers and neurons at all levels of the central and peripheral nervous system, and NGF was evaluated because it is one of the most widely studied neurogenic growth factors. For both markers, immunohistochemistry was performed as previously reported (15), using rabbit polyclonal antihuman PGP9.5 (1:1,000; Dako K4003, Glostrup, Denmark) and antihuman NGF (1:3,000; sc-548, Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies, before incubation with the secondary antibody (EnVision, Dako Glostrup, Denmark). Human intestinal tissue was used as a positive control for PGP9.5 and NGF immunostaining, but no expression was detected in the absence of the primary antibody. NFD was calculated by counting the number of PGP9.5-positive fibers per area of tissue (mm^2), whereas NGF was evaluated by determining the ratio between stained and total area in glands and stroma of each sample. Hence, the area of NGF coverage was ascertained by establishing the ratio between DAB-labeled area and total area in each sample using the Tissue IA system (Leica Biosystems, Dublin, Ireland).

Digital Acquisition

All sections were scanned using the Leica SCN400 scanner (Leica Biosystems, Wetzlar, Germany), and image acquisition was achieved with the Tissue IA system (Leica Biosystems, Dublin, Ireland). ImageJ software (National Institutes of Health, Bethesda, MD) was applied for morphological analysis and immunohistochemical quantification of E-cadherin, β-catenin, and N-cadherin adhesion markers, using the color deconvolution plugin to isolate the DAB channel.

Statistical Analysis

Results are expressed as means ± SE. The Mann–Whitney test was used for statistical analysis (GraphPad Prism 5 software; GraphPad, San Diego, CA) to compare two data groups (center vs. front of lesions), and Spearman's correlation test for comparison of groups and gland analysis. A value of $P<.05$ was considered to be statistically significant.

RESULTS

Morphological Analysis

Glands located at the front (most invasive area) of lesions were found to be significantly thinner (7.1623 ± 0.1094 μm; $P<.0001$) than those located in the center (area situated close to the posterior part of the cervix) (14.92 ± 0.1682) (Fig. 1A).

Proliferation

Ki67 staining was found to be significantly more extensive at the invasion front ($1.48 \pm 0.12 \times 10^{-3}$ nuclei/surface area; $P<.0001$) than in the center ($0.65 \pm 0.16 \times 10^{-4}$ nuclei/surface area) (Fig. 1B). We also noted a significant correlation ($P<.0001$; $r = -0.7815$) between proliferation and gland thickness when all glands were taken into account (Supplemental Fig. 2A), demonstrating that the thinner the glands, the higher the proliferation. This correlation was also significant when considering the center ($P<.05$; $r = -0.1800$) and the front ($P<.0001$; $r = -0.4540$) separately (Supplemental Fig. 2A).

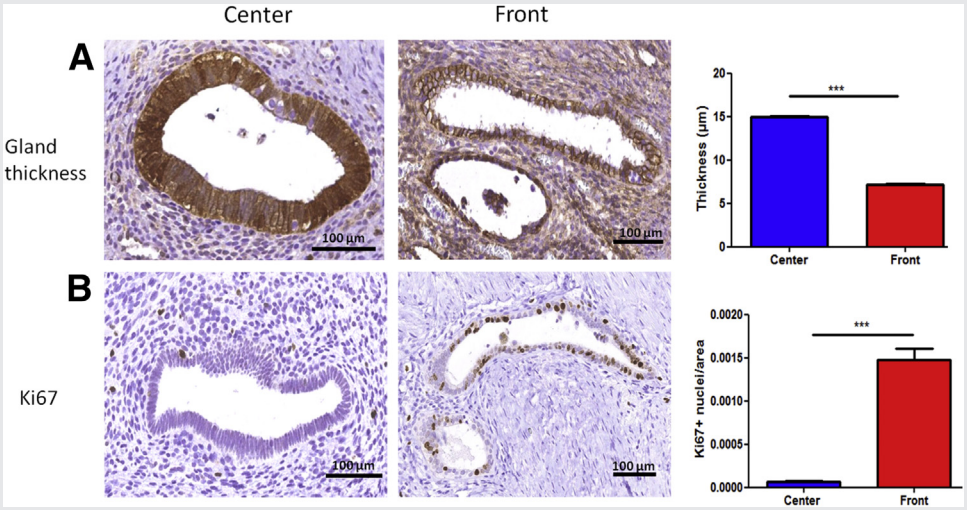
Adhesion Molecules

Concerning the tight junction protein β-catenin, no significant difference ($P=.1743$) was detected between glands at the front of lesions (99.93 ± 3.035 pixels/surface area) and those in the center (92.85 ± 4.255 pixels/surface area) (Fig. 2A). Immunostaining levels of the strong epithelial cell–cell adhesion marker E-cadherin were found to be significantly lower ($P<.0001$) at the invasion front (66.51 ± 2.247 pixels/surface area) than in the center (136.64 ± 4.822 pixels/surface area) (Fig. 2B).

A significant correlation ($P<.0001$; $r = 0.7442$) was observed between E-cadherin values and gland thickness when all glands were taken into account, indicating that the lower the levels of E-cadherin, the thinner the glands (Supplemental Fig. 2B). This correlation was also encountered at the front ($P<.0001$; $r = 0.5413$) and center ($P=.0216$; r=0.3344) of lesions when analyzed separately (Supplemental Fig. 2B).

Immunoreactivity for the mesenchymal adhesion marker N-cadherin showed significantly higher ($P=.0002$) levels of this cell–cell adhesion marker in glands at the front of lesions (105.05 ± 2.630 pixels/surface area) than those in the center (65.51 ± 1.837 pixels/surface area) (Fig. 2C). Expression of N-

FIGURE 1



Morphology and proliferation analysis of deep nodular endometriotic glands. **(A)** Gland thickness measurement. Glands located at the front (n = 88) of the lesion are significantly thinner than those in the center (n = 126). ***P<.0001. **(B)** Ki67 immunohistochemical analysis of glands. Glands in the front (n = 88) show significantly more Ki67 staining than glands at the center (n = 126). ***P<.0001.

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cadherin plays an important role in initiating pro-migratory signaling and providing strong yet flexible cell cohesion essential for migration of cell groups (22, 23).

We also observed a significant correlation (P<.0001; r = −0.8374) between levels of N-cadherin and gland thickness when all glands were taken into account. This demonstrates that the thinner the glands, the higher the levels of N-cadherin (Supplemental Fig. 2C). Glands in the center (P<.0001; r = −0.5609) and at the front (P<.0001; r = −0.7436) of lesions displayed the same correlation when analyzed separately (Supplemental Fig. 2C).

Matrix Metalloproteinase-9

Endometriotic glands at the invasion front expressed significantly higher levels (44.91 ± 2.91%; P<.0001) of MMP-9 than those located in the center of lesions (11.85 ± 2.31%) (Fig. 3A1 and B1). Immunoreactivity for MMP-9 was also significantly higher (P<.0001) in stromal cells at the front of lesions (8.17% ± 1.37%) compared to those in the center (2.26% ± 0.53%) (Fig. 3A2 and B2).

PGP9.5 and NGF Detection

NGF labeling of glandular epithelial cells was found to be significantly (P<.0001) stronger at the front (40.68% ± 4.37%) than in the center (19.57% ± 6.93%) of lesions (Fig. 4A). NGF staining of stromal cells was also significantly (P=.0023) more extensive at the invasion front (34.39% ± 6.44%) than in the center (8.48% ± 3.13%) (Fig. 4B).

NFD was evaluated by counting the number of PGP9.5-positive nerve fibers in the stromal area of lesions. A significantly (P<.0001) higher density was encountered at the front (71.53 ± 17.30 fibers/mm^2) compared to the center (8.66 ± 2.417 fibers/mm^2) (Fig. 4C).

DISCUSSION

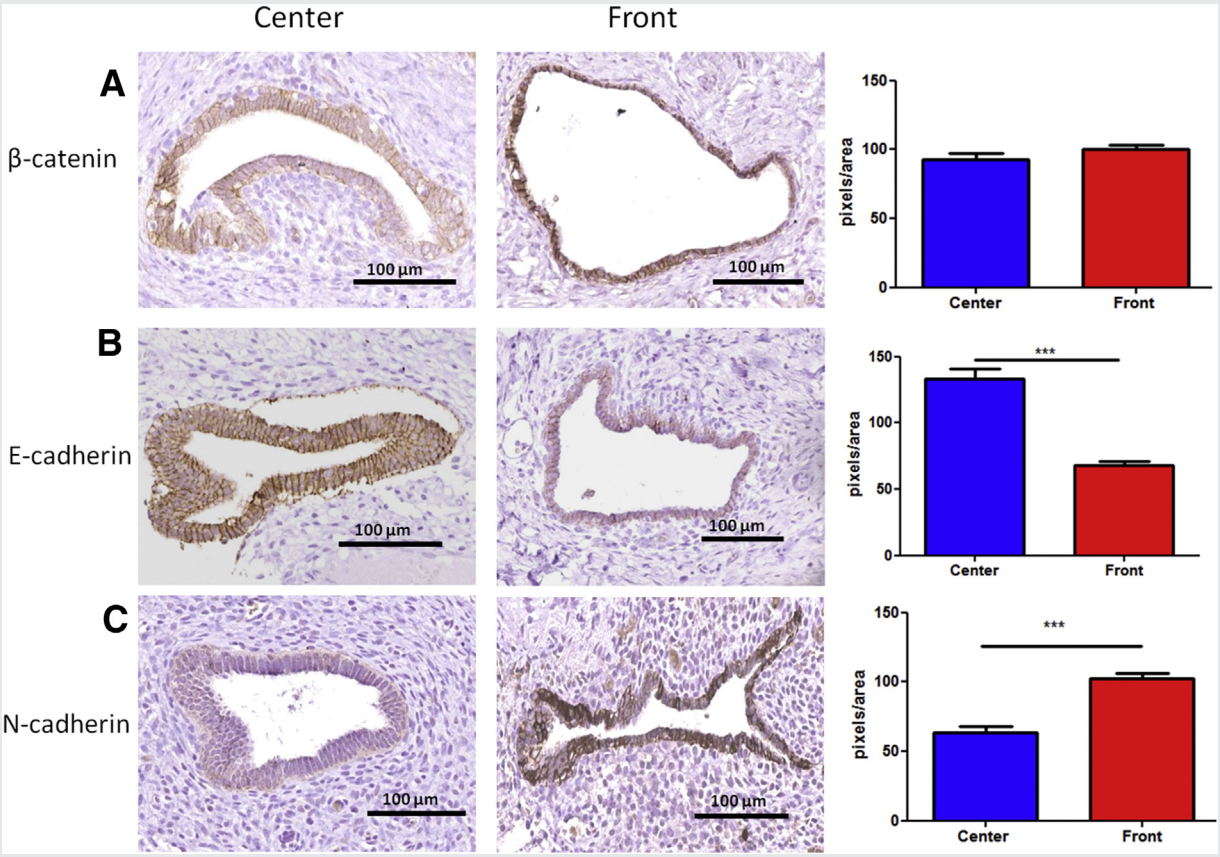
The present study focuses specifically on type III nodules (according to our classification) (10, 24), which are the most aggressive form of deep endometriosis, originating from the posterior side of the cervix and extending cranially to the anterior wall of the rectum (10, 24). Previous studies in our baboon model suggest that the process supporting and promoting invasion of these lesions is dominated by CCM (18). In human nodules, this invasion phenomenon has never before been studied and remains unelucidated, which is why we set out to investigate the possible mechanism(s) of invasion leading to infiltration of the rectal wall in deep endometriotic lesions observed in women.

Lesion Morphology

Our results demonstrate that endometriotic glands located at the front of lesions are significantly thinner, with significantly higher levels of proliferation, than those in the center. This indicates that acquisition of an invasive phenotype may enhance the migratory capacity of endometriotic glands, as seen in the baboon model, facilitating invasion of the recto-vaginal septum and eventually infiltration of the anterior wall of the rectum.

The morphology of these lesions resembles that observed in some malignant diseases, in which cells from the native primary tumor remain differentiated, while a secondary group is able to colonize the neighborhood, leading to cancer metastasis (25–27). These features are highly suggestive of CCM. In this migration process, cells that remain at the leading edge and establish contact with the ECM generate traction. These cells sense the microenvironment and guide the motion of so-called follower cells, defined as a group of closely connected cells accompanying this movement (19, 28). Reduced

FIGURE 2



β-Catenin, E-cadherin, and N-cadherin immunostaining of glands. **(A)** β-Catenin immunostaining. No significant difference are found in β-catenin values between center (n = 69) and front (n = 73). P=.174. **(B)** E-cadherin immunostaining. Glands in the front (n = 44) of lesions show significantly lower E-cadherin staining than those in the center (n = 40). ***P<.0001. **(C)** N-cadherin immunostaining. Glands in the front (n = 52) expressed higher levels of N-cadherin compared to glands in the center (n = 69). ***P=.0002.

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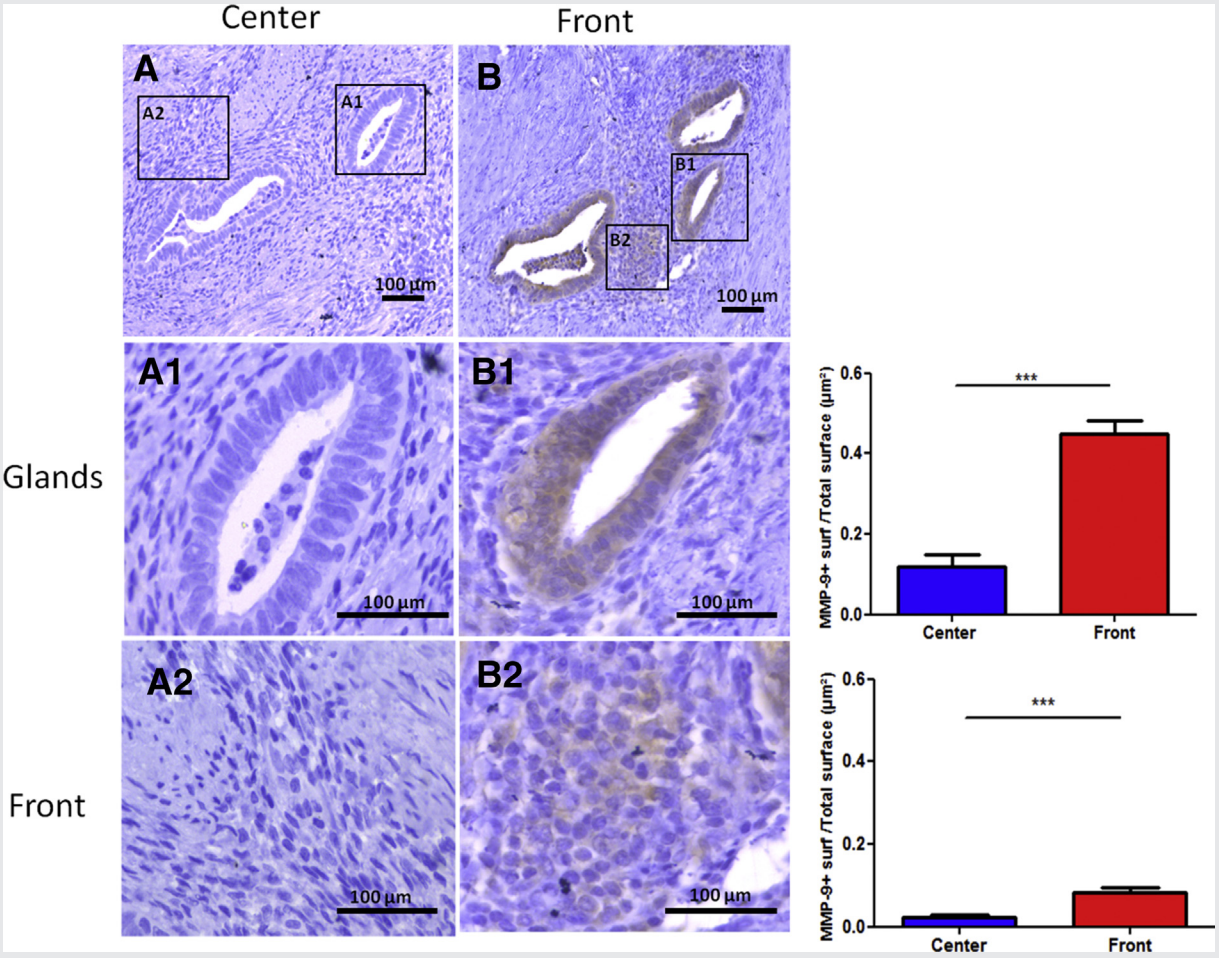
gland thickness at the front of lesions may be a consequence of these traction forces generated during the invasion process, and could have an impact on contact made with surrounding tissues, reducing inertia and thereby encouraging movement of glands. CCM has also been described in some development events, such as branching of the mammary ducts. In this case, "pushing" forces and migration across the ECM may result from extensive growth driven by proliferation of cells within and adjacent to the leading edge (28, 29). Transference of these forces would therefore allow leader cells at the edge to drag a relatively passive mass of follower cells (19). In our study, this could explain the higher proliferation rates observed at the invasion front, suggesting the involvement of CCM in the invasion process of human endometriotic glands.

Adhesion Molecules

Type I cadherins (such as E-cadherin) have a large extracellular domain that binds to the same molecule on adjacent cells, and a cytoplasmic domain that binds intracellular β-catenin, thus creating a protein complex that connects two neighboring cells (30). Maintenance of this cell–cell contact throughout the invasion process is one of the main features of CCM (31). In our baboon model, invasive lesions exhibited lower levels of β-catenin and E-cadherin than noninvasive lesions 6 months after endometriosis induction (17). However, after 1 year, we found homogeneous expression of both adhesion markers, with no difference between the center and front of lesions (15). In the present study in humans, we did not detect any difference in β-catenin staining between endometriotic glands located in the center of lesions and those at the invasion front. We did, nevertheless, observe lower levels of E-cadherin and higher levels of N-cadherin expression in deep endometriotic glands located at the front of lesions compared to the center.

Human lesions are assumed to be older than lesions induced in baboons. Indeed, time from disease onset to its diagnosis has been estimated to be 8–11 years (32), so our results suggest time-dependent expression of adhesion markers. Although expression of β-catenin appears to stabilize after 1 year, decreased levels of E-cadherin at the front of human lesions may indicate cyclic expression of this cell–cell adhesion molecule during the invasive process of the disease.

FIGURE 3



Matrix metalloproteinase-9 (MMP-9) immunostaining of deep endometriotic lesions. (**A1** and **B1**) MMP-9 immunostaining of glands. Glands in the front (n = 47) displayed significantly higher MMP-9 expression than those in the center (n = 65). ***$P<.0001$. (**A2** and **B2**) Stromal areas in the front (n = 17) are significantly more stained for MMP-9 than those in the center (n = 28).***$P<.0001$.

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Moreover, functional loss of expression of the epithelial cell–cell adhesion marker E-cadherin in glandular cells sheds light on the idea of acquisition of an invasive phenotype, whereby cells in glands may partially lose their epithelial conformation to promote migration and invasiveness.

Some authors have suggested involvement of a process known as EMT. During this process, stationary polarized epithelial cells lose their cell–cell adhesions and convert to highly motile mesenchymal cells, allowing single-cell migration and boosting their migratory and invasive capacities. Functional loss of expression of the epithelial cell–cell adhesion marker E-cadherin and concomitant increased expression of the mesenchymal marker N-cadherin are considered a hallmark of this process (33, 34). However, in our study, there was only a decline (not a complete loss) of E-cadherin levels along the invasion front, and cells remained attached to one another in a gland conformation without losing contact, which is the main feature of CCM. This process occurs when groups of cells that retain their cell–cell junctions move together to invade surrounding organs (18, 30).

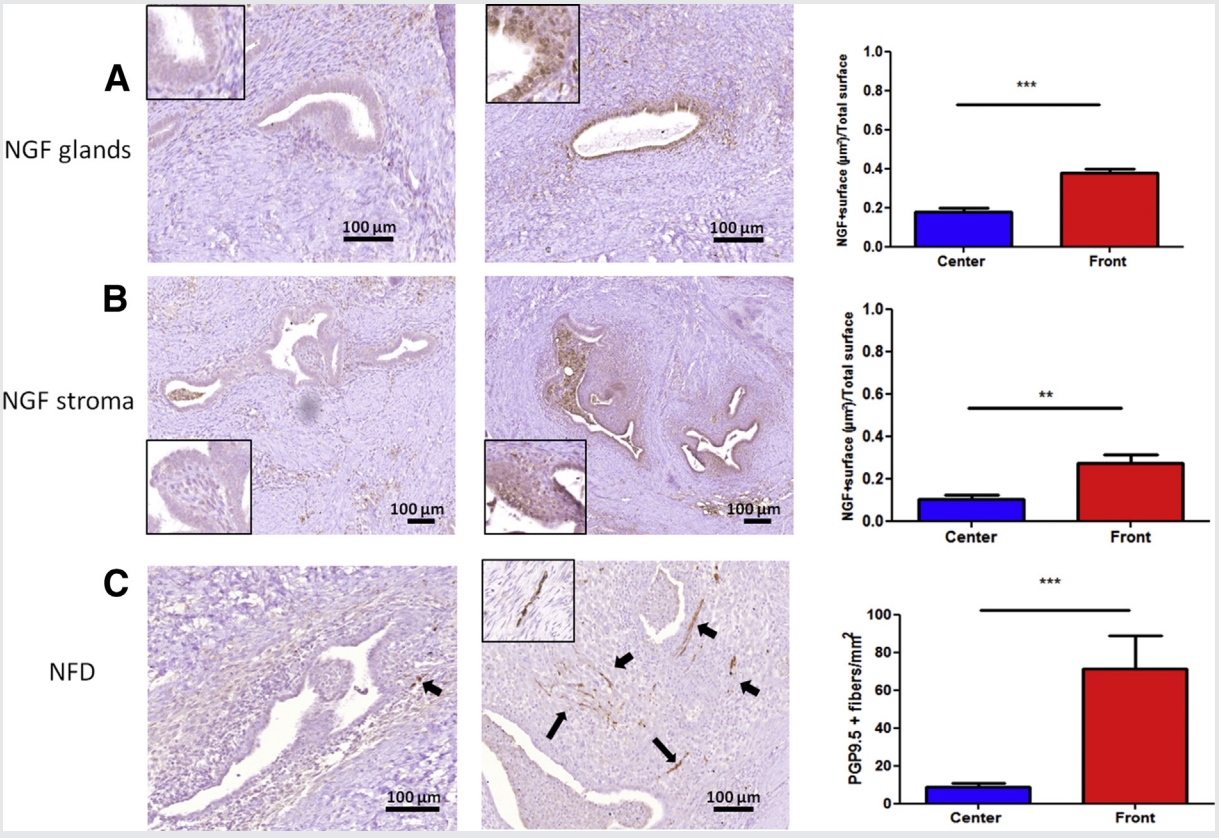
Moreover, reduced E-cadherin levels during EMT are generally associated with release of β-catenin from its interaction membrane proteins (35), resulting in translocation to the nucleus to exert its transcription factor–like function together with other transcription factors, such as LEF1/TCF. This process dictates gene expression associated with cell migration (36) and appears to be crucial for maintenance of EMT (37). We did not observe nuclear localization of β-catenin in any of the analyzed glands, and so cannot propose this mechanism to account for the reported gland migration.

Taken collectively, these results suggest that CCM is the main mechanism responsible for cell migration in human deep nodular endometriotic lesions.

ECM and MMPs

The process of cell migration also requires degradation of the ECM. In this context, MMPs are able to degrade multiple ECM components and were previously reported to be involved in endometriosis development (38). N-cadherin may promote

FIGURE 4



Nerve growth factor and protein gene product (PGP9.5 immunostaining of deep endometriotic lesions. **(A)** NGF immunostaining of glands. Glands in the front (n = 63) show significantly higher NGF staining than those in the center (n = 78) of lesions. ***$P<.0001$. **(B)** NGF immunostaining of stromal cells. Levels of NGF were significantly higher in the stromal areas of the front (n = 27) compared to stromal areas in the center (n = 39).**$P=.0015$. **(C)** PGP9.5 analysis (black arrows: positive nerve staining) revealed significantly higher nerve fiber density in the stromal area surrounding endometriotic glands of the front (n = 14) compared with the center (n = 29) ***$P<.0001$.

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motility and invasion by expression of MMP-9, an ECM-degrading enzyme with collagenolytic activity (33, 39). Transfection of N-cadherin into a weakly metastatic cell line, MCF-7, was found to be sufficient to increase cell migration and invasion across a collagen-rich matrix (40). Transfected cells coexpressed N-cadherin and MMP-9 (40). Indeed, this adhesion molecule activates the MAPK-ERK pathway, which results in increased transcription of MMP-9, hence dramatically increasing cellular invasiveness (33). In the present study, we encountered significantly stronger MMP-9 expression in glands and stromal cells at the invasion front than in the center of deep nodular endometriotic lesions, making it the first study to demonstrate increased expression of this protease throughout the invasion process of deep lesions. This supports the role of MMP-9 in the development and progression of deep nodular endometriosis, facilitating migration across the ECM by means of its proteolytic activity.

NGF and Innervation

Anaf et al were the first to report a close histological relationship between nerves and deep nodular endometriotic lesions (41). However, the mechanism of innervation in these lesions is still poorly understood. In the baboon model, we hypothesized that NGF, a neurotrophic factor involved in survival of neurons and axonal growth, is implicated in recruitment of nerves from neighboring tissue by means of axonal guidance (15). Anaf et al found that Trk-A receptor, the receptor for NGF, is expressed in nerves lying within or in the vicinity of deep endometriotic lesions (42), suggesting that NGF could act as a positive chemotaxin for neurons, thereby facilitating their contact with target tissues through interaction with its receptors (42).

In the present study, we found significantly stronger NGF expression in glands and stromal cells located at the invasion front of lesions (approaching bowel infiltration) than in the center. Innervation was also significantly greater at the front. These findings may further endorse the role of NGF as a nerve recruiter from surrounding organs, and could explain why deep nodular endometriosis is much more painful and innervated than other forms of the disease (8).

Clinical Implications

Although medical treatment of rectovaginal endometriosis can alleviate the symptoms, in the majority of cases, surgical

excision is the most effective approach to relieve pain. A number of surgical techniques can be used to remove type III deep nodular endometriosis. There is an ongoing debate between supporters of the most radical approach, which involves bowel resection, and the most conservative approach, which entails use of the shaving technique. The 17 deep endometriotic nodules analyzed in our study were removed by the shaving technique, so residual endometriotic foci may have been left behind on the bowel. Here we suggest, for the first time, involvement of CCM in the mechanism of invasion of human deep nodular endometriosis. During this process, a connection between the center and invasion front is essential to promote gland migration, so when the center is removed, residual glands may be unable to evolve. This could explain the low rates of recurrence observed after conservative surgery, further advocating a more conservative approach as first-line therapy in case of type III nodules, as proposed by Donnez and Roman (21).

In conclusion, although some data in the literature point to features of EMT in human deep nodular endometriosis, our study clearly suggests that gland invasion in these lesions is dominated by CCM. Moreover, our results shed further light on the mechanism of innervation of deep nodular endometriosis, suggesting that NGF could act as a positive chemotaxin for neurons, facilitating their contact with target tissues. We therefore propose that innervation of these lesions may be a consequence of nerve recruitment from surrounding organs.

Acknowledgments: The authors thank Dr. Mathieu Luyckx for help with sample collection and clinical input, Olivier van Kerk for technical support, and Guillaume E. Courtoy and Catherine Vander Linden for scientific contribution. They also thank Deborah Godefroidt for administrative support and Mira Hryniuk, B.A., for revising the English language of the manuscript. This study was supported by grants from the Fonds National de la Recherche Scientifique de Belgique (FNRS) grant 5/4/150/5 awarded to MMD and donations from the Ferrero family.

REFERENCES

1. Wheeler JM. Epidemiology of endometriosis-associated infertility. J Reprod Med 1989;34:41–6.
2. Giudice LC, Kao LC. Endometriosis. Lancet 2004;364:1789–99.
3. Nisolle M, Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. Fertil Steril 1997;68:585–96.
4. Donnez J, Nisolle M, Casanas-Roux F, Brion P, Da Costa Ferreira N. Stereometric evaluation of peritoneal endometriosis and endometriotic nodules of the rectovaginal septum. Hum Reprod 1996;11:224–8.
5. Van Langendonckt A, Luyckx M, Gonzalez MD, Defrere S, Donnez J, Squifflet J. Differential expression of genes from the homeobox A cluster in deep endometriotic nodules and peritoneal lesions. Fertil Steril 2010; 94:1995–2000.
6. Anglesio MS, Papadopoulos N, Ayhan A, Nazeran TM, Noe M, Horlings HM, et al. Cancer-associated mutations in endometriosis without cancer. N Engl J Med 2017;376:1835–48.
7. Anaf V, Chapron C, El Nakadi I, De Moor V, Simonart T, Noel JC. Pain, mast cells, and nerves in peritoneal, ovarian, and deep infiltrating endometriosis. Fertil Steril 2006;86:1336–43.
8. Donnez O, Soares M, Defrère S, van Kerk O, van Langendonckt A, Donnez J, et al. Nerve fibers are absent in disease-free and eutopic endometrium, but present in endometriotic (especially deep) lesions. J End Pelvic Pain Disord 2013;5:68–76.
9. Donnez J, Squifflet J. Complications, pregnancy and recurrence in a prospective series of 500 patients operated on by the shaving technique for deep rectovaginal endometriotic nodules. Hum Reprod 2010;25:1949–58.
10. Donnez J, Squifflet J. Laparoscopic excision of deep endometriosis. Obstet Gynecol Clin North Am 2004;31:567–80.
11. Vigano P, Candiani M, Monno A, Giacomini E, Vercellini P, Somigliana E. Time to redefine endometriosis including its pro-fibrotic nature. Hum Reprod 2018;33:347–52.
12. Furuya M, Masuda H, Hara K, Uchida H, Sato K, Sato S, et al. ZEB1 expression is a potential indicator of invasive endometriosis. Acta Obstet Gynecol Scand 2017;96:1128–35.
13. Matsuzaki S, Darcha C. Epithelial to mesenchymal transition-like and mesenchymal to epithelial transition-like processes might be involved in the pathogenesis of pelvic endometriosis. Hum Reprod 2012;27:712–21.
14. Matsuzaki S, Darcha C, Pouly JL, Canis M. Effects of matrix stiffness on epithelial to mesenchymal transition-like processes of endometrial epithelial cells: Implications for the pathogenesis of endometriosis. Sci Rep 2017;7: 44616.
15. Orellana R, Garcia-Solares J, Donnez J, van Kerk O, Dolmans MM, Donnez O. Important role of collective cell migration and nerve fiber density in the development of deep nodular endometriosis. Fertil Steril 2017;107:987–95.
16. Donnez O, Van Langendonckt A, Defrere S, Colette S, Van Kerk O, Dehoux JP, et al. Induction of endometriotic nodules in an experimental baboon model mimicking human deep nodular lesions. Fertil Steril 2013;99: 783–9.
17. Donnez O, Orellana R, Van Kerk O, Dehoux JP, Donnez J, Dolmans MM. Invasion process of induced deep nodular endometriosis in an experimental baboon model: similarities with collective cell migration? Fertil Steril 2015; 104:491–7.
18. Friedl P, Locker J, Sahai E, Segall JE. Classifying collective cancer cell invasion. Nat Cell Biol 2012;14:777–83.
19. Mayor R, Etienne-Manneville S. The front and rear of collective cell migration. Nat Rev Mol Cell Biol 2016;17:97–109.
20. Donnez O, Soares M, Defrere S, Dehoux JP, van Langendonckt A, Donnez J, et al. Nerve fiber density in deep nodular endometriotic lesions induced in a baboon experimental model. Fertil Steril 2013;100:1144–50.
21. Donnez O, Roman H. Choosing the right surgical technique for deep endometriosis: shaving, disc excision, or bowel resection? Fertil Steril 2017;108: 931–42.
22. Shih W, Yamada S. N-cadherin-mediated cell-cell adhesion promotes cell migration in a three-dimensional matrix. J Cell Sci 2012;125:3661–70.
23. Shih W, Yamada S. N-cadherin as a key regulator of collective cell migration in a 3D environment. Cell Adh Migr 2012;6:513–7.
24. Squifflet J, Feger C, Donnez J. Diagnosis and imaging of adenomyotic disease of the retroperitoneal space. Gynecol Obstet Invest 2002;54(Suppl 1):43–51.
25. Lv ZD, Kong B, Li JG, Qu HL, Wang XG, Cao WH, et al. Transforming growth factor-beta 1 enhances the invasiveness of breast cancer cells by inducing a Smad2-dependent epithelial-to-mesenchymal transition. Oncol Rep 2013; 29:219–25.
26. Cao C, Chen Y, Masood R, Sinha UK, Kobielak A. alpha-Catulin marks the invasion front of squamous cell carcinoma and is important for tumor cell metastasis. Mol Cancer Res 2012;10:892–903.
27. Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA, et al. Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc Natl Acad Sci U S A 2001; 98:10356–61.
28. Khalil AA, Friedl P. Determinants of leader cells in collective cell migration. Integr Biol 2010;2:568–74.
29. Andrew DJ, Ewald AJ. Morphogenesis of epithelial tubes: Insights into tube formation, elongation, and elaboration. Dev Biol 2010;341:34–55.
30. Poncelet C, Leblanc M, Walker-Combrouze F, Soriano D, Feldmann G, Madelenat P, et al. Expression of cadherins and CD44 isoforms in human

endometrium and peritoneal endometriosis. Acta Obstet Gynecol Scand 2002;81:195–203.
31. Goodwin M, Yap AS. Classical cadherin adhesion molecules: coordinating cell adhesion, signaling and the cytoskeleton. J Mol Histol 2004;35:839–44.
32. Sinaii N, Cleary SD, Ballweg ML, Nieman LK, Stratton P. High rates of autoimmune and endocrine disorders, fibromyalgia, chronic fatigue syndrome and atopic diseases among women with endometriosis: a survey analysis. Hum Reprod 2002;17:2715–24.
33. Hazan RB, Qiao R, Keren R, Badano I, Suyama K. Cadherin switch in tumor progression. Ann N Y Acad Sci 2004;1014:155–63.
34. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 2009;9:265–73.
35. Orsulic S, Huber O, Aberle H, Arnold S, Kemler R. E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. J Cell Sci 1999;112:1237–45.
36. Muller T, Bain G, Wang X, Papkoff J. Regulation of epithelial cell migration and tumor formation by beta-catenin signaling. Exp Cell Res 2002;280:119–33.
37. Li J, Zhou BP. Activation of beta-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters. BMC Cancer 2011;11:49.
38. Henriet P, Mon KS, Marbaix E. Are matrix metalloproteinases and their inhibitors reliable diagnosis biomarkers and attractive therapeutic targets in endometriosis? Metalloprotein Med 2016;3:81–92.
39. Suyama K, Shapiro I, Guttman M, Hazan RB. A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. Cancer Cell 2002;2:301–14.
40. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol 2000;148:779–90.
41. Anaf V, Simon P, El Nakadi I, Fayt I, Buxant F, Simonart T, et al. Relationship between endometriotic foci and nerves in rectovaginal endometriotic nodules. Hum Reprod 2000;15:1744–50.
42. Anaf V, Simon P, El Nakadi I, Fayt I, Simonart T, Buxant F, et al. Hyperalgesia, nerve infiltration and nerve growth factor expression in deep adenomyotic nodules, peritoneal and ovarian endometriosis. Hum Reprod 2002;17:1895–900.

La invasión de lesiones nodulares humanas de endometriosis profunda se asocia con la migración colectiva de células y desarrollo nervioso

Objetivo: Estudiar los mecanismos de invasión e inervación de la endometriosis profunda en mujeres.

Diseño: Análisis morfológico e inmunohistoquímico de lesiones humanas de endometriosis.

Ámbito: Unidad de Investigación Universitaria.

Paciente(s): Diecisiete muestras de biopsia de lesiones endometriósicas profundas fueron recogidas de pacientes que se sometieron a cirugía por endometriosis profunda.

Intervención(es): Las muestras endometriósicas se dividieron en dos partes: la frontal (área más invasiva de las lesiones, próximas a la infiltración rectal) y la central (área próxima a la parte posterior del cérvix).

Variable de estudio: Caracterizar: morfología glandular, proliferación y expresión de moléculas de adhesión (beta-catenina, E-cadherina, y N-cadherina) para determinar el posible rol de la migración colectiva de células (CCM) en el proceso de invasión; y los valores de factor de crecimiento nervioso (NGF) y densidad de fibras nerviosas (NFD) para clarificar los mecanismos de inervación.

Resultados: Las glándulas de la parte frontal tuvieron un grosor significativamente reducido, pero una proliferación significativamente mayor. La expresión de beta-catenina fue similar en las regiones frontales y centrales. Los niveles de E-caherina fueron significativamente más bajos y los de N-cadherina significativamente más altos en las glándulas de la parte frontal. La expresión de metaloproteinasa de matriz-9 fue significativamente mayor en glándulas y células estromales localizadas en la parte frontal. La expresión de NFD y NGF fue significativamente más alta también en la región frontal.

Conclusión: Aunque algunos datos de la literatura sugieren rasgos de transición epitelial a mesenquimal en nódulos de endometriosis profunda humana, nuestro estudio sugiere que la invasión glandular de dichas lesiones está principalmente dominada por CCM. La inervación de los nódulos profundos de endometriosis podría ser una consecuencia del reclutamiento nervioso desde los órganos circundantes.

Palabras clave: Endometriosis profunda nodular, migración colectiva de células, inervación, invasión.