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M2 macrophages enhance endometrial cell invasiveness by promoting collective cell migration in uterine adenomyosis



BIOGRAPHY

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KEY MESSAGE

M2 macrophages accumulate in endometrium and ectopic lesions from adenomyosis patients and enhance the invasion capacity of endometrial epithelial and stromal cells by promoting collective cell migration. These findings indicate that M2 macrophages play a key role in disease pathogenesis and could be targeted to develop novel therapeutic options.

ABSTRACT

Research question: Are M2 macrophages implicated in endometrial invasiveness in adenomyosis?

Design: Seventeen formalin-fixed paraffin-embedded uterine samples and 16 fresh endometrial biopsies were collected from women with or without adenomyosis. Double immunofluorescence was performed to determine the predominant macrophage population in adenomyosis between M1 and M2 phenotypes. The invasion capacity of endometrial cells was assessed by invasion assays and quantitative polymerase chain reaction for genes involved in cell motility and epithelial–mesenchymal transition (EMT). Specific mechanisms of invasion were investigated by immunohistochemistry for E-cadherin, N-cadherin and matrix metalloproteinase 9 (MMP9).

Results: Only M2 macrophages were found to accumulate in adenomyosis, in higher numbers in both eutopic endometrium ($P = 0.0109$) and lesions ($P = 0.0267$) than healthy tissue. Co-culture with M2 macrophages significantly boosted invasion capacity in endometrial epithelial ($P = 0.0002$; $P = 0.002$) and stromal cells ($P = 0.0469$; $P = 0.0047$) from both adenomyosis patients and healthy controls. No gene expression differences indicating EMT were noted, either between co-cultured and control cells, or between healthy and adenomyotic cells. E- and N-cadherin protein expression did not differ significantly between endometrium from adenomyosis subjects and healthy tissue but MMP9 expression was increased in eutopic stroma from adenomyosis patients ($P = 0.0492$). In adenomyosis, both E-cadherin ($P = 0.0379$) and N-cadherin ($P = 0.0196$) were more extensively expressed in basal glands than functional glands.

Conclusions: M2 macrophages accumulate in adenomyosis and enhance invasion capacity of adenomyotic and even healthy endometrial cells, implying that macrophage infiltration alone may be sufficient to promote the disease. This study failed to detect any changes pointing to EMT, suggesting an alternative mode of invasion. Strong E- and N-cadherin-positive intercellular junctions in basal (invasive) glands suggest the involvement of collective cell migration in the invasion process of endometrium.

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KEYWORDS

Adenomyosis
Collective cell migration
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Epithelial–mesenchymal transition
M2 macrophages

INTRODUCTION

Despite growing numbers of diagnoses, uterine adenomyosis remains a poorly understood disease, with no definitive medical treatment to date. It is now estimated that it affects close to 20% of gynaecological patients, potentially accounting for more than 40% of total hysterectomies performed (*Donnez et al., 2018*; *García-Solares et al., 2018b*; *Krentel and De Wilde, 2022*). Adenomyosis is a serious chronic condition, markedly impairing a woman's quality of life and putting strain on healthcare systems worldwide (*Donnez et al., 2021*). It is therefore vital to decipher the mechanisms underlying the pathogenesis and symptoms of the disease in order to explore novel, safer and more effective therapeutic options.

The most widely accepted hypothesis on the origin of adenomyosis suggests invasion of the myometrium by the endometrial basalis via a traumatized endometrial–myometrial junctional zone (*García-Solares et al., 2018b*; *Leyendecker et al., 2009*). However, the exact mechanism by which endometrial cells become invasive is still unknown. In the literature, especially in the field of cancer, two main mechanisms of cell invasion have been described, namely epithelial–mesenchymal transition (EMT) and collective cell migration (CCM) (*Dongre and Weinberg, 2019*; *Friedl and Gilmour, 2009*). The former is characterized by destabilization of strong E-cadherin-positive cell–cell junctions, before being replaced with N-cadherin and eventually disassembling and allowing single cells to change their polarity and shape into mesenchymal cells with motile capacity (*Dongre and Weinberg, 2019*). EMT is known to occur during disease metastasis and also fibrosis, because it results in alpha smooth muscle actin (αSMA)-positive myofibroblasts capable of secreting large amounts of collagen (*Wynn, 2008*). CCM, on the other hand, depends on cells keeping their junctions intact and collaborating to form an invasive group of cells with the capacity to progress, attach and rearrange the surrounding extracellular matrix (ECM) (*Friedl and Gilmour, 2009*). Indeed, both EMT and CCM have previously been implicated in the pathogeneses of endometriosis and adenomyosis (*Chen et al., 2010*; *Donnez et al., 2015*; *García-Solares et al., 2018a*; *Matsuzaki and Darcha, 2012*), but there is still a lack of firm evidence, especially on the first step of invasion of the myometrium by eutopic endometrium observed in adenomyosis.

Macrophages are potent mediators of tumour progression, inducing ECM remodelling, enhancing cell motility and promoting angiogenesis via a range of pro- and anti-inflammatory molecules they secrete (*Condeelis and Pollard, 2006*). They also show differential properties during disease evolution, as they are capable of reversibly shifting their phenotype between M1 and M2 with distinct surface markers and capacities (*Shapouri-Moghaddam et al., 2018*).

Recent data support the role of immunological dysregulation in adenomyosis, primarily altered macrophage infiltration and function (*Bourdon et al., 2021*; *Donnez et al., 2021*). Indeed, a number of studies have reported increased accumulation of these cells in endometrium and lesions from adenomyosis patients, implicating them in disease invasiveness, pain and impaired embryo receptivity (*Bourdon et al., 2021*). Excess macrophage infiltration in adenomyosis has previously been confirmed (*Stratopoulou et al., 2021a*) and so it was decided to dig deeper into the role of these cells in disease development. The specific goals of the present study were to determine which macrophage phenotype is involved in adenomyosis, and if and how these cells can actually trigger endometrial invasiveness during disease onset.

MATERIALS AND METHODS

Study participants and tissue collection

Human tissue for this study was used in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Cliniques Universitaires Saint-Luc (CUSL) and Université Catholique de Louvain on 31 August 2020 (ref: 2020/14AOU/410).

Fresh endometrial biopsies were retrieved from CUSL patients by means of a Novak curette. The adenomyosis group included six patients diagnosed by magnetic resonance imaging or transvaginal ultrasound (TVUS), with histological confirmation on surgical specimens, where available (*Chapron et al., 2020*). Four of the six women were undergoing hysterectomy for symptomatic adenomyosis. The other two patients presented for diagnostic biopsy in the context of adenomyosis, having had prior confirmation of the presence of the disease via TVUS. The healthy control group involved 10 patients with no sign of adenomyosis, endometriosis or any endometrial pathology. Endometrial samples were collected either after hysterectomy for leiomyomas (30%), or diagnostic biopsy in the context of infertility or abnormal uterine bleeding (70%). All patients were premenopausal and had not been given hormones or selective steroid receptor modulators for at least 2 months prior to intervention. One small tissue fragment was fixed in paraformaldehyde for histological evaluation of the menstrual phase (*Noyes et al., 1975*), while the rest was used for endometrial cell isolation.

For immunohistochemistry (IHC)/immunofluorescence analyses, another 17 samples were recovered from the anatomopathology archives of the CUSL in the form of formalin-fixed paraffin-embedded tissue blocks. They derived from eight adenomyosis patients and nine healthy controls, according to the same inclusion criteria as described above.

A summary of patient characteristics can be found in Supplementary Table 1. The source of endometrial tissue used for each experimental procedure is shown in Supplementary Figure 1.

Immunohistochemistry/immunofluorescence

IHC was performed on 5-μm tissue sections as previously documented (*Mosele et al., 2021*; *Stratopoulou et al., 2021a*). E- and N-cadherin were used as markers of cell–cell junctions in order to assess activation of the EMT cascade, while matrix metalloproteinase 9 (MMP9) was selected as an indicator of invasion activity. Tissue sections were digitized with the Pannoramic SCAN II, Sysmex,Terhulpen, Belgium at 20 × magnification.

Analyses were conducted using the Visiopharm 2022.01, Hamamatsu, Mont-Saint-Guiber, Belgium to calculate the staining index in areas of interest. The staining index was defined as % stained area multiplied by (255 – mean intensity in the stained area), where 255 represents the maximum intensity for 8-bit images. Foci consisting of endometrial glands and stroma inside the myometrium at a distance of at least 2.5 mm from the

junctional zone were deemed ectopic (*Levgur et al., 2000*). For E- and N-cadherin, only the epithelial compartment was considered, and for MMP9, only the stromal compartment.

CD80/CD163 double immunofluorescence was conducted to detect M1 and M2 macrophages, respectively. The same steps as for IHC were followed up to addition of the first secondary antibody, after which the slides were incubated with Alexa Fluor™ 555 Tyramide Reagent (Invitrogen, Eugene, OR, USA, #B40955, 1/200) for 15 min. They were then microwaved submerged in citrate buffer (pH 6) for antibody stripping, prior to overnight incubation with the second primary antibody. The next day, the sections were incubated with the second secondary antibody for 1 h and subsequently with the Alexa Fluor™ 488 Tyramide Reagent for 15 min, before mounting. Whole sections were digitized with the Pannoramic P250 Flash III at 20 × magnification.

Macrophage accumulation was quantified as the percentage of cells with cytoplasmic positivity per total cells, excluding the glandular and myometrial compartments, using QuPath 0.3.0 software (*Bankhead et al., 2017*).

The complete list of antibodies and specific dilutions is shown in Supplementary Table 2, while representative pictures of positive and negative controls can be found in Supplementary Figure 2. Positive controls included tissue samples where the markers of interest are known to be expressed, namely sarcoidosis for CD80/CD163, intestine for E-cadherin, liver for N-cadherin and spleen for MMP9. For negative controls, endometrial sections were incubated with FLEX rabbit or mouse negative controls (IS 600—IS 750, Dako North America, CA, USA) instead of the primary antibody.

Endometrial cell isolation, culture and co-culture

Biopsies retrieved from the operating room were immediately placed in cold sterile phosphate-buffered saline and transferred to the research laboratory. They were processed according to a previously published protocol (*Defrère et al., 2005*) with some modifications. Briefly, the tissue was finely minced and digested in 1 mg/ml collagenase (Sigma, St. Louis, MO, USA, #C9263) for 1 h. The suspension was filtered through a 300-μm sieve to exclude undigested fragments, followed by 40 μm to separate the two cell populations. Stromal cells passing through the sieve were centrifuged for 5 min at 160 g and resuspended in DMEM/F-12 (Life Technologies, Paisley, UK, #21041025) containing 10% fetal bovine serum (FBS) (Life Technologies, #16140071) and 1% Anti-Anti (Life Technologies, #15240062). After 20 min, long enough for stromal but not epithelial cells to adhere, the medium was aspirated and replaced with fresh medium to rule out the presence of any residual epithelial cells. Epithelial cells remaining stuck to the top of the 40-μm sieve were retrieved by turning it over and washing it in DMEM/F-12 containing 5% FBS, 1% Anti-Anti and 10 μg/ml insulin (Sigma, #I9278). The cells were then placed in the incubator for 40 min, before transfer to a new flask, making sure all stromal cells were left behind. They were then cultured for 7—10 days at 37°C with 5% CO_2 prior to beginning co-culture, with medium replacement every 2 days.

Successful isolation of the two cell populations was confirmed by immunocytochemistry on seven representative samples using pan-cytokeratin (CK22) as a marker of epithelial cells and CD10 as a specific marker of endometrial stromal cells. Cell counting determined the purity of both types of culture to range between 94% and 98% (Supplementary Figure 3).

Co-culture was performed with endometrial cells placed at the bottom of 6-well plates and differentiated M2 macrophages deposited on top of them on 0.4-μm PET inserts. The selected duration was 48 h, a period thought to be sufficient to observe the effects of co-culture (*Smith et al., 2015*; *Watanabe et al., 2004*), while avoiding dedifferentiation of M2 macrophages into monocytes in the absence of differentiating factors. At the end of the co-culture, endometrial cells were harvested using trypsin-EDTA (Life Technologies, #25200072) and used for invasion assays and RNA extraction.

THP-1 cell culture and differentiation into M2 macrophages

THP-1 (acute monocytic leukaemia) cells were kindly provided by Professor Dominique Lison (Louvain Centre for Toxicology and Applied Pharmacology, Université Catholique de Louvain, Belgium). This cell line has long been established as a reliable in-vitro model of human monocytes and tissue macrophages upon differentiation in phorbol 12-myristate 13-acetate (PMA) (*Chanput et al., 2014*; *Smith et al., 2015*; *Tsuchiya et al., 1982*).

Cells were cultured in RPMI 1640 (Life Technologies, #21875034) supplemented with 10% FBS, 1% Anti-Anti, 1 mmol/l sodium pyruvate (Life Technologies, #11360039) and 10 mmol/l HEPES (Life Technologies, #15630106), as previously reported (*Lison et al., 2021*). The medium was changed to DMEM/F-12 with 10% FBS and 1% Anti-Anti for the purposes of differentiation and co-culture. For differentiation, 150,000 cells were exposed to 200 nmol/l PMA (Sigma, #P8139) for 48 h, followed by 24 h in culture medium. Macrophages were then exposed to 25 ng/ml interleukin (IL)-4 (Peprotech, Cranbury, NJ, USA, #200-04) and 25 ng/ml IL-13 (Peprotech, #200-13) for a further 24 h to induce their polarization into the M2 phenotype (*Smith et al., 2015*). Finally, the cells were washed and placed in fresh medium, with the inserts deposited on top of the wells containing endometrial cells. Successful differentiation and polarization were confirmed by positive immunostaining for CD68 (universal macrophage marker) and CD163 (specific marker of the M2 subtype) on cytospin slides (Supplementary Figure 4).

Transwell invasion assays

At the end of co-culture, endometrial cells were detached using trypsin-EDTA, and 100,000 were set aside for invasion assays, while the rest were used for RNA extraction. Invasion assays were performed in 24-well plates on 8-μm PET inserts covered with 100 μl Matrigel solution (Life Sciences, #356234) at a dilution of 300 μg/ml. Upon centrifugation for 10 min at 230 g, the cells were resuspended in 100 μl DMEM/F-12 and placed on top of the inserts, with 500 μl FBS-containing medium at the bottom of the wells to act as a cell chemoattractant.

The cells were allowed to invade for 48 h, after which they were fixed in paraformaldehyde for 5 min and permeabilized in methanol for 15 min, before staining with crystal violet solution (Sigma, #V5265). Any non-invasive cells on top of the inserts were rubbed away using a wet cotton bud. Inserts containing invasive cells that reached the bottom of the membrane were photographed by light microscopy at 20 × magnification for counting purposes.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany, #74104) according to the manufacturer's instructions. Genomic DNA was eliminated by incubating with DNase I mixture (QIAGEN, #79254) for 15 min. The quantity and purity of extracted RNA were determined using the NanoDrop 2000 spectrophotometer, prior to reverse transcription into complementary DNA (cDNA) with the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA, #A5000), as instructed by the manufacturer.

Expression of mRNA of interest was then investigated by qPCR using TaqMan™ probes. Genes selected as representative of the EMT mechanism in epithelial cells included the two cadherins, namely E-cadherin (CDH1) and N-cadherin (CDH2), as well as SNAIL (SNAI1) and SLUG (SNAI2), two transcriptional factors known to promote EMT. In stromal fibroblasts, expression levels of αSMA (ACTA2) as a myofibroblast marker, vimentin (VIM) as a stromal fibroblast marker, and connective tissue growth factor (CTGF) and collagen I (COL1A1) as markers of fibrogenesis were examined.

Quantitative PCR reactions were achieved using the TaqMan™ Gene Expression Master Mix (Life Technologies, #4369016) in a final volume of 25 μl, containing 5 μl cDNA (25 ng). All reactions were performed in duplicate. Thermal cycling and signal detection were carried out using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, USA) in standard conditions.

Relative gene expression levels were estimated at −ΔCt (*Livak and Schmittgen, 2001*) upon normalization against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ABL proto-oncogene 1 (ABL1) for epithelial and stromal cells, respectively. References for all probes used can be found in Supplementary Table 3.

Statistical analysis

All statistical analyses were conducted on GraphPad Prism 9.1.2 (GraphPad Software Inc., San Diego, CA, USA). Paired or unpaired *t*-tests were applied to compare findings from different conditions or groups, respectively. Where data did not follow a normal distribution, Wilcoxon signed-rank test and Mann–Whitney *U*-test were used for paired and unpaired comparisons, respectively. *P*-values below 0.05 were considered significant.

RESULTS

Accumulation of M2 macrophages in endometrium

By trying to identify the main macrophage phenotype in adenomyosis, immunofluorescence analysis demonstrated that CD163-positive M2 macrophages are predominant in both adenomyosis and healthy endometrium, as CD80-positive M1 macrophages were only detected in one of the 17 samples examined (data not shown). M2 macrophage accumulation was noted in all but two samples, showing higher rates in both endometrium ($P = 0.0109$) and lesions ($P = 0.0267$) from adenomyosis patients than endometrium from healthy patients (FIGURE 1A, B).

Invasion capacity of endometrial cells with or without the influence of M2 macrophages

To assess the involvement of macrophages in endometrial invasiveness, the invasion capacity of endometrial cells with or without prior interaction with M2 macrophages was compared. The analysis revealed that eutopic epithelial cells from both healthy ($P = 0.002$) and adenomyosis ($P = 0.0002$) patients became significantly more invasive when interacting with M2 macrophages compared to corresponding controls. Moreover, cells from adenomyotic endometrium were in turn significantly more invasive than healthy cells in the same co-culture conditions ($P = 0.0312$) (FIGURE 2A). Eutopic stromal cells also acquired enhanced invasion capacity when co-cultured with M2 macrophages in both the healthy ($P = 0.0047$) and adenomyosis ($P = 0.0469$) groups (FIGURE 2B).

Expression of EMT-related genes in endometrial cells upon interaction or not with macrophages

To understand whether EMT is the mechanism underlying enhanced cell invasiveness, mRNA expression was analysed in a series of genes involved in this cascade and its subsequent fibrogenesis. A slight decrease in CDH2 was observed in healthy epithelial cells co-cultured with M2 macrophages ($P = 0.0133$), while CDH1, SNAI1 and SNAI2 remained unchanged in all conditions (FIGURE 3A). In terms of stromal cells, M2 macrophages caused a significant decline in ACTA2 levels in both healthy cells ($P = 0.0017$) and eutopic cells from adenomyosis patients ($P = 0.0034$), along with increased VIM expression in healthy cells ($P = 0.0462$). No differences were recorded in CTGF or COL1A1 levels, either between the different conditions or between adenomyosis and healthy cells (FIGURE 3B).

Protein expression of MMP9, E- and N-cadherin in endometrial tissue

Given that the mRNA expression analysis did not reveal any activation of the EMT cascade, protein expression levels of E- and N-cadherin were investigated to characterize intercellular junctions, and MMP9 to identify tissue invasiveness. No significant differences were detected in E-cadherin expression between healthy endometrium and endometrium and lesions from adenomyosis patients. Epithelial N-cadherin expression was similar in endometrium from healthy and adenomyosis subjects, but appeared to be stronger in ectopic glands than those in healthy endometrium ($P = 0.0135$) (FIGURE 4A, B). Stromal expression of MMP9 was found to be higher in eutopic endometrium from adenomyosis patients than healthy participants ($P = 0.0492$), with no other differences identified between the groups (FIGURE 4A, B).

Protein expression of E- and N-cadherin in the different layers of endometrial tissue in adenomyosis patients

As adenomyosis is generally thought to derive from invasion of the myometrium by the endometrial basalis, it was decided to examine potential differences in this layer that favour this event. Expression of E- and N-cadherin were compared between the functional layer, the basalis (glands adjacent to the junctional zone) and adenomyotic lesions. Both E-cadherin ($P = 0.0379$) and N-cadherin ($P = 0.0196$) levels were elevated in basal glands compared with those in functional endometrium. N-cadherin was also significantly higher in ectopic glands than those in the functional layer ($P = 0.0021$) (FIGURE 5A, B).

DISCUSSION

Uterine adenomyosis is widely believed to originate from invasion of the endometrial basalis, establishing ectopic glandular and stromal lesions inside the myometrium (*García-Solares et al., 2018b*; *Leyendecker*

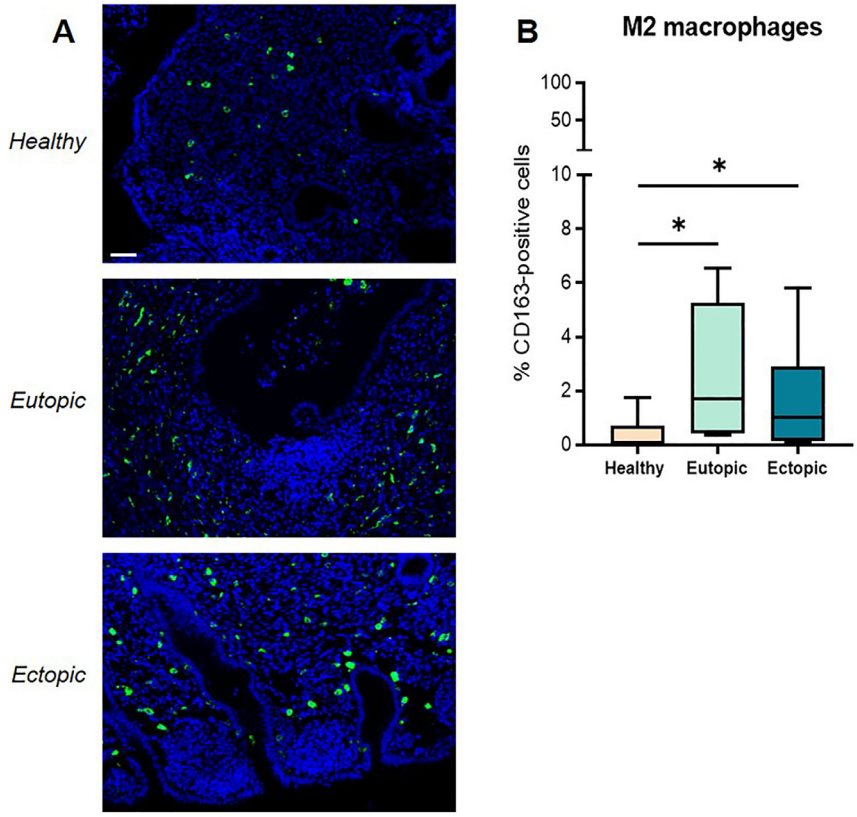


FIGURE 1 M2 macrophage accumulation in adenomyosis. (A) Representative pictures of CD163-positive M2 macrophages in endometrium: healthy; eutopic endometrium from adenomyosis patients; ectopic lesions. Magnification 20 ×. Scale bar represents 50 μm. (B) Boxplots showing rates of CD163-positive cells per total cells in the stromal compartment (*P = 0.0109; 0.0267). n = 9 for healthy and n = 8 for adenomyosis.

et al., 2009). However, the process of disease invasion has been poorly characterized experimentally, mainly due to a lack of reliable models.

Our team and others have previously shown macrophage accumulation to be a key factor in the pathogenesis of adenomyosis (*Bourdon et al., 2021*; *Stratopoulou et al., 2021a*). This hypothesis is corroborated by the fact that anti-inflammatory drugs are frequently prescribed for adenomyosis, primarily to target dysmenorrhoea and pelvic pain symptoms (*Stratopoulou et al., 2021b*). We can now report that it is specifically M2 macrophages that reside both in endometrium and lesions from adenomyosis patients, and may therefore play a crucial role in disease development and/or progression. M2 macrophages are known to be the predominant phenotype in many tumours as well, and have been linked to heightened metastatic activity and poorer clinical outcomes (*Komohara et al., 2016*). Preliminary data on adenomyosis also suggest that endometrial cells themselves could drive macrophage polarization into the M2 phenotype, further confirming the pivotal role of this subpopulation in the pathogenesis of the disease (*An et al., 2017*).

M2 macrophages are well-known mediators of tissue repair in physiological and pathological conditions. In this context, they release abundant anti-inflammatory cytokines, mediating cell proliferation, (neuro)angiogenesis and ECM deposition to heal the injury. Nonetheless, excess M2 macrophage accumulation, as in the case of chronic inflammation, can lead to disease progression and fibrosis (*Condeelis and Pollard, 2006*; *Shapouri-Moghaddam et al., 2018*). Given that adenomyosis is characterized by chronic trauma to the junctional zone (*Leyendecker et al., 2009*), excess M2 macrophage accumulation and its consequences would not be unexpected and may indeed be the trigger for disease initiation and the onset of symptoms.

This study has demonstrated that both the epithelial and stromal compartments of endometrium have invasion capacity *in vitro*, suggesting that they are probably both involved in disease development. Upon interacting with M2 macrophages, endometrial cells from both adenomyosis and healthy women acquired a more invasive phenotype, indicating that M2 macrophages alone may be sufficient to promote the disease. Nevertheless, epithelial cells from adenomyosis patients became significantly more invasive when interacting with macrophages compared with their healthy counterparts, so these cells may be more susceptible to the action of local macrophages. This observation implies that both macrophages and abnormal endometrial cells constitute determining factors for disease development in adenomyosis patients.

This study did not reveal any gene expression changes consistent with EMT and fibrosis in eutopic endometrial cells from adenomyosis patients compared with healthy subjects. EMT has previously been blamed for epithelial cell invasiveness in adenomyotic lesions, with IHC showing lower E-cadherin expression than seen in corresponding eutopic endometrium (*Chen et al., 2010*). This study failed to

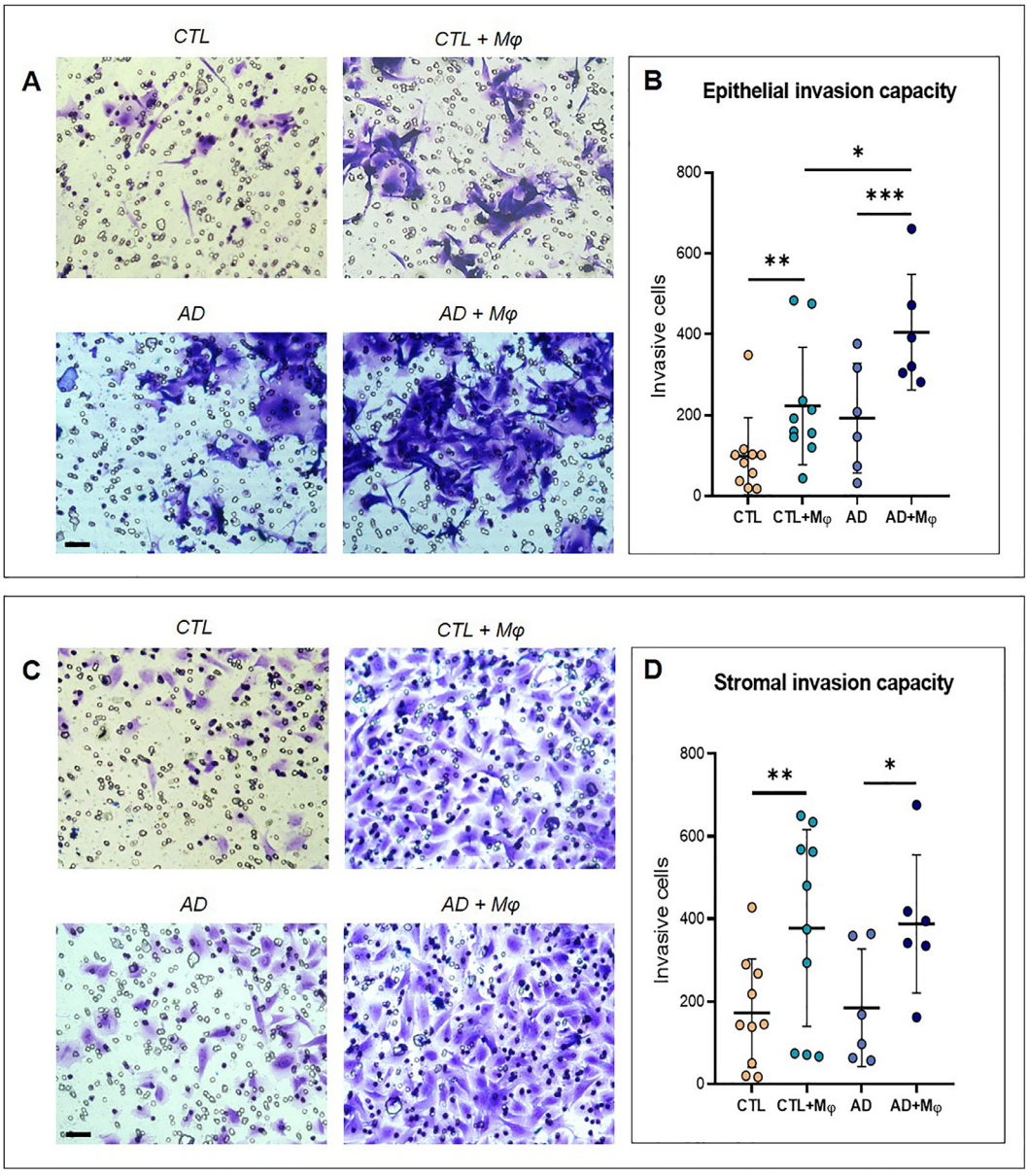


FIGURE 2 Endometrial epithelial and stromal cell invasiveness. (A) Epithelial cell invasiveness. Scatterplots showing numbers of invasive cells (**P = 0.002; ***P = 0.0002; *P = 0.0312). Results are presented as mean ± SD. Magnification 20 ×. Scale bar represents 50 μm. (B) Stromal cell invasiveness. Scatterplots showing numbers of invasive cells (**P = 0.0047; *P = 0.0469). Results are presented as mean ± SD. Magnification 20 ×. Scale bar represents 50 μm. CTL = cells from healthy controls; CTL + Mφ = cells from healthy controls co-cultured with macrophages; AD = cells from adenomyosis patients; AD + Mφ = cells from adenomyosis patients co-cultured with macrophages. n = 10 for healthy and n = 6 for adenomyosis.

confirm this hypothesis, having observed higher epithelial N-cadherin in ectopic lesions, but not accompanied by any significant decrease in E-cadherin. In terms of eutopic endometrial cells, neither the action of macrophages nor adenomyosis itself appeared to promote EMT at mRNA or protein levels. On the contrary, co-culture with macrophages had the opposite effect in some cases, reducing CDH2 expression in healthy epithelial cells, enhancing VIM expression in healthy stromal cells and clearly lowering ACTA2 levels in stromal cells from both healthy and adenomyotic endometrium.

ACTA2, a gene encoding αSMA, is a well-known indicator of fibrosis, because it identifies activated collagen-producing myofibroblasts (*Hinz et al., 2007*). We and others have previously confirmed fibrosis to be a key element of both endometriosis and adenomyosis pathogeneses (*Mosele et al., 2021*; *Stratopoulou et al., 2021a*; *Viganò et al., 2020*). However, it was only possible to detect increased collagen accumulation in ectopic lesions, while eutopic endometrium from the same patients did not differ substantially from healthy tissue (*Mosele et al., 2021*; *Stratopoulou et al., 2021a*). It is therefore likely that fibrogenesis is triggered at a later stage, upon disease initiation and lesion establishment. The precise impact of macrophages on this process in ectopic lesions needs to be further investigated, given that the inflammatory activity of these cells can often result in fibrosis (*Viganò et al., 2020*; *Wynn, 2008*).

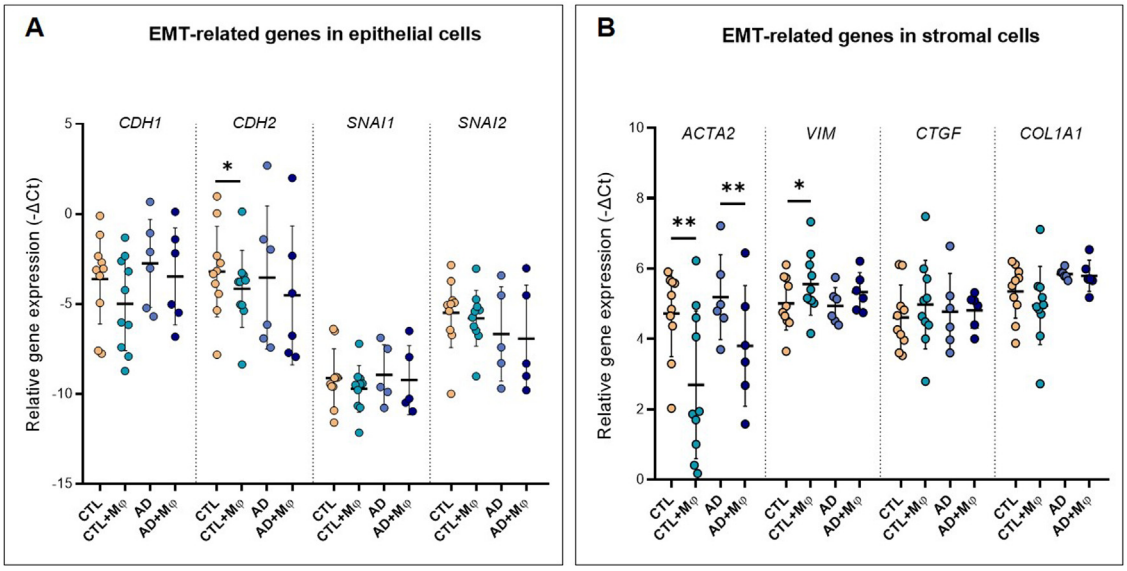


FIGURE 3 Expression of EMT-related genes. (A) Scatterplots showing relative gene expression (−ΔCt) in epithelial cells (*P = 0.0133). (B) Scatterplots showing relative gene expression (−ΔCt) in stromal cells (**P = 0.0017 for healthy cells, 0.0034 for eutopic cells from adenomyosis patients; *P = 0.0462). Results are presented as mean ± SD. Note that one adenomyosis sample was excluded from analysis of SNAI1 and SNAI2 due to insufficient RNA. CTL = cells from healthy controls; CTL + Mφ = cells from healthy controls co-cultured with macrophages; AD = cells from adenomyosis patients; AD + Mφ = cells from adenomyosis patients co-cultured with macrophages. n = 10 for healthy and n = 6 for adenomyosis.

In contrast to some previous reports, this study reveals that CCM may be the mechanism behind endometrial invasiveness. The hallmarks of CCM are maintenance of tight cell–cell junctions and, at the same time, motility and capacity to rearrange the surrounding ECM (*Friedl and Gilmour, 2009*). It has been demonstrated that endometrial cells do possess invasion capacity *in vitro*, irrespective of undergoing EMT and losing their cohesion molecules. At the histological level, an increase in MMP9 in eutopic endometrium from adenomyosis patients was observed, in line with previous reports on the involvement of this protein in the endometrial invasion process (*Collette et al., 2006*). Finally, E- and N-cadherin-positive cell junctions were maintained in endometrium from adenomyosis subjects and looked even more pronounced in glands adjacent to the junctional zone, favouring motility of entire clusters of cells rather than single cells individually.

Consistent with these findings, a recent transcriptomics-based study reported CCM to be the main mechanism responsible for eutopic endometrium invasiveness in adenomyosis (*Zhai et al., 2022*). The authors also found that MMP9 expression was markedly higher in adenomyosis patients, along with a series of genes involved in cell–cell cohesion and coupling. Moreover, previous research has already proved CCM to be an essential component of the invasion process in deep endometriotic nodules, a closely associated disease (*Donnez et al., 2015*; *García-Solares et al., 2018a*; *Stratopoulou et al., 2021a*). In this context, both baboon and human endometriotic nodules acquired an invasive phenotype and ECM rearrangement properties, while at the same time maintaining their epithelial phenotype and some cell–cell junctions, even along the most progressive front edge of the disease (*Donnez et al., 2015*; *García-Solares et al., 2018a*). These data collectively provide further proof that CCM may be the main mechanism underlying endometrial invasiveness in adenomyosis (*García-Solares et al., 2018b*).

On the other hand, N-cadherin is widely used as a marker of mesenchymal cells in EMT (*Dongre and Weinberg, 2019*), and this study did indeed find this protein to be more strongly expressed in the invasive fraction (basal and ectopic) of endometrial glands in adenomyosis. However, as previous research has shown, N-cadherin may in fact identify endometrial epithelial progenitor cells, as it was abundantly expressed in deep basal glands with clonogenic potential (*Nguyen et al., 2017*). In line with the latter study, we also found the same glands to simultaneously express high levels of E-cadherin, making them highly unlikely to have undergone EMT, as this process would require loss of strong E-cadherin-positive junctions. The most recent evidence suggests that N-cadherin may be implicated in endometrial receptivity too (*Verma et al., 2022*), so its function cannot be limited to EMT and requires further investigation.

The present study has both strengths and limitations. Its strong points include specific use of M2 macrophages, proved here to express the same surface marker as those native to endometrium and lesions. It was therefore possible to more accurately mimic interactions between endometrial cells and local macrophages in adenomyosis. The study also isolated both epithelial and stromal cells, demonstrating for the first time that both populations have similar invasion capacity *in vitro*. These findings lead us to believe that both these cell populations participate actively in disease invasiveness and may play distinct roles that need to be explored in greater depth. The main drawback of the study is the 2D nature of the cultures, which cannot fully reconstitute in-vivo cell arrangement or cell–cell interactions, or allow CCM to be studied in real time. To overcome this limitation, our current research is focused on implementing 3D organoids as a more accurate in-vitro model of adenomyosis. Moreover, a

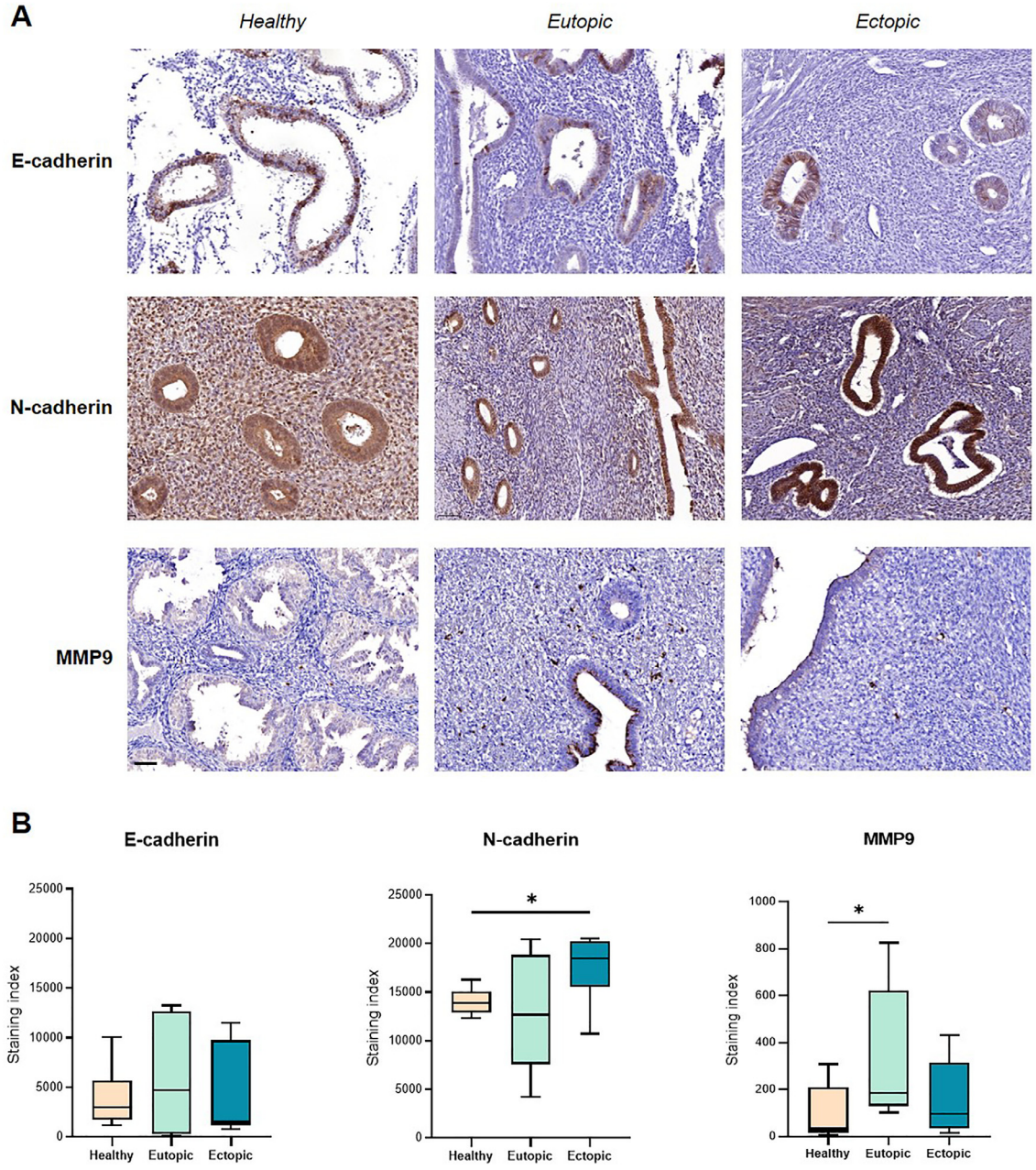


FIGURE 4 Immunohistochemical analysis of E-cadherin, N-cadherin and MMP9. (A) Representative pictures of E-cadherin, N-cadherin and MMP9 expression in endometrium: healthy; eutopic endometrium from adenomyosis patients; ectopic lesions. Magnification 20 × . Scale bar represents 50 μm. (B) Boxplots showing staining index ranges for E-cadherin, N-cadherin (*P = 0.0135) and MMP9 (*P = 0.0492). n = 9 for healthy and n = 8 for adenomyosis.

cancerous cell line was used to model endometrial macrophages, which implies a malignant background with potentially differential responses at times compared to normal somatic cells in their natural environment. Nevertheless, THP-1 cells are a well-established model that has proved suitable for the study of interactions between macrophages and other cell types, thereby adding value to the current results (*Chanput et al., 2014*). This model also lacks interpatient cell population heterogeneity and cannot reveal potential differences in macrophage characteristics between adenomyosis patients and disease-free women. While technically challenging, due to small numbers of endometrial macrophages, future studies should concentrate on native populations and potential patient-to-patient variability in their characteristics.

In conclusion, this study has shown for the first time that M2 macrophages accumulate in adenomyosis, and their interaction with endometrial epithelial and stromal cells is sufficient to promote invasiveness in both adenomyotic and healthy endometrium. It has also established that M2 macrophages do not induce EMT, but actually appear to reverse it in cultured endometrial cells, favouring cell–cell cohesion. Finally, although eutopic endometrium from adenomyosis patients does exhibit features of invasiveness, invasive glands were found to

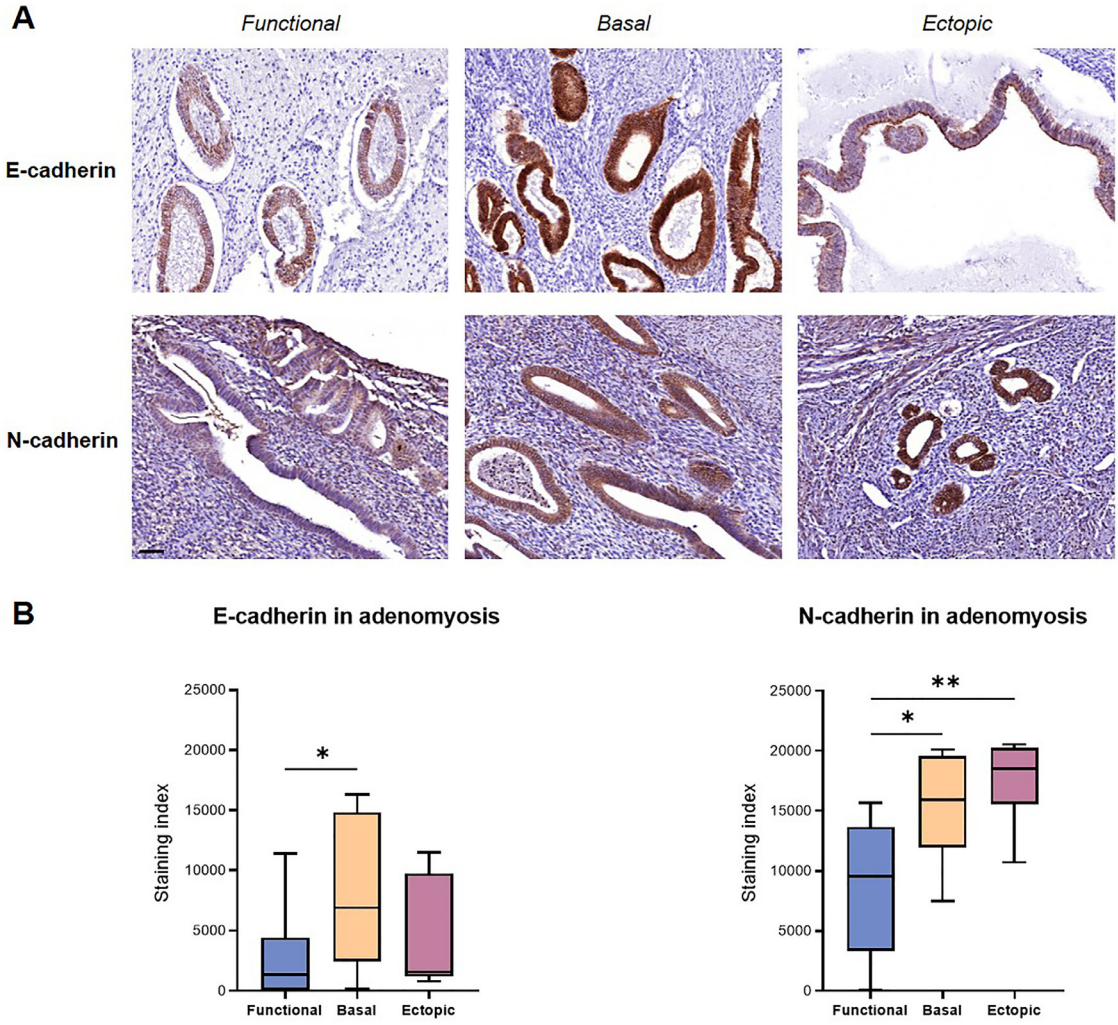


FIGURE 5 E- and N-cadherin expression in different areas of endometrial tissue. (A) Representative pictures of E- and N-cadherin expression in endometrium from adenomyosis patients: functional; basal; ectopic glands. Magnification 20 × . Scale bar represents 50 μm. (B) Boxplots showing staining index ranges for E-cadherin (*P = 0.0379) and N-cadherin (*P = 0.0196; **P = 0.0021). n = 8 for adenomyosis.

be characterized by strong intercellular junctions, suggesting a mechanism of CCM of endometrium into the myometrium during disease initiation. More research is needed to elucidate this mechanism and its driving force in adenomyosis.

DATA AVAILABILITY

Data will be made available on request.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2023.01.001.

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