Reduced $\alpha$-2,6 sialylation regulates cell migration in endometriosis

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STUDY QUESTION: Is endometriosis associated with aberrant sialylation patterns and what is the potential impact of such anomalies on cell migratory properties?

SUMMARY ANSWER: The reduced $\alpha$-2,6 sialylation patterns in the peritoneal fluid of endometriosis-affected women and in stromal and epithelial cells from endometriotic lesions could be associated with enhanced cell migration.

WHAT IS KNOWN ALREADY: Endometriosis is considered to be a benign disease although, like cancer, it has the characteristic of being an invasive disease with cells that have an enhanced capacity to migrate. Aberrant sialylation has been reported in various malignancies and it has been linked to tumour invasion and metastasis.

STUDY DESIGN, SIZE, DURATION: We conducted a prospective laboratory study in a tertiary-care university hospital. We investigated non-pregnant patients who were <42 years of age ($n = 273$) when they underwent surgery for a benign gynaecological condition.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study population consisted of 102 women with histologically proven endometriosis and 71 endometriosis-free controls, who underwent a complete surgical exploration of the abdominopelvic cavity. Peritoneal fluids were collected during the surgical procedures, and endometrial and endometriotic biopsies were performed on all of the patients to generate stromal and epithelial primary cell cultures. The expression of $\alpha$-2,6-sialyltransferase (ST6GALNAC1) was studied in eutopic and ectopic endometria of endometriosis patients and in eutopic endometria of controls by reverse transcription followed by quantitative real-time polymerase chain reaction (RT-qPCR). The $\alpha$-2,6 sialylation levels were measured by ELISA in the peritoneal fluids of patients and controls and by western-blot in primary endometrial and endometriotic cell cultures using Sambucus nigra agglutinin (SNA), an $\alpha$-2,6 sialic acid-binding lectin. A transwell migration assay after incubation of the cells with neuraminidase was also performed to evaluate the impact of desialylation on endopic endometrial stromal cell migration.

MAIN RESULTS AND THE ROLE OF CHANCE: ST6GALNAC1 gene expression was significantly lower in endometriotic lesions compared to that in eutopic endometrium of endometriosis-affected patients and healthy endometrium (16-fold for both; $P < 0.01$). We observed a significant reduction in SNA levels in the peritoneal fluids of endometriosis-affected women compared to control women (median

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Introduction

Endometriosis is a benign, chronic, gynaecological disorder defined by the presence of endometrial tissue (glands and stroma) outside the uterine cavity (Sampson, 1927). Three different types of endometriosis can coexist: superficial peritoneal endometriosis (SUP), ovarian endometrioma (OMA) and deeply infiltrating endometriosis (DIE). The prevalence of endometriosis is 6–10% in the general female population; in women with pain or infertility, the prevalence is as high as 35–50% (Giudice and Kao, 2004). Despite its high prevalence, the pathogenesis of the disease remains unclear among numerous theories including retrograde menstruation, coelomic metamplasia and Müllerian remnants. Immunological, hormonal, genetic/epigenetic and environmental factors may also be involved (Vercellini et al., 2014; Sofos et al., 2015; Maniglio et al., 2016; Gordts et al., 2017; Laganà et al., 2017). The retrograde flux of endometrial cells through the oviducts is the most commonly accepted mechanism for the arousal of endometriosis lesions (Sampson, 1927). Once they reach the peritoneal cavity, they can implant on pelvic structures through a multistep process involving adhesion, invasion, angiogenesis, proliferation and steroidogenesis. The inflammatory environment and aberrant immune responses appear to be directly associated with the initiation and progression of the disease. Indeed, a large body of evidence indicates that reduced apoptosis of endometriotic cells and increased apoptosis of peritoneal fluid immune cells may induce a permissive peritoneal environment for the initiation and progression of endometriotic lesions (Leavy, 2015; Laganà et al., 2016; Vetticka et al., 2016; Luckow Invitti et al., 2018).

One striking feature regarding the disease is its ability to invade into the surrounding tissue and grow locally, forming endometriotic lesions in the abdominal cavity, which is a feature that resembles cancer. Previous studies have shown that endometriotic cells display significant activation of growth-related signalling pathways, especially the MAP kinases and mTOR/Akt pathways (McKinnon et al., 2016) which are also observed in malignant tumour cells. However, in endometriosis, cell proliferation and invasion are controlled and stop at a certain stage, and the occurrence of endometriotic lesions outside the peritoneal cavity is exceptional (Borghese et al., 2008; Santulli et al., 2015). Therefore, endometriosis is ultimately not fatal, which makes it a benign disease.

Aberrant sialylation has been reported for decades in various malignancies and it has been linked to tumour invasion and metastasis (Vajaria et al., 2016). During neoplastic transformation, the activity of sialyltransferases (STs) is generally de-regulated and cancer cells consequently exhibit abnormal expression of tumour-associated carbohydrate antigens at their surface, while also exhibiting greater adhesion and invasion (Pinho and Reis, 2015). Surprisingly, the sialylation pattern of endometriotic cells has been investigated very little. Indeed, there has only been a single study to date, by Miller et al. (2010) and it reported lower levels of α-2,3 sialylation in endometrial biopsies of endometriosis patients compared with healthy controls. Therefore, in this study we investigated whether sialylation patterns were altered in endometriosis, by comparing peritoneal fluid samples and in-vitro culture of eutopic endometrial cells and ectopic endometriotic cells obtained from patients with histologically proven endometriosis and eutopic endometrial cells of endometriosis-free women. We also studied the potential impact of such anomalies on cell migratory properties.

Materials and Methods

Patients, tissue and peritoneal fluid collection

This study was approved by the local institutional review board (approval number 05-2006 provided by the ‘Comité de Protection des Personnes et des Biens dans la Recherche Biomédicale’ of Paris Cochin) and all participants gave written informed consent. The samples were obtained from 102 patients with painful endometriosis who underwent surgery for complete removal of the endometriotic lesions between January 2012 and March 2018. Endometriosis was categorized according to a surgical classification based on the location of the worst endometriotic lesion: SUP, OMA and DIE, as described previously (Chapron et al., 2006). Endometriosis was scored according to the ASRM classification (Revised American Society for Reproductive Medicine classification of endometriosis, 1997). For surgical samples of ovarian lesions, endometriotic tissues were collected from OMA cyst walls under macroscopic view after cystectomy and rinsed in physiological saline to discard chocolate fluid and any remaining red blood cells. Control endometrial specimens were collected from 71 non-pregnant women without any

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Our control group involved patients operated for benign gynecological conditions (e.g. tubal infertility, uterine fibroids or ovarian cysts) which may also be associated with altered sialylation patterns.

WIDER IMPLICATIONS OF THE FINDINGS: The hyposialylation pattern of endometriotic cells appeared to be associated with enhanced migratory abilities, which might contribute to the establishment of early endometriotic implants. Further research is needed to confirm these findings, as this could lead to new potential therapeutic targets for this complex disorder.

STUDY FUNDING AND COMPETING INTEREST(S): No external funding was received and there are no conflicts of interest.

Key words: α-2,6 sialylation / endometriosis / migration / pathogenesis / Sambucus nigra agglutinin.
macroscopic endometriotic lesion as verified by thorough examination of the abdominopelvic cavity. Indications for surgery in these control patients were the following: tubal infertility, non-endometriotic ovarian cysts or uterine myoma. Women with cancer or chronic viral infections and women who did not provide consent were excluded from the study.

The study analysis used a prospectively managed database. For each patient, their personal history data were obtained during face-to-face interviews, which were conducted by the surgeon in the month prior to the surgery. A highly structured previously published questionnaire was used for all of the patients (Chapron et al., 2010). The following items were recorded: age, parity, gravidity, height, weight, BMI, past history of hormonal and/or surgical treatment of endometriosis, existence of gynaecological pain symptoms (dysmenorrhea, deep dyspareunia, non-cyclic chronic pelvic pain (NCCPP)), and gastrointestinal and lower urinary tract symptoms. In order to evaluate the pain intensity preoperatively, the 10-cm visual analog scale was used (Huskisson, 1974). Patients who had no hormonal treatment at the time of surgery had undergone a minimum wash-out period of three months.

Patients with endometriosis provided both eutopic and ectopic endometria. Endometrial biopsy specimens were collected from control patients. All of the samples were histologically characterized. The phase of the menstrual cycle was confirmed by histologic analysis of endometrial biopsies.

Peritoneal fluid (PF) was also collected during surgery from all of the patients in the study. The samples were centrifugated at 800 g for 10 min at 4°C and the supernatants were collected. Aliquots of the samples were stored at −80°C until they were needed for the analysis.

**Total RNA extraction and reverse transcription**

After surgical resection, one part of each sample was immediately frozen in liquid nitrogen. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. The RNA quality was checked by agarose gel electrophoresis and by spectrophotometry. Total RNA quantification was quantified using spectrophotometry by measurement of the absorbance at 260 nm.

The RNA was treated with deoxyribonuclease (DNase; Invitrogen) to remove any contaminating DNA. A 4 μg sample of total RNA was reverse transcribed using random primers and M-MLV Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. A negative control without RNA was included in each series of reverse transcription reactions. Each sample was resuspended in the presence of RNaseOut (Invitrogen).

**Analysis of ST6GALNAC1 and ST6GALNAC5 gene expression by quantitative real-time PCR of tissue samples**

STs expression was studied in both eutopic and ectopic endometria of endometriosis patients, and in the eutopic endometrium of controls, by quantitative real-time PCR. These two target enzymes were chosen based on the results of a previous transcraptomic study (Borghese et al., 2008) that showed that their gene expression levels were the most highly dysregulated in the eutopic endometrium of endometriosis patients compared with other STs. A set of five genes, including two target genes (ST6GALNAC1 and ST6GALNAC5) and three reference genes (SDHA, RPLP0 and HNRNPA1), was analyzed by quantitative real-time PCR using cDNA synthetized from each sample. The primers for the real-time PCR analysis were chosen using the PRIMER3 software, based on published sequences (Borghese et al., 2008) (Supplementary Table SI). All of the selected primers were aligned with the BLAST software to avoid non-specific annealing and cross-amplifications. The primers were purchased from Eurogentec.

Quantitative PCR was carried out with a Light Cycler 480, 96-well apparatus (Roche Diagnostics, Indianapolis, IN, USA). We used a Light Cycler 480 SYBR Green I Master (Roche) amplification kit, according to the manufacturer’s instructions. The PCR protocol consisted of an initial denaturation of 5 min at 95°C followed by 40 cycles of 95°C for 10 s, annealing temperature for 10 s, 72°C for 10 s, and a final melting curve. All of the primer sets exhibited a good linear correlation and equal priming efficiencies over a wide range of known cDNA concentrations compared with their cycle threshold (Ct) values. The real-time PCR efficiency for all the genes was estimated to be ≥90%.

The relative change of abundance for each target gene compared with a set of internal controls was determined using the formula 2−ΔΔCt (Livak and Schmittgen, 2001). The set of internal controls included a geometric means of the three reference genes. The results were analysed with the LightCycler software using the ΔΔCt method (Santulli et al., 2012).

The experiments were performed with samples from 16 patients and 15 controls of the cohort.

**Characterization of glycoforms in the peritoneal fluid by enzyme-linked immunosorbent assay test using biontylated lectins**

Abnormal glycosylation was investigated in the PF samples of all the patients by ELSA using three different lectins: Concanavalin A (Con-A) which is a mannose and glucose-binding lectin; and two sialic acid-binding lectins, Maackia amurensis leucoagglutinin (MAACKIA) which binds to α-2,3 sialic acids and Sambucus nigra agglutinin (SNA) which binds to α-2,6 sialic acids.

The protein concentration of the peritoneal fluids was measured for all the samples using the spectoroscopic Bradford protein assay. A 96-well plate (Falcon, Corning, NY, USA) was coated with 25 μg/well of peritoneal fluid protein at 37°C for 16 h. The wells were rinsed three times with 0.2 mL of phosphatebuffered saline containing 0.5% Tween 20 (PBS-Tween). Biotinylated lectins (Vector Laboratory, Burlingame, Canada; 0.1 μL), diluted in PBS-Tween to obtain a final concentration of 0.01 mg/mL, were added to the wells and incubated at room temperature for 30 min. The wells were rinsed three times with 0.2 mL of PBS-Tween before and after HRP-conjugated streptavidin (0.1 mL of a 0.43 μg/mL solution in PBS-Tween; Thermo Fisher Scientific) was added and incubated for 1 h at room temperature. The chromogenic peroxidase substrate 2,2’-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) was used to detect the bound HRP-streptavidin. The absorbance at 405 nm was measured after 30 min.

**Cell isolation and culture**

Primary endometrial and ectopic endometriotic cell cultures were prepared from biopsy samples. Biopsy specimens were rinsed and minced into small pieces and then digested with 5% dispase and collagenase (2 mg/mL, Gibco Invitrogen, Cergy Pontoise, France) for 1 h at 37°C and separated using serial filtration. Red blood cells were removed by hypotonic lysis (using 0.15 M NaCl, 1 mM KHCO3, 0.1 mM Na2 EDTA). Debris was removed using sieves with 100 μm apertures; the epithelial cells were retained on sieves with 40 μm apertures while the stromal cells remained in the filtrate. Both of these cell types were plated in Primaria flasks (Becton Dickinson Labware, Le Pont de Claix, France) and cultured in DMEM (Gibco Invitrogen, Cergy Pontoise, France) with 10% foetal calf serum (FCS). For each sample, two populations of cells were obtained: stromal cells and epithelial cells. For the endometriosis group, the epithelial and stromal cell cultures were derived from biopsies of both the eutopic and ectopic endometria. For the control
group, the epithelial and stromal primary cell cultures were derived from biopsies of the eutopic endometrium.

The purity of the stromal and the epithelial cell suspensions was assessed by staining with a 1:100 dilution of FITC-labelled anti-cytokeratin and Cy3-labelled anti-vimentin antibodies, respectively (Sigma-Aldrich, St Louis, MI, USA). The fluorescence signal was imaged using an Olympus fluorescent microscope (Hamburg, Germany) and images were captured using a Cell Imaging station (Olympus). Both of the cell populations were negative for CD3 (T cells), CD45 (leucocytes) and CD11b (monocytes and granulocytes) staining. The cell samples were only used when they were at least 90% pure.

Each cell type was cultivated in its specific medium until the cells were 90% confluent, which occurred between 7 and 14 days after collection of the samples.

Characterization of α-2,6 sialylation by immunoblot-based measurement of *Sambucus nigra* agglutinin in cell lysates

Cells were lysed in ice-cold RIPA buffer (10 mM Tris–HCl, pH 7.5, 5 mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with a 25 mM sodium fluoride, 0.5 mM sodium orthovanadate and 1% antiproteinase cocktail. Equal amounts of protein (40 μg) were loaded and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to nitrocellulose membranes, the membranes were blocked with 5% of bovine-serum albumin (BSA) (Sigma-Aldrich) overnight at 4°C, and then incubated 1 h at room temperature with biotinylated lectin *Sambucus nigra* (Vector Laboratories Inc.). The membranes were then washed twice, and SNA was detected with a 1:2000 dilution of HRP-conjugated streptavidin (Thermo Fisher Scientific) and visualized using an Enhanced Chemiluminescence system (Advansta, Menlo Park, CA, USA).

The experiments were performed with cells obtained from six patients and six controls.

Transwell migration assay after treatment of eutopic endometrial stromal cells with neuraminidase

The impact of desialylation on the capacity of the eutopic endometrial stromal cells to migrate was assessed using neuraminidase, which is a glycohydrolytic enzyme that cleaves the glycosidic linkages between sialic acid and mucopolsaccharides.

Cells were harvested with PBS-EDTA (PBS; Invitrogen; EDTA; Sigma-Aldrich; 2.5 mM) for 30 minutes at 37°C, and then washed with PBS containing 1% FCS and 1% penicillin/streptomycin (wash buffer) and resuspended in medium without FCS (106 cells/200 μL). Half of the cells were treated with 1 unit of neuraminidase (*Sambucus nigra*) for 2 h at 37°C, and the other half (control cells) were incubated in the medium without FCS under the same conditions. A six-well transwell chamber (8 μm, BD Matrigel® Invasion Chamber, product #354481—Becton Dickinson, Bedford, MA, USA) was used to evaluate the motility of the eutopic endometrial stromal cells. The cells were seeded at a concentration of 5 x 10⁴ cells per well in the upper part of BD Biocoat Matrigel® Invasion Chambers in DMEM. The lower chamber was filled with DMEM containing 5% BSA as chemoattractant. After 22 h of incubation at 37°C, the cells that had not migrated through the membrane were removed with a cotton swab. The cells on the lower surface of the membrane were fixed with methanol for 2 min, stained with 1% toluidine blue for 2 min and washed three times in PBS. The stained cells that had migrated to the lower surface were visualized using a microscope and photographed. For each experiment, the cells were counted in six different microscope fields and the results expressed as means ± SEM of these counts. The experiments were performed with the cells obtained from six patients and six controls.

Effective cell desialylation by neuraminidase was verified using endometrial stromal cells from one of the control patients. A 96-well plate (Falcon) was used with an amount of 2.10⁴ cells/well at 37°C overnight. The cells were washed twice with PBS; one half of the wells were incubated with 0.25 units of neuraminidase (Sigma-Aldrich) for 30 min to 1 h at 37°C, and control cells were incubated in the medium with 2.5% FCS under the same conditions. After three washes with PBS, biotinylated SNA (Vector Laboratories; 0.1 mL, diluted in PBS-Tween to obtain a final concentration of 0.01 mg/mL, was added to the wells and incubated at room temperature for 30 min. The wells were rinsed three times with 0.2 mL of PBS-Tween before and after HRP-conjugated streptavidin (0.1 mL of a 0.43 μg/mL solution in PBS-Tween; Thermo Fisher Scientific) was added and incubated for 1 h at room temperature. The chromogenic peroxidase substrate 2,2'-azonio-bis(3-ethylbenzthiazoline-6-sulfonic acid) was used to detect the bound HRP-streptavidin. The absorbance at 405 nm was measured after 30 min.

Cell viability was evaluated using the crystal violet assay after the same incubation protocol with neuraminidase or control medium. The cells were stained with 0.05% crystal violet and 2% ethanol in PBS for 30 min at room temperature. After four washes with PBS, the stain was dissolved in methanol and measured at 550 nm using a Fusion Spectrophotometer (Packard Bell). Each of these experiments was performed in duplicate.

Statistical analysis

All of the data were collected in a computerized database and subsequently analysed using SPSS and GraphPad software (SPSS Inc.; Chicago, IL, USA; GraphPad; La Jolla, CA, USA). The Student’s t test or the Mann–Whitney U test was used for quantitative variables and the Pearson’s Chi-square or Fisher’s exact test for qualitative variables, as appropriate.

When more than two groups were compared, we used the Kruskal–Wallis test. When the group medians were significantly different by the Kruskal–Wallis test (P < 0.05), pairwise comparisons were performed with Dunn’s multiple comparison test.

Correlations between SNA levels in the peritoneal fluid and clinical and anatomical characteristics of disease severity measured by semi-quantitative variables were examined using the non-parametric Spearman’s rank correlation test.

Comparison of transwell migration in eutopic endometrial stromal cells of patients and controls was performed using a non-parametric test for matched samples (Wilcoxon matched pairs test).

P-values <0.05 were considered significant.

Results

Study population

There were 102 women with endometriosis and 71 disease-free women included in the study. The patients’ characteristics are presented in Table I. No differences were found in terms of age, gravidity and parity between the study group and the control group. The BMI was significantly lower in the endometriosis group (P = 0.01). The percentages of patients in the secretory and in the proliferative phases were similar in the two study groups. The percentage of patients undergoing hormonal treatment was also similar between the endometriotic patients and the controls.
Abnormal ST6GALNAC1 and ST6GALNAC5 expression in the ectopic endometrium of endometriosis patients

The baseline characteristics of the patients used for the PCR assays are presented in Supplementary Table SII. The two groups were comparable for all the variables. The basal levels of expression of the two STs studied in the endometrium of disease-free women are presented in Supplementary Table SIII. The level of ST6GALNAC1 mRNA was more than a thousand times higher than the level of ST6GALNAC5 mRNA.

Figure 1 and Supplementary Table SIV depict the relative changes in gene expression of the enzymes in the eutopic and ectopic endometria of endometriosis patients compared with the endometrium of healthy women. ST6GALNAC1 expression was significantly lower in ectopic endometrium compared with healthy tissue and eutopic endometrium of endometriosis-affected patients (16-fold for both, $P < 0.01$). Conversely, ST6GALNAC5 expression was significantly higher in ectopic endometrium compared with eutopic endometrium of disease-free women and endometriosis patients (42.2- and 34.3-fold, respectively, $P < 0.01$, Fig. 1).

Reduced $\alpha$-2,6 sialylation in the peritoneal fluid of endometriosis-affected women

The amount of lectins recognizing specific glycoforms in the peritoneal fluid of the endometriosis patients and the controls is presented in Figs 2–4. There was no difference in the amount of mannose-specific lectin (Con-A, Fig. 2) or in the $\alpha$-2,3 sialic acid-specific lectin (MAACKIA, Fig. 3), between the two groups. By contrast, we observed that the amount of $\alpha$-2,6 sialic acid-specific lectin (SNA) in the endometriosis-affected group was significantly lower ($P < 0.01$, Fig. 4). Subgroup analysis according to the endometriotic phenotype revealed that the lower level in the endometriosis patients was mainly due to the low values in the DIE group (Fig. 4).

In order to assess whether SNA levels could be a suitable biomarker of disease severity, we evaluated its correlation with various clinical parameters. SNA levels in the peritoneal fluid did not correlate with deep dyspareunia ($r = -0.226; P = 0.05$) or NCCPP ($r = -0.144; P = 0.20$). However, there were significant negative correlations between peritoneal fluid SNA levels and dysmenorrhea ($r = -0.462; P < 0.001$), gastrointestinal symptoms ($r = -0.384; P = 0.001$), the total ASRM score ($r = -0.268; P = 0.012$), and the ASRM adhesion score ($r = -0.301; P = 0.005$). Low $\alpha$-2,6

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### Table I Baseline characteristics of patients.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Endometriosis ($n = 102$)</th>
<th>Controls ($n = 71$)</th>
<th>P-value$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)$^a$</td>
<td>$30.8 \pm 5.3$</td>
<td>$31.5 \pm 5.3$</td>
<td>$0.460$</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)$^a$</td>
<td>$21.4 \pm 2.9$</td>
<td>$22.8 \pm 3.1$</td>
<td>$0.010$</td>
</tr>
<tr>
<td>Parity$^a$</td>
<td>$0.2 \pm 0.6$</td>
<td>$0.4 \pm 0.6$</td>
<td>$0.062$</td>
</tr>
<tr>
<td>Gravidity$^a$</td>
<td>$0.4 \pm 0.9$</td>
<td>$0.7 \pm 0.9$</td>
<td>$0.050$</td>
</tr>
<tr>
<td>Hormonal treatment before surgery (n, %)</td>
<td></td>
<td></td>
<td>$0.070$</td>
</tr>
<tr>
<td>Yes</td>
<td>$44 (43.1%)$</td>
<td>$21 (29.6%)$</td>
<td>$0.327$</td>
</tr>
<tr>
<td>Nature of the hormonal treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined hormonal contraceptives</td>
<td>$12 (27.3%)$</td>
<td>$4 (19.1%)$</td>
<td></td>
</tr>
<tr>
<td>Progestin-only contraceptives</td>
<td>$11 (25%)$</td>
<td>$2 (9.5%)$</td>
<td></td>
</tr>
<tr>
<td>GnRH-agonists</td>
<td>$13 (29.5%)$</td>
<td>$10 (47.6%)$</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>$8 (18.2%)$</td>
<td>$5 (23.8%)$</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>$58 (56.9%)$</td>
<td>$50 (70.4%)$</td>
<td></td>
</tr>
<tr>
<td>Cycle phase if no hormonal treatment</td>
<td></td>
<td></td>
<td>$0.141$</td>
</tr>
<tr>
<td>Proliferative phase</td>
<td>$8 (13.8%)$</td>
<td>$13 (26%)$</td>
<td></td>
</tr>
<tr>
<td>Secretory phase</td>
<td>$13 (22.4%)$</td>
<td>$14 (28%)$</td>
<td></td>
</tr>
<tr>
<td>Missing data</td>
<td>$37 (63.8%)$</td>
<td>$23 (46%)$</td>
<td></td>
</tr>
<tr>
<td>Surgical classification of endometriosis (n, %)$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUP</td>
<td>$35 (34.3%)$</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>OMA</td>
<td>$27 (26.5%)$</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>DIE</td>
<td>$40 (39.2%)$</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Total ASRM score$^a$</td>
<td>$23 \pm 24.7$</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Data are presented as mean ± standard deviation.

$^b$ According to a previously published surgical classification for deeply infiltrating endometriosis by Chapron et al. (2006).

$^c$ Statistical analysis was performed using Fisher’s exact test or Pearson’s Chi-square for qualitative variables and Student’s $t$ test for quantitative variables.
sialylation levels, therefore, correlated with the pain scores and surgical features that are indicative of the extent and the severity of the disease (Fig. 5).

Reduced α-2,6-sialylation in primary cultures of endometriotic cells

SNA levels were assessed by western blot in all of the different primary cell cultures derived from eutopic endometrium of the controls and eutopic and ectopic endometria of the endometriosis patients, as shown in Fig. 6.

For stromal cells, the SNA levels were significantly lower in the ectopic endometriotic cells compared with the eutopic cells of the controls ($P < 0.05$), whereas no statistical difference was found with the eutopic cells of endometriosis patients and the eutopic cells of controls.

For epithelial cells, the SNA levels were lower in both the eutopic and ectopic cells of endometriosis patients compared

Figure 1 Analysis of ST6GALNAC1 and ST6GALNAC5 gene expression in eutopic and ectopic endometrium of endometriosis patients compared with healthy endometrium of disease-free women. The relative amount of messenger RNA in eutopic and ectopic endometrium of endometriosis patients ($n = 16$) is shown compared to healthy endometrium of controls ($n = 15$). The relative expression level was normalized using the geometric mean of three housekeeping genes. The data are presented as means± the standard error of the mean. The statistical analysis was performed using the Kruskal–Wallis test with Dunn’s Multiple Comparison Test. Statistical significance: $P < 0.05$. ST6GALNAC1, α-N-acetylgalactosaminide α-2,6-sialyltransferase 1; ST6GALNAC5, α-N-acetylgalactosaminide α-2,6-sialyltransferase 5.

Figure 2 Peritoneal Concanavalin A levels measured by enzyme-linked immunosorbent assay in endometriosis patients and controls, and according to the surgical classification of endometriosis. The levels of Con-A in the peritoneal fluid of endometriosis patients ($n = 102$; (SUP = 35, OMA = 27, DIE = 40)) are shown compared to controls ($n = 71$). The data are presented as medians, interquartile ranges and extremes. The statistical analysis was performed using the Mann–Whitney U test or the Kruskal–Wallis test with Dunn’s Multiple Comparison Test. None of the comparisons were statistically significant. Con-A, concanavalin A (specific mannose-binding lectin); ENDO, endometriosis; SUP, superficial endometriosis; OMA, endometrioma; DIE, deep infiltrating endometriosis.
with the eutopic cells of controls \((P < 0.05\) and \(P < 0.05\), respectively).

**Enhanced migratory capacities of eutopic endometrial stromal cells of endometriosis patients after desialylation**

The cell viability, assessed using the crystal violet assay, was not altered after neuraminidase treatment (Supplementary Fig. S1). Effective desialylation after neuraminidase treatment was confirmed, as indicated by SNA levels that declined over time (Supplementary Fig. S2).

The impact of desialylation on stromal cell migration was evaluated using a transwell chamber assay (Fig. 7A). The migratory properties of the endometrial cells of the control patients in the lower chamber through Matrigel-coated polycarbonate filters were similar in the presence or in the absence of neuraminidase. By contrast, significantly more of the eutopic endometrial cells of the endometriosis patients migrated into the lower chamber in the presence of neuraminidase.

**Figure 3** Peritoneal *Maackia amurensis* leucoagglutinin levels measured by enzyme-linked immunosorbent assay in endometriosis patients and controls, and according to the surgical classification of endometriosis. The levels of MAACKIA in the peritoneal fluid of endometriosis patients \((n = 102; (SUP = 35, OMA = 27, DIE = 40))\) are shown compared to controls \((n = 71)\). The data are presented as medians, interquartile ranges, and extremes. The statistical analysis was performed using the Mann–Whitney U test or the Kruskal–Wallis test with Dunn’s Multiple Comparison Test. None of the comparisons were statistically significant. MAACKIA, *Maackia amurensis* leucoagglutinin (specific α-2,3 sialic acid-binding lectin); ENDO, endometriosis; SUP, superficial endometriosis; OMA, endometrioma; DIE, deep infiltrating endometriosis.

**Figure 4** Peritoneal *Sambucus nigra* agglutinin levels measured by enzyme-linked immunosorbent assay in endometriosis patients and controls, and according to the surgical classification of endometriosis. The levels of SAMBUCUS in the peritoneal fluid of endometriosis patients \((n = 102; (SUP = 35, OMA = 27, DIE = 40))\) are shown compared to controls \((n = 71)\). The data are presented as medians, interquartile ranges, and extremes. The statistical analysis was performed using the Mann–Whitney U test or the Kruskal–Wallis test with Dunn’s Multiple Comparison Test. *Significantly different from controls \((P < 0.01)\). **Significantly different from OMA and SUP \((P < 0.01)\). ***Significantly different from controls \((P < 0.001)\). SAMBUCUS, *Sambucus nigra* agglutinin (specific α-2,6 sialic acid-binding lectin); ENDO, endometriosis; SUP, superficial endometriosis; OMA, endometrioma; DIE, deep infiltrating endometriosis.
(104.7 ± 28.3 versus 13 ± 5.1 cells per field, respectively, $P = 0.017$) (Fig. 7B). Thus, desialylation increased the migratory potential of the eutopic endometrial stromal cells of the endometriosis patients.

**Discussion**

This study has highlighted a potential role for abnormal sialylation in the pathogenesis of endometriosis, by analysing peritoneal fluids, tissue samples, and stromal and epithelial primary cell cultures extracted from biopsies of ectopic and eutopic endometria of patients with and without endometriosis. We showed that the expression of ST6GALNAC1, an $\alpha$-2,6 ST, was down-regulated in the ectopic endometrium of endometriosis patients. Concomitantly, we found that there was a low level of $\alpha$-2,6 sialylation in the peritoneal fluid of endometriosis-affected women and in stromal and epithelial cells from endometriotic lesions compared with eutopic cells from disease-free women. Moreover, the level of $\alpha$-2,6 sialylation appears to be negatively correlated with the severity of the disease. Finally, treatment with neuraminidase (a sialidase enzyme) increased the in-vitro ability of eutopic endometrial stromal cells of endometriosis patients to migrate, thus suggesting a potential role for reduced sialylation in the establishment of early endometriotic lesions.

The originality of the subject and the methodological design are the main strengths of this study. First, as far as we can tell, this is the first study to examine aberrant sialylation and its potential pathogenetic role in endometriosis. Second, we only included patients who had undergone complete surgical evaluation of the peritoneal cavity and for whom there was histological proof of the disease, and thus the endometriosis state was accurately assessed according to a previously described classification (Chapron et al., 2006). Third, control patients underwent surgical examination as well, and therefore, clinical and histopathological findings were also recorded. Moreover, our control population displayed a diversity of pathologies that reflect the most common benign conditions in gynaecology. Fourth, we chose an in-vitro model based on primary cell cultures derived from endometrial and endometriotic lesions biopsies that is routinely used in our...
Reduced α-2,6 sialylation in endometriosis

Figure 6 Sambucus nigra agglutinin levels measured by western blot in stromal and epithelial endometrial cells of control women and endometriosis patients. Differences in SNA levels among stromal and epithelial cells of controls (n = 6), eutopic endometrial cells and ectopic endometriotic cells of endometriosis-affected women (n = 6) assessed by western blot of cell lysates. The data are presented as means ± standard error of the mean. The statistical analysis was performed using the Kruskal–Wallis test with Dunn’s Multiple Comparison Test. Statistical significance: P < 0.05. SAMBUCUS, Sambucus nigra agglutinin (SNA) (specific α-2,6 sialic acid-binding lectin).

Figure 7 Effect of desialylation on the migration of eutopic endometrial stromal cells, studied by the transwell migration assay after neuraminidase treatment, in endometriosis patients compared to controls. (A) Eutopic endometrial stromal cells of endometriosis patients (n = 6) and endometrial stromal cells of control women (n = 6) migrating through the Matrigel-coated membrane, stained with toluidine blue. Left panels: after incubation in control medium, for 2 h at 37°C. Right panels: after incubation with 1 unit of neuraminidase, for 2 h at 37°C. (B) Quantitative analysis of the migrated cells: the mean number ± standard error of mean of the migrated cells in six microscope fields was calculated for control endometrial and eutopic endometrial stromal cells after incubation in neuraminidase or in control medium. The statistical analysis was performed using the Wilcoxon test. Statistical significance: P < 0.05.

To the best of our knowledge, this is the first report that draws attention to a reduced α-2,6 sialylation in the peritoneal fluid and endometriotic cells of endometriosis patients. Indeed, few studies to
date (Jones et al., 2009; Miller et al., 2010; Berkes et al., 2013; Kocbek et al., 2014) have investigated the glycosylation profile in patients with endometriosis. Berkes et al. (2013) compared the plasma N-glycome of 92 patients with endometriosis and 62 controls undergoing diagnostic/operative laparoscopy, and they identified alterations (a decrease or an increase) in several glycan peaks. Another team found an increase in the levels of biglycan in peritoneal fluids from ovarian endometriosis patients (Kocbek et al., 2014). In regard to endometriotic cell glycosylation patterns, two studies based on endometrial biopsies from endometriosis patients and healthy controls have highlighted reduced fucosylation, α-2,3 sialylation and α-N-acetylgalactosaminylat-
on (Jones et al., 2009; Miller et al., 2010). None of these studies, however, focused on the potential link between aberrant glycosylation and the pathogenesis of the disease. Indeed, there is a paucity of data in the literature in this regard. As far as we can tell, there have only been two studies to date. Rodgers et al. studied the attachment of endometrial cells to peritoneal mesothelial cells according to the level of N- and O-glycosylation of CD44, a transmembrane glycoprotein that aids in cell migration and adhesion in the peritoneal cavity. They found that glycosylation inhibition reduced endometrial cell attachment to the peritoneum (Rodgers et al., 2011). Wimalachandra et al. (2018) investigated the role of glucosylceramides, which are bioactive sphingolipids known to be abundantly present in the endometrial tissue of endometriosis patients and that enhance signals for cell migration, and they showed that inhibition of ceramides glycosylation attenuated cell motility. Yet, both studies used immortalized human endometrial cell lines instead of primary cell cultures from endometriosis patients, which can be considered to be a bias when exploring the pathogenesis of endometriosis.

As tumour cell sialylation has long been linked with cancer progression, cancer studies could shed light on how reduced sialylation could be associated with increased endometriotic cell migration and invasiveness. Unfortunately, mechanistic studies of such cell surface modifications have also been limited and the data are conflicting. One hypothesis was provided by Liu et al. (2011), who studied epidermal growth factor receptor (EGFR) sialylation and its impact on the receptor function in different lung cancer cell lines. EGFR, a transmembrane glycoprotein overexpressed in various cancers (De Luca et al., 2008), participates in the regulation of cancer invasion and is also known to be up-regulated in endometriosis (Aghajanova and Giudice, 2011; Liu et al., 2015; Chatterjee et al., 2018). The study showed that reduced EGFR sialylation enhanced the receptor activation, thereby increasing EGFR-mediated cell invasion. One could presume that reduced sialylation in endometriotic cells similarly activates the EGFR-pathway and thus, increases cell invasiveness. Another explanation has been provided by Bassagañas et al. (2014), who investigated the impact of sialylation on the regulation of two glycoprotein functions, α2β1 integrin and E-cadherin, both of which are involved in the processes of cancer cell migration, using a pancreatic adenocarcinoma cell model. α2β1 integrin is the main transmembrane receptor for type I collagen and it interacts with intracellular signal transducers that in turn activate key pathways involved in the cell migration. E-cadherin is centrally involved in cell-cell contacts by orchestration of adhesions junctions. Both of these adhesion molecules are thought to play important roles in the development of endometriotic lesions (Lin et al., 2018; Rutherford et al., 2018; Zhu et al., 2018). The results of Bassagañas et al. indicate that a lower α-2,6 sialic acid content on both of these molecules leads to reduced cell-cell adhesiveness, thereby endowing the cells with a more invasive phenotype. A similar pathogenetic mechanism might occur during the development of endometriosis.

However, most studies of this topic in the cancer research literature indicate that there is, in fact, a correlation between increased sialylation and tumour aggressiveness, metastatic potential, and poor prognosis (Pinho and Reis, 2015). Indeed, cancer cells often have high levels of ST expression and elevated levels of sialylated glycan, which is opposite to our findings in endometriosis. These patterns are often associated with malignancy and a poor prognosis in patients (Nakamori et al., 1993; Amado et al., 1998). For instance, Wen et al. (2017) were able to show that a high level of expression of ST3GAL1 was associated with peritoneal dissemination of human ovarian cancer cells and an advanced stage of epithelial ovarian cancer. Tamura et al. (2016) found that RNA interference-mediated gene silencing of ST6GALNAC1 suppressed the metastatic potential of gastric cancer cells. Bos et al. (2009) have reported that ST6GALNAC5 expression in breast cancer cells enhanced their passage through the blood–brain barrier and was associated with brain metastasis. All these data are in favour of a link between hypersialylation and metastatic potential. Our results regarding the hyposialylation pattern of endometriotic cells are strikingly different and are in keeping with the fact that endometriosis is a benign disease with features that are distinct from cancer, notably local invasion and the absence of metastasis. Moreover, in a transcriptomic study of endometriotic lesions (Borghese et al., 2008), genes controlling the cell cycle were shown to be systematically downregulated, preventing endometriotic cells from attaining a more aggressive phenotype. These findings are consistent with the natural history of this chronic disease, in which malignant transformation occurs rarely (one case out of every 1000 patients with endometriosis during 10 years of follow-up) (Saavalainen et al., 2018) and dissemination outside the peritoneal cavity is very uncommon (Wang et al., 2016).

In conclusion, we have shown that the expression of ST6GALNAC1 was down-regulated in ectopic endometriotic lesions, and this was associated with a reduced level of α-2,6 sialylation, using an *in vitro* model of primary cell cultures provided by biopsies of eutopic and ectopic endometria of patients with and without endometriosis. The hyposialylation pattern of endometriotic cells appears to be associated with an enhanced capacity of cells to migrate, which may contribute to the establishment of early endometriotic implants. This was the first study to focus on aberrant sialylation in endometriosis and its possible role in the pathogenesis of the disease. Further investigations are needed to confirm these findings and to provide new insights into the pathophysiology of this enigmatic disease, thereby possibly leading to new therapeutic targets for this complex disorder.

**Supplementary data**

Supplementary data are available at *Human Reproduction* online.

**Authors’ roles**

P.S., C.C. and F.B. conceived of and designed the study. P.S., L.M., S.C., I.G., L.D., M.J., P.G. and F.R. collected the data and performed the experiments. P.S. developed the statistical analyses. C.M., P.S. and F.B. authored the article. All of the authors read and approved the final version of the article.
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Conflict of interest
There are no conflicts of interest.

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