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Density of small diameter sensory nerve fibres in endometrium: a semi-invasive diagnostic test for minimal to mild endometriosis

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BACKGROUND: The aim of our study was to test the hypothesis that multiple-sensory small-diameter nerve fibres are present in a higher density in endometrium from patients with endometriosis when compared with women with a normal pelvis, enabling the development of a semi-invasive diagnostic test for minimal–mild endometriosis.

METHODS: Secretory phase endometrium samples ($n = 40$), obtained from women with laparoscopically/histologically confirmed minimal–mild endometriosis ($n = 20$) and from women with a normal pelvis ($n = 20$) were selected from the biobank at the Leuven University Fertility Centre. Immunohistochemistry was performed to localize neural markers for sensory C, Aδ, adrenergic and cholinergic nerve fibres in the functional layer of the endometrium. Sections were immunostained with anti-human protein gene product 9.5 (PGP9.5), anti-neurofilament protein, anti-substance P (SP), anti-vasoactive intestinal peptide (VIP), anti-neuropeptide Y and anti-calcitonine gene-related polypeptide. Statistical analysis was done using the Mann–Whitney *U*-test, receiver operator characteristic analysis, stepwise logistic regression and least-squares support vector machines.

RESULTS: The density of small nerve fibres was ~14 times higher in endometrium from patients with minimal–mild endometriosis (1.96 ± 2.73) when compared with women with a normal pelvis (0.14 ± 0.46, $P < 0.0001$).

CONCLUSIONS: The combined analysis of neural markers PGP9.5, VIP and SP could predict the presence of minimal–mild endometriosis with 95% sensitivity, 100% specificity and 97.5% accuracy. To confirm our findings, prospective studies are required.

Key words: endometriosis / semi-invasive diagnosis / nerve fibres

Introduction

Endometriosis is a common, chronic gynaecological disease defined by the ectopic presence of endometrial glands and stroma, most commonly in the pelvis. It is symptomatically associated with infertility and pelvic pain including dysmenorrhoea, dyspareunia, dyschezia and chronic pelvic pain (Milingos *et al.*, 2003; Sinaii *et al.*, 2008). Endometriosis-associated pain can be caused by peritoneal inflammation, adhesion formation and specific innervation of endometriotic lesions and is correlated with the presence of deep infiltrating disease (Anaf *et al.*, 2002; Berkley *et al.*, 2005; Mechsner *et al.*, 2007; Wang *et al.*, 2009). However, there is a poor correlation between pain and the degree of endometriosis (Chapron *et al.*, 2003) (minimal–mild–moderate–severe), as determined according to the revised staging system of American Society for Reproductive Medicine (American Society for Reproductive Medicine, 1997).

For a definitive diagnosis of endometriosis, visual inspection of the pelvis at laparoscopy is the 'gold standard' investigation, ideally combined with histological confirmation (Kennedy *et al.*, 2005). However, laparoscopy is a surgical procedure with rare but significant potential risks for the patients (Slack *et al.*, 2007).

Owing to the lack of a no- or semi-invasive diagnostic tool, the delay between onset of pain symptoms and surgically confirmed endometriosis can be as long as 8 years in the UK and USA (Hadfield *et al.*,

1996; Sinaii et al., 2008). The current delay in diagnosis and treatment contributes to years of suffering and potential infertility if the disease is left untreated. Clearly, a simple non-invasive diagnostic method may greatly help to reduce this delay, especially for minimal–mild endometriosis which cannot be diagnosed by clinical examination or ultrasound.

Attempts for non-invasive diagnosis of endometriosis based on the analysis of biomarkers in peripheral blood have been limited by insufficient sensitivity and specificity (D'Hooghe et al., 2004; Kyama et al., 2006, 2008). On the basis of the fact that eutopic endometrium from women with endometriosis is biologically different from women with a normal pelvis (Evans et al., 2007; Berbic et al., 2009; Tran et al., 2009), a semi-invasive diagnostic test for endometriosis can potentially be developed in endometrium obtained after transcervical endometrial biopsy. Whatever method is used, the most important property of any diagnostic test is high sensitivity in order to ensure that no women with endometriosis or other significant pelvic pathology are missed who might benefit from surgery for infertility and/or pain (D'Hooghe et al., 2006).

In recent studies, a higher density of small unmyelinated nerve fibres has been shown in the functional layer of endometrium from women with confirmed endometriosis when compared with women without endometriosis, especially in the secretory phase of the cycle (Tokushige et al., 2006, 2007). Indeed, sensory nerve fibres can be identified in functional layer endometrium by immunohistochemical analysis of various neural transmitters such as substance P (SP), vasoactive intestinal polypeptide (VIP) or neural proteins like protein gene product 9.5 (PGP9.5), neurofilament (NF), neuropeptide Y (NPY) and calcitonine gene-related protein (CGRP). The detection of endometrial nerve fibres has been proposed as a diagnostic tool for endometriosis in a recent pilot study (Al-Jefout et al., 2007). However, this study was limited by the lack of uniform histological confirmation of endometriosis, inclusion of variable numbers of patients from all stages of the disease and by cycle phase-related changes of endometrium.

In the present study, we tested the hypothesis that women with minimal and mild endometriosis express a higher density of sensory small-diameter nerve fibres in the functional layer of endometrium than women with a normal pelvis in order to develop a possible semi-invasive diagnostic tool for minimal to mild endometriosis.

Materials and Methods

Tissue collection

In this study, 40 endometrial samples were selected from the biobank at the Leuven University Fertility Centre where tissues from women undergoing laparoscopies for infertility and/or pain have been stored since 1998. Endometrial biopsies were obtained after hysteroscopy and before laparoscopy using a Pipelle (Pipelle de Cornier, Paris, France), which is a sterile and disposable plastic cannulae for sampling endometrium (Mutch et al., 2007). All patients had signed a written informed consent before recruitment, and the study protocol had been approved by the Institutional Ethical and Review Board of University Hospital Gasthuisberg.

Endometrial samples were selected based on cycle phase, on the presence/absence of endometriosis and on the absence of medical treatment for endometriosis within 3 months before sample collection. Menstrual cycle stage was reported as per the patient's report of last menstrual period and by histological evaluation of the endometrial tissues according to the criteria of Noyes et al. (1975).

Only samples collected during the secretory phase of the cycle were selected, since the density of multiple small nerve fibres is higher during this phase than during other phases of the cycle (Tokushige et al., 2006). Twenty endometrial samples were selected from women with laparoscopically and histologically confirmed minimal ($n = 10$) or mild ($n = 10$) endometriosis (mean age 33 ± 10 years), staged according to the revised staging system of American Society for Reproductive Medicine (American Society for Reproductive Medicine, 1996). Another 20 endometrial samples were selected from women with a laparoscopically confirmed normal pelvis (mean age 32 ± 5 years). The prevalence of dysmenorrhoea, dyspareunia and chronic pelvic pain was comparable in patients with and without endometriosis (Table I).

Demographic data of our study population are shown in Table I.

Histology

All biopsies had been fixed in 10% neutral-buffered formalin immediately after collection for at least 24 h, processed, paraffin embedded and stored at room temperature until further use. For this study, paraffin blocks were sectioned at 4 μm thickness on a Leica microtome (type 2055 Autocut, Nussloch, Germany). One hundred serial sections were collected in sets of four subsequent sections on 25 silane-coated slides and were air-dried at 37°C. Every 10th slide of this series was stained with haematoxylin–eosin for morphological evaluation. For immunohistochemical evaluation, we selected sections that exhibited clear histological features consistent with a normal secretory phase.

Immunohistochemistry

Tissue sections were preheated for 2 h at 55°C, then deparaffinized and rehydrated. After rinsing in 0.01 M tris-buffered saline (TBS), the tissue sections were heat retrieved in 0.01 M TBS pH 9 with 0.001 M EDTA. Serial sections were incubated overnight at 4°C with monoclonal mouse anti-human NF (ready to use; Dako, Glostrup, Denmark) polyclonal rabbit anti-PGP9.5 (diluted 1:900; Dako), polyclonal rabbit anti-SP (diluted 1:2000; Serotec, Raleigh, NC, USA), monoclonal mouse anti-CGRP (diluted 1:2000; Sigma, St Louis, MO, USA), polyclonal rabbit anti-VIP (diluted 1:1400; Chemicon, Temecula, CA, USA) and polyclonal rabbit anti-NPY (diluted 1:2000, Chemicon) respectively. The antibodies were detected with REAL Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse (Dako) according to the manufacturer's instructions. Non-specific immunoglobulin binding was blocked with a mixture of BSA (2%), Tween-80 (0.1%) and non-fat dried milk (1%) applied for 15–45 min before the first and the second antibody incubations; 0.01 M TBS was used for all dilutions and rinsing steps throughout the staining procedure and all steps were carried out at room temperature except when otherwise stated. Sections were counterstained lightly with Mayer's haematoxylin and mounted in glycerine jelly. We used normal human skin as a positive control as it reliably contains myelinated and unmyelinated nerve fibres expressing PGP9.5, VIP, SP, CGRP, NPY and NF. Rabbit and mouse immunoglobulin fractions were used as respective negative controls, and the concentrations were matched with the concentrations of the antibodies.

Assessment of nerve fibre density was performed using image analysis software KS400 3.0 (Zeiss, Göttingen, Germany) linked to a Zeiss microscope (Axioskop 50) fitted with a Zeiss color camera (Axiocam MRc5). The evaluation of all immunohistochemical staining was done blindly by the first author (A.B.) who evaluated the whole surface of each section on high-power images (objective 40x, optovar 1, resolution 860×644 Px) of adjacent non-overlapping fields from left to right and from top to bottom. Each high-power field (HPF) covered a maximal area of

Table I Demographic characteristics of the study population ($n = 40$)

	Endometriosis ($n = 20$)	Controls ($n = 20$)
Age (years, mean ± SD)	33 ± 10	32 ± 5
Gravidity/parity (mean ± SD)	0.1 ± 0.3/0.05 ± 0.22	0.35 ± 0.87/0.15 ± 0.7
Primary/secondary infertility [*n* (%)]	18 (90)/2 (10)	17 (85)/3 (15)
Chronic pelvic pain [*n* (%)]	0 (0)	0 (0)
Dysmenorrhoea [*n* (%)]	3 (15)	2 (10)
Dyspareunia [*n* (%)]	0 (0)	1 (5)
Concurrent hormonal medication [*n* (%)]	0 (0)	0 (0)
Previous treatment for infertility [*n* (%)]	3 (15)	4 (20)
Ovulation induction	1 (5)	0 (0)
Laparoscopic surgery	2 (10)	4 (20)
Indication for surgery [*n* (%)]		
Infertility	2 (10)	4 (20)
Pelvic pain	0 (0)	0 (0)
Ethnicity [*n* (%)]		
Caucasian	20 (100)	19 (95)
Asian	0 (0)	1 (5)

0.0789 mm^2 from which all irrelevant zones (i.e. artefactual or not belonging to the actual tissue) were subtracted before the measurement of the actually assessed field area. Within these HPFs, all nerve fibre profiles expressing neural markers were counted with exclusion of those crossing the right or the bottom side of the field frame, respectively, thus avoiding to count these fibre profiles twice. After summation of the nerve fibre counts and the HPF area values for the whole section, the total number of nerve fibres was divided by the total surface area of the examined endometrium to obtain the nerve fibre density for the current section. The average duration of screening of one specimen was 30 ± 10 min.

Statistical analysis

Data are presented as mean (SD) number of nerve fibres/mm^2. Numerical data were analysed using Excel (version 5.0; Microsoft Corporation, Redmond, WA, USA). Variables were tested for normality using the Kolmogorov–Smirnov Lilliefors and the Shapiro–Wilk tests before univariate analysis of the data using the Mann–Whitney *U*-test. All marker data were used as continuous variables. The cut-offs (Table III) were calculated during the calculation of specificity and sensitivity using the statistical software Prism 5.0. (GraphPad Software Inc., La Jolla, CA, USA). For the least-squares support vector machines (LS-SVM) modelling, no discretization or categorization was performed. The continuous values of the markers were used when building models (i.e. multivariate logistic regression and LS-SVM).

The differences of nerve fibre density between eutopic endometrium from women with and without endometriosis were tested for significance by the Mann–Whitney *U*-test, using the statistical package Prism 5.0 (GraphPad Software Inc.).

Multivariate analysis was done using stepwise logistic regression (SAS 9.1.3 for Windows, Cary, NC, USA) and stepwise logistic regression and LS-SVM (MATLAB scripts were downloaded from LS-SVMlab version 1.5 http://www.esat.kuleuven.ac.be/sista/lssvmlab/). For stepwise logistic regression, only variables with significant odds ratios (P-value < 0.05) were allowed in the model.

For LS-SVM analysis, feature selection was performed based on leave-one-out cross-validation (LOO-CV) analysis. Briefly, in each LOO-CV, the neural markers were ranked according to their P-value (Mann–Whitney *U*-test). Then, the top '*n*'-features were selected where '*n*' ranged from 1 to 6 (corresponding to all neural markers). The '*n*' with the lowest LOO-CV error was selected to build a model on the full data set. The models were evaluated based on their area under the receiver operator characteristic (ROC) curve (AUC) (Hanley and McNeil, 1982).

Additionally, an operating point on the ROC curve was chosen corresponding to the maximum of the sum of sensitivity and specificity. Then, models were also evaluated by their sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV).

Results

In 90% (18/20) of women with endometriosis, nerve fibres were observed in the endometrium (Table II). In this group, immunohistological staining was positive for PGP9.5, SP, CGRP, VIP and NPY but not for NF, except in one patient, suggesting that almost all small nerve fibres were unmyelinated and represent a mixture of sensory C, adrenergic and (in smaller amount) myelinated sensory Aδ and cholinergic nerve fibres (Fig. 1). However, these nerve fibres were not distributed homogeneously throughout the endometrium. The density of nerve fibres was markedly skewed, with few specimens showing counts above 30/mm^2 and with most between 0 and 10/mm^2. There was no significant difference between the nerve fibre densities in women with confirmed minimal endometriosis (2.1 ± 2.87) and those with mild endometriosis (1.84 ± 2.59, $P = 0.46$).

In only 40% (8/20) of women without endometriosis, only small numbers of PGP9.5-stained nerve fibres were present and were positive for SP, CGRP, VIP and NPY but not for NF, except in two patients (Table II).

Univariate and multivariate analyses are shown in Tables III and IV, respectively. No multivariate logistic regression model could be built

Table II Quantitative assessment of the endometrial nerve fibre density stained against different neural markers in patients with and without endometriosis

Marker	EM nerve fibre density: total number of nerve fibres/mm^2 EM surface area screened [median (range), mean ± SD]		Total number of nerve fibres present in total EM surface area screened/patient [median (range), mean ± SD]		Total EM surface area screened (mm^2)/patient [median (range), mean ± SD]
	Endo (n = 20)	Control (n = 20)	Endo (n = 20)	Control (n = 20)	
PGP9.5	2.30 (0–9.23), 2.62 ± 2.19‡	0.0 (0–0.79), 0.21 ± 0.28‡	9 (0–32), 11.10 ± 7.92‡	0 (0–5), 1.20 ± 1.73‡	5.74 (2.13–10.55), 5.65 ± 2.02
NPY	1.73 (0–18.05), 2.52 ± 3.91‡	0.0 (0–0.62), 0.15 ± 0.23‡	5 (0–31), 7.7 ± 7.76‡	0 (0–5), 1.05 ± 1.70‡	5. 84 (1.57–9.82), 5.68 ± 2.44
CGRP	1.58 (0–4.9), 1.94 ± 1.58‡	0.0 (0–0.68), 0.08 ± 0.19‡	5 (0–31), 6.85 ± 7.2‡	0 (0–3), 0.45 ± 0.99‡	5.54 (1.89–10.05), 5.53 ± 2.70
SP	1.50 (0–8.45), 2.29 ± 2.2‡	0.0 (0–0.56), 0.1 ± 0.2‡	6 (0–27), 7.95 ± 7.04‡	0 (0–3), 0.55 ± 1.05‡	5. 92 (1.43–10.07), 5.54 ± 2.5
VIP	0.71 (0–16.79), 2.37 ± 3.77‡	0.0 (0–0.43), 0.06 ± 0.15‡	4.5 (0–22), 7.75 ± 6.9‡	0 (0–3), 0.85 ± 1.95‡	5.84 (1.01–9.81), 5.63 ± 2.12
NF	0.0 (0–0.45), 0.02 ± 0.10¶	0.0 (0–4.68), 0.25 ± 1.04¶	0 (0–1), 0.05 ± 0.22¶	0 (0–30), 1.60 ± 6.70¶	6.19 (1.73–9.99), 5.92 ± 2.32

EM, endometrium; Endo, patients with endometriosis; Control, women with a normal pelvis.
‡$P < 0.0001$.
¶NS.

that corresponded to our criteria ($P < 0.05$ on odds ratios). Using LOO-CV analysis with LS-SVM modelling (Table IV), the best result was obtained when selecting the top three neural markers on the basis of their P-value (Mann–Whitney U-test). A LS-SVM model, built on the complete data set with the top three neural markers VIP, PGP9.5 and SP had an AUC of 0.99 (SE 0.01) (Fig. 2). After choosing an operating point, this model allowed the diagnosis of endometriosis with a sensitivity of 95%, specificity of 100%, accuracy of 97.5%, PPV of 100% and NPV of 95%, corresponding to one endometriosis patient classified as control by the model (i.e. false negative).

Discussion

Other authors provided initial evidence that the assessment of nerve fibre density in eutopic secretory endometrium can be used as a diagnostic test for endometriosis (Al-Jefout *et al*., 2007; Tokushige *et al*., 2008). Here, we provide further evidence that this technique can be used as a diagnostic test for minimal to mild endometriosis (American Society for Reproductive Medicine, 1996) with high sensitivity (95%) and high specificity (100%). Only women with endometriosis who had not received any hormonal treatment of endometriosis within 3 months of endometrial biopsy were included, since it has been reported that hormonal medical treatment significantly decreases the multiple small nerve fibre density in the functional layer of endometrium (Tokushige *et al*., 2008).

Our study design differs from that of previous fundamental studies (Al-Jefout *et al*., 2007) in a number of important ways. First, only patients with the highest need for a non-invasive diagnostic test, i.e. patients with minimal–mild endometriosis were included, whereas in previous studies (Al-Jefout *et al*., 2007), a mixed population of women with minimal–severe endometriosis was studied. It is well accepted that women with moderate–severe endometriosis are less in need of a non-invasive diagnostic test since this degree of endometriosis can be diagnosed clinically and by imaging methods fairly accurately (Kennedy *et al*., 2005).

Second, all cases with endometriosis were confirmed by both laparoscopy and histology, whereas in the previous study, histological confirmation was not available in all cases (Al-Jefout *et al*., 2007).

Third, only secretory phase endometrium was selected since the highest density of nerve fibres is observed during this phase and since we wanted to rule out cycle phase-dependent changes in endometrial nerve fibre density (Tokushige *et al*., 2006), whereas in previous studies endometrium from mixed unspecified phases of the cycle was studied (Al-Jefout *et al*., 2007). In our study, the choice to analyse only secretory phase endometrial samples may reduce the utility of this test, since many women have quite inaccurate recall of their cycle phase. The rationale for this choice was the observation that the expected density of sensory nerve fibres is higher in the secretory phase than in the other phases of menstrual cycle (Tokushige *et al*., 2006). We recognize that additional studies are needed to confirm that our model is also applicable during other phases of the menstrual cycle.

Fourth, taking into consideration our observation and previous reports showing that nerve fibres are not distributed homogeneously in the functional layer of endometrium (Tokushige *et al*., 2006, 2007; Al-Jefout *et al*., 2007), we examined the whole surface of all specimens to avoid inevitable bias resulting from randomly chosen fields, whereas previous investigators only assessed randomly chosen fields

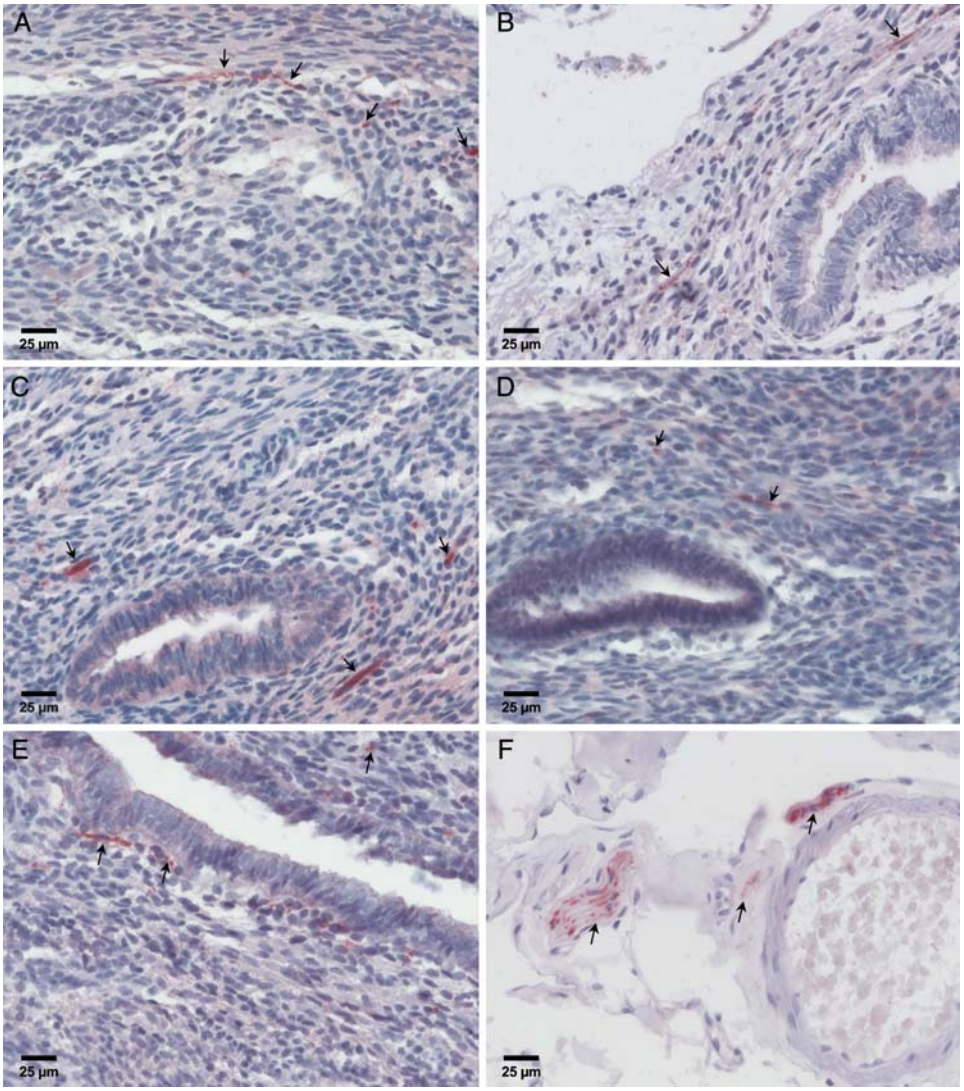

Figure 1 Small-diameter nerve fibres in eutopic endometrium in women with minimal and mild endometriosis. Eutopic endometrium stained with PGP9.5 (**A**), VIP (**B**), SP (**C**) NPY (**D**) and CGRP (**E**). Arrows denote tiny positive multiple nerve fibres. Eutopic endometrium from woman with endometriosis stained with NF (**F**). Arrows denote perivascular myelinated nerve fibres. Magnification ×400. Scale bars represent 25 µm.

Table III Univariate analysis of different endometrial neural markers for the semi-invasive diagnosis of minimal and mild endometriosis

Marker	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	Cut-off value for nerve fibre density	AUC (95% CI)
PGP9.5	95 (75.13–99.87)	75 (50.90–91.34)	79.19	93.75	0.49	0.94 (0.86–1.02)
VIP	95 (75.13–99.87)	80 (56.34–94.27)	82.6	84.21	0.08	0.94 (0.87–1.00)
CGRP	90 (68.30–98.77)	85 (62.11–96.79)	85.71	89.47	0.23	0.92 (0.83–1.01)
SP	95 (75.13–99.87)	80 (56.34–94.27)	82.6	84.21	0.2	0.90 (0.85–1.01)
NPY	95 (75.13–99.87)	65 (40.78–84.61)	72	86.66	0.13	0.90 (0.80–0.99)
NF	95 (75.13–99.80)	10 (1.23–31.70)	0.33	48.64	0.19	0.52 (0.34–0.70)

Data are presented as percentages. PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve for diagnostic accuracy.

Table IV Selecting the number of neural markers for LS-SVM modelling based on LOO-CV

Number of neural markers	AUC (SE)
1	0.56 (0.10)
2	0.84 (0.07)
3	0.98 (0.02)
4	0.94 (0.05)
5	0.96 (0.03)
6 (all)	0.94 (0.04)

AUC, area under the ROC curve; SE, standard error.

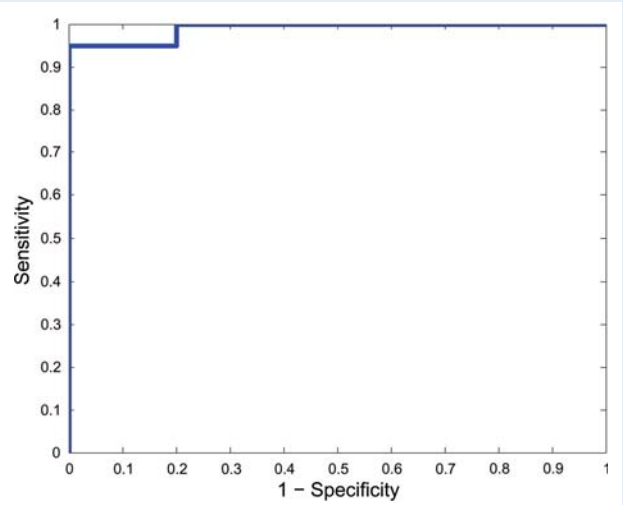

Figure 2 ROC curve of the LS-SVM model built using PGP9.5, VIP and SP.

(Tokushige *et al*., 2006, 2007; Al-Jefout *et al*., 2007).Using this meticulous approach, it is not surprising that the range of nerve fibre densities was considerably lower in our study (0–18.05/mm^2) than in previous studies (1.6–125/mm^2) (Al-Jefout *et al*., 2007) and that the mean nerve fibre density for PGP9.5 positive nerve fibres in secretory phase endometrium was six times lower in our patients with minimal to mild endometriosis (2.62 $\pm$ 2.19/mm,^{2} mean $\pm$ SD) compared with the patients with mixed stages of endometriosis investigated in a previous study (13 $\pm$ 6/mm,^{2} mean $\pm$ SD) (Tokushige *et al*., 2006). This methodology may also explain why our diagnostic model could not confirm previously reported (Al-Jefout *et al*., 2007) results showing 100% sensitivity and 100% NPV for the diagnosis of minimal–severe endometriosis.

Fifth, the multivariate statistical methods used in our study were superior to the univariate analyses described by previous investigators (Al-Jefout *et al*., 2007).

It can be questioned whether LS-SVM or SVM modelling in general is already considered widely applicable to clinical settings. In our opinion, advanced mathematical analysis using these models has penetrated clinical research (De Smet *et al*., 2006; Pochet and Suykens, 2006) and has been widely accepted. Indeed, the application of a Bayesian network for diagnosis of pregnancies of unknown location has been published previously by our group (Gevaert *et al*., 2006). Logistic regression, artificial neural networks and support vector machines have also been used in the diagnosis of malignancy of ovarian masses (Van Holsbeke *et al*., 2009). Additionally, the use of LOO-CV has been widely accepted as a replacement for an independent test set for small data sets (G. Cawley, IJCNN 2006, pp. 1661–1668) in several clinical applications (Hedenfalk *et al*., 2001; Hoshida *et al*., 2008). However, we acknowledge that no technique can completely replace an independent test set and our results will have to be confirmed on prospectively collected data.

In our study, we selected a panel of neural biomarkers that are known (Tokushige *et al*., 2006, 2007; Al-Jefout *et al*., 2007) to identify and differentiate nerve fibres. We used PGP9.5 that is a pan-neuronal marker (Lundberg *et al*., 1988; Quinn, 2007), whereas SP and CGRP are sensory nerve fibre markers (Le Greves *et al*., 1985), which can be present in both Aδ and C fibres (Heinrich *et al*., 1986). VIP is a specific marker for parasympathetic neurons and can be present in both sensory and cholinergic nerve fibres (Lynch *et al*., 1980), whereas NPY is a specific marker for sympathetic neurons and can be present in both sensory and adrenergic nerve fibres (Heinrich *et al*., 1986). NF is specific marker for myelinated nerve fibres (Schlaepfer, 1987). Our findings confirm the previously reported data from pioneers (Tokushige *et al*., 2006, 2007; Tariverdian *et al*., 2007) that the endometrium of patients with minimal to mild endometriosis is predominantly innervated by multiple small-diameter sensory (mostly C fibres), adrenergic and, in smaller amount, Aδ and cholinergic nerve fibres. Our data have potentially high impact on the clinical diagnosis and management of endometriosis. Transcervical endometrial biopsy represents an acceptable semi-invasive technique, much less invasive than a laparoscopy, but still possibly associated with some degree of pelvic pain at the time of biopsy. An early semi-invasive diagnosis of minimal–mild endometriosis in women with or without pain who try to conceive should enable gynaecologists to select them for laparoscopic excision of endometriosis that improves pain and fertility (Kennedy *et al*., 2005) and may prevent progression of endometriosis to a moderate to severe stage, since endometriosis is a progressive disease in at least 50% of the cases (D'Hooghe and Debrock, 2002).

Our proposed semi-invasive diagnostic test for minimal–mild endometriosis seems to be rather complex. However, we have previously reported that both stepwise logistic regression and LS-SVM can be used to develop models for diagnostic testing in clinical reality (Timmerman *et al*., 2005; De Smet *et al*., 2006; Gevaert *et al*., 2006; Pochet and Suykens, 2006). We believe that, after validation in an independent patient population, our method can be developed into a valuable and relatively simple tool for the clinical diagnosis of minimal–mild endometriosis using autostainers for immunohistochemistry, automated histopathological image analysis and mathematical formula in Excel (Microsoft Corporation, Redmond, WA, USA) that can be used for diagnostic testing.

Although the sample size of our study was relatively small, it was comparable with the sample size of 37 patients used in a previous study (Al-Jefout *et al*., 2007). Nevertheless, due to this limitation, we had to resort to LOO-CV techniques to estimate the independent test set performance. Although LOO-CV is accepted as a

cross-validation technique for small data sets, over fitting is still a danger and the developed models need to be further tested on independent data. We plan additional research to validate and confirm our results in a prospective controlled study.

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Authors' contributions

A.B., C.M.K., T.D., A.F. and A.V. had substantial contributions to conception and design. A.B., C.M.K., O.G., B.D.M. and V.F. contributed to acquisition of data. A.B., L.V., O.G., B.D.M. and T.D. carried out the analysis and interpretation of data. All authors contributed in drafting the article or revising it critically for important intellectual content and final approval of the version to be published.

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