

# Diagnosis of endometriosis by detection of nerve fibres in an endometrial biopsy: a double blind study

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**BACKGROUND:** Diagnosis of endometriosis currently requires a laparoscopy and this need probably contributes to the considerable average delay in diagnosis. We have reported the presence of nerve fibres in the functional layer of endometrium in women with endometriosis, which could be used as a diagnostic test. Our aim was to assess efficacy of nerve fibre detection in endometrial biopsy for making a diagnosis of endometriosis in a double-blind comparison with expert diagnostic laparoscopy.

**METHODS:** Endometrial biopsies, with immunohistochemical nerve fibre detection using protein gene product 9.5 as marker, taken from 99 consecutive women presenting with pelvic pain and/or infertility undergoing diagnostic laparoscopy by experienced gynaecological laparoscopists, were compared with surgical diagnosis.

**RESULTS:** In women with laparoscopic diagnosis of endometriosis ( $n = 64$ ) the mean nerve fibre density in the functional layer of the endometrial biopsy was 2.7 nerve fibres per  $\text{mm}^2$  ( $\pm 3.5$  SD). Only one woman with endometriosis had no detectable nerve fibres. Six women had endometrial nerve fibres but no active endometriosis seen at laparoscopy. The specificity and sensitivity were 83 and 98%, respectively, positive predictive value was 91% and negative predictive value was 96%. Nerve fibre density did not differ between different menstrual cycle phases. Women with endometriosis and pain symptoms had significantly higher nerve fibre density in comparison with women with infertility but no pain (2.3 and 0.8 nerve fibre per  $\text{mm}^2$ , respectively,  $P = 0.005$ ).

**CONCLUSIONS:** Endometrial biopsy, with detection of nerve fibres, provided a reliability of diagnosis of endometriosis which is close to the accuracy of laparoscopic assessment by experienced gynaecological laparoscopists.

This study was registered with the Australian Clinical Trials Registry (ACTR) 00082242 (registered: 12/12/2007). The study was approved by the Ethics Review Committee (RPAH Zone) of the Sydney South West Area Health Service (Protocol number X05-0345) and The University of Sydney Human Research Ethics Committee (Ref. No. 10761) and all women gave their informed consent for participation.

**Key words:** endometriosis diagnosis / laparoscopy / immunohistochemistry / endometrial biopsy / nerve fibres

## Introduction

Endometriosis is a benign gynaecological disease defined as the presence of endometrial-like glands and stroma outside the uterine cavity, most commonly implanted over visceral and peritoneal surfaces within the female pelvis. Endometriosis exhibits disturbances of cellular proliferation, cellular invasion and neoangiogenesis (Giudice *et al.*, 1998). Although the exact prevalence of endometriosis in the general population is not clear, the prevalence in women of reproductive age is

estimated to range between 10 and 15% (Lebovic *et al.*, 2001). Endometriosis is commonly associated with a range of pelvic pain symptoms such as chronic dysmenorrhoea, premenstrual abdominal and pelvic pain, back pain, dysuria, dyschezia and dyspareunia. However, the relationship between different pains and endometriosis is not well understood and there is poor correlation between the severity of pain symptoms and anatomical staging of the disease (Chapron *et al.*, 2003).

The diagnosis of endometriosis is a major stumbling block for both clinical management and research studies of this enigmatic disease.

At the moment, there is no simple, reliable, non-invasive way to diagnose endometriosis, although there are a number of studies currently underway to try and identify 'biomarkers' of this disease. Symptoms like chronic pelvic pain and infertility may suggest the presence of endometriosis; however, laparoscopy is still required for confirmation or exclusion (Kennedy *et al.*, 2005). Unfortunately, there is still a substantial delay in the diagnosis of endometriosis in most countries. The length of time from the onset of symptoms to the definite diagnosis is often quite long, with an average of 6–10 years in many centres. This delay is even longer in young age patients and in severe cases (Hadfield *et al.*, 1996; Zrubek *et al.*, 1999; Ballard *et al.*, 2006; Matsuzaki *et al.*, 2006). A whole range of problems may result from this delay including deterioration of patient's quality of life, progression of the disease, the impact of absence of an explanation of pain, and the financial burden on the budget of medical services. Moreover, laparoscopy is still a surgical procedure and can be associated with uncommon complications (Slack *et al.*, 2007). A simple diagnostic test is urgently needed.

We have previously reported the results of a pilot study using endometrial biopsy for the diagnosis of endometriosis (Al-Jefout *et al.*, 2007). The concept of using endometrial biopsy was possible after reports of the novel finding of multiple small unmyelinated sensory C nerve fibres in the functional layer of eutopic endometrium in all women with endometriosis, although women without endometriosis did not have any nerve fibres in the functional layer (Tokushige *et al.*, 2006a, b). Women with endometriosis also had a highly significantly increased density of nerve fibres in the basal layer of endometrium and in the myometrium compared with women without endometriosis (Tokushige *et al.*, 2006a). These nerve fibres were also found in ectopic peritoneal endometriotic lesions and in deep infiltrating endometriosis (Wang *et al.*, 2009) and expressed a wide range of neural function markers (Tokushige *et al.*, 2006b). The unmyelinated nerve fibres in the functional layer of eutopic endometrium were found to express vasointestinal peptide, neuropeptide Y, Substance P and calcitonin gene-related peptide (Tokushige *et al.*, 2006b), suggesting that these fibres were sensory C, adrenergic and cholinergic.

The results of our pilot study (Al-Jefout *et al.*, 2007) showed that it is possible to make the diagnosis of endometriosis using endometrial biopsy. However, the results of the pilot study needed to be verified by a double-blind study with a larger number of patients and the involvement of more surgeons.

We set up a double-blind prospective study to compare the relative efficacy of a narrow, disposable 'Endosampler' endometrial biopsy suction cannula in comparison with laparoscopy. The endometrial biopsy was used to detect small unmyelinated nerve fibres immunohistochemically [using the pan-neuronal marker protein gene product 9.5 (PGP9.5)] in the functional layer of endometrium in women undergoing diagnostic laparoscopy for pelvic pain or infertility.

## Materials and Methods

### Participants

Inclusion criteria for the study included women in the reproductive age group undergoing laparoscopy for pelvic pain and/or infertility, and not currently receiving hormonal treatment for at least 3 months prior to laparoscopy. Exclusion criteria for the study included suspected pregnancy

and unwillingness to participate in the study. Written informed consent was obtained from all participants [mean age 33.9 years (range: 20–50)].

Patients were recruited and treated at The Royal Prince Alfred Hospital, Sydney. A detailed clinical history was obtained from the participants prior to the procedure. Tissue processing and staining were carried out at the laboratories of the Queen Elizabeth II Research Institute for Mothers and Infants, University of Sydney.

### Surgical procedures and sampling technique

The reference standard in this study for diagnosing endometriosis was laparoscopy and visualization of endometriotic lesions with surgical staging of the disease by five gynaecologists with extensive experience in endometriosis, using the revised American Fertility Society (rAFS) scoring system (rAFS, 1979). Biopsy confirmation of lesions was available in almost all cases.

Endometrial biopsies were obtained prior to laparoscopy using Endosampler (Medgyn® Products Inc., Lombard, Illinois, USA). Meticulous attention was paid to the technique of endometrial biopsy in order to ensure that a narrow but deep endometrial strip was obtained. 'Endosampler' provides a reasonably reliable quality of endometrial sampling (Fig. 1). A solid column of endometrium was much easier to assess immunohistochemically than a superficial and fragmented biopsy.

The quality of endometrial biopsy was histologically assessed prior to nerve fibre counting and four specimens were discarded owing to unsatisfactory quality (very superficial and fragmented). These cases were not included in the study. The quality of the specimens was assessed as: good  $n = 25$ , satisfactory  $n = 51$  and partially fragmented  $n = 23$  (and unsatisfactory, superficial and fragmented,  $n = 4$ ); total = 103. Hence, only 99 biopsies were available for study.

### Histological assessment

All samples underwent haematoxylin and eosin (H&E) staining for conventional histological assessment, and dating was performed blindly by one experienced gynaecological pathologist at The Royal Prince Alfred Hospital, Department of Anatomical Pathology, according to the criteria of Noyes *et al.* (1950).



**Figure 1** Hysteroscopic view of human uterine cavity after endometrial biopsy in the secretory phase.

## Immunohistochemistry

After surgical removal, the specimens were immediately fixed in 10% neutral buffered formalin for ~18–24 h, processed and embedded in paraffin wax according to a standard protocol. Each section was cut at 4  $\mu$ m and routinely stained with H&E. Immunohistochemistry was performed using polyclonal rabbit anti-PGP9.5 (Dako, Australia), a highly specific pan-neuronal marker, which recognizes all types of nerve fibres. Antigen retrieval techniques were used for PGP9.5. Sections were immunostained using the antibody for PGP9.5 (dilution 1:1400). Alkaline Phosphatase/RED, Link, Biotinylated Secondary Antibodies (AB2) and REAL Detection System, Alkaline Phosphatase/RED, Streptavidin Alkaline Phosphatase (AP), and stained with REAL Detection System, chromogen (Fast Red) (Dako, Sydney, Australia). The functional layer of an average of 2–3 sections per specimen was examined for nerve fibres.

All immunostaining was carried out on a Dako Autostainer Model S3400 (Dako, Sydney, Australia). Images of the sections were captured using an Olympus microscope BX51 and digital camera DP70 (Olympus, Tokyo, Japan). We used normal skin as a positive control, as it reliably contains myelinated and unmyelinated nerve fibres expressing PGP9.5. The functional layer of eutopic endometrium from women without endometriosis was used as a negative control as it does not contain any nerve fibres expressing PGP9.5. Rabbit immunoglobulin fraction was also used as a negative control, the concentration being matched with the concentration of the antibodies.

Each patient was given a code and this was not broken until after the analysis at the end of the study. Identification and counting of nerve fibres was undertaken blindly by two different people, both of them with good experience in nerve fibre identification. Blinded counting gave close (98%) correlations between the two individuals. The surgical assessment of endometriosis carried out at laparoscopy was maintained separately by a third party until the blinded assessments of nerve fibres were available and correlations were then made.

## Statistics

An assessment of nerve fibre density was carried out as previously described in our pilot study (Al-Jefout *et al.*, 2007). The distribution of the data was examined prior to analysis. Statistical significance was established at  $P$ -values of  $<0.05$ , and using the Mann–Whitney  $U$ -test for comparison of two groups (denoted by M–W  $U$  z), and the Kruskal–Wallis chi-square test (denoted by K–W  $\chi^2$ ) for the comparison.

## Results

During the period from 12 December 2007 to 10 December 2008, 103 endometrial biopsies were obtained from 103 women undergoing laparoscopy, however, four were discarded due to poor quality so total number of patients included in this double blind study is 99 (women with laparoscopically confirmed endometriosis  $n = 64$ , women with laparoscopic exclusion of endometriosis  $n = 35$ ). Laparoscopy was indicated for a complaint of pain symptoms alone ( $n = 52$ ), infertility alone ( $n = 24$ ), pelvic pain plus infertility ( $n = 20$ ) and no pain and no infertility ( $n = 3$ ). All endometrial samples exhibited conventional histological features consistent with normal menstrual cycle phases (menstrual  $n = 15$  cases; proliferative  $n = 39$ ; mid-cycle  $n = 14$ ; secretory  $n = 31$ ) (Table I). Women in this study with laparoscopically proven endometriosis ( $n = 64$ ) had varying severities of disease according to the rAFS Scoring System (Stage I:  $n = 21$ ; Stage II:  $n = 12$ ; Stage III:  $n = 7$ ; Stage IV:  $n = 24$  women) (Table II).

Superficial peritoneal endometriosis (including one case with diaphragmatic endometriosis) was 50% of all cases with endometriosis at laparoscopy, and bowel and ovarian endometrioma and deep infiltrating endometriosis were found in the other 50% of cases,  $n = 32$  (Table II).

**Table I** Demographic characteristics of women undergoing diagnostic laparoscopy for pelvic pain and/or infertility

Categories	Subcategories (N)	Total 99	Endometriosis diagnosis at laparoscopy	
			Yes 64	No 35
Age (years), mean $\pm$ SE (range)		34.0 $\pm$ 0.6 (20–50)		
Menstrual phase, % (n)	Menstrual	15.2 (15)	17.2 (11)	11.4 (4)
	Proliferative	39.4 (39)	32.8 (21)	51.4 (18)
	Mid cycle	14.1 (14)	17.2 (11)	8.6 (3)
	Secretory	31.3 (31)	32.8 (21)	28.6 (10)
History of endometriosis, % (n)		20.2 (20)	28.1 (18)	5.7 (2)
Symptoms, % (n)	Pain alone	52.5 (52)	62.5 (40)	34.3 (12)
	Infertility alone	24.2 (24)	12.5 (8)	45.7 (16)
	Pain and infertility	20.2 (20)	25.0 (16)	11.4 (4)
	No pain or infertility	3.0 (3)	0.0 (0)	8.6 (3)
Pain symptoms, % (n)	Dysmenorrhea	43.3 (43)	48.4 (31)	34.3 (12)
	Dyspareunia	10.1 (10)	10.9 (7)	8.6 (3)
	Dysmenorrhea/dyspareunia	14.1 (14)	15.6 (10)	11.4 (4)
	Dyschezia	1.0 (1)	1.6 (1)	0.0 (0)
	Dysmenorrhea/dyspareunia/dyschezia	8.1 (8)	12.5 (8)	0.0 (0)
	Cyclical shoulder pain	1.0 (1)	1.6 (1)	0.0 (0)
	No pain symptoms	22.2 (22)	9.4 (6)	45.7 (16)

**Table II** Nerve fibre density per mm<sup>2</sup> in the functional layer of endometrial biopsy in women with and without endometriosis, as diagnosed at laparoscopy

Category	Subcategory	N (*)	Mean fibre density	SD	Range	Statistics
Findings at laparoscopy	Endometriosis diagnosis at laparoscopy	64 (63)	2.7	3.4	0–24.7	M–W $U z = -6.35$ , $P < 0.001$
	No endometriosis	35 (6)	3.1**	1.7	0–8	
Types of Endometriosis	Superficial endometriosis	32	2.7	4.4	0.6–24.7	M–W $U z = -1.88$ , $P = 0.060$
	Endometrioma, bowel and Deeply infiltrating endometriosis	32	2.7	2.2	0.0–10.3	
Quality of endometrial biopsy	Good quality specimen	25	2.9	5.2	0–24.7	K–W $\chi^2 = 1.34$ , $df = 2$ , $P = 0.51$
	Satisfactory	51	1.7	1.9	0–8.0	
	Fragmented	23	1.8	2.0	0–10.0	
Symptoms	Some pain symptoms	77	2.3	3.4	0–24.7	M–W $U z = -2.82$ , $P = 0.005$
	No pain symptoms	22	0.8	1.2	0–3.60	
	Infertility	44	1.3	1.5	0–8.0	M–W $U z = -1.30$ , $P = 0.19$
	No infertility	55	2.5	3.9	0–24.7	
	Both pain and infertility	25	1.7	1.7	0–8.0	K–W $\chi^2 = 2.21$ , $df = 2$ , $P = 0.33$
	Pain or infertility	71	2.1	3.5	0–24.0	
No pain or infertility	3	0.4	0.7	0–1.20		
Menstrual phase	Menstrual	15	2.0	2.5	0–10.5	K–W $\chi^2 = 0.97$ , $df = 3$ , $P = 0.81$
	Proliferative	39	2.2	4.3	0–24.7	
	Mid cycle	14	1.9	2.4	0–8.0	
	Secretory	31	1.7	1.5	0–5.0	
History of endometriosis	No history of endometriosis	79	1.6	2.1	0–10.3	M–W $U z = -2.47$ , $P = 0.01$
	Endometriosis history	20	3.5	5.4	0–24.7	
Stage of disease at laparoscopy	Stage I	21	2.6	5.1	0.8–24.7	K–W $\chi^2 = 6.78$ , $df = 3$ , $P = 0.08$
	Stage II	12	3.2	2.5	0.6–10.0	
	Stage III	7	2.4	1.4	1.0–5.0	
	Stage IV	24	2.7	2.4	0–10.3	

\*Number with positive nerve fibres.

\*\*Nerve fibre density for the six cases where active endometriosis was not identified at laparoscopy.

M–W  $U z$ : Mann–Whitney  $U$  test.

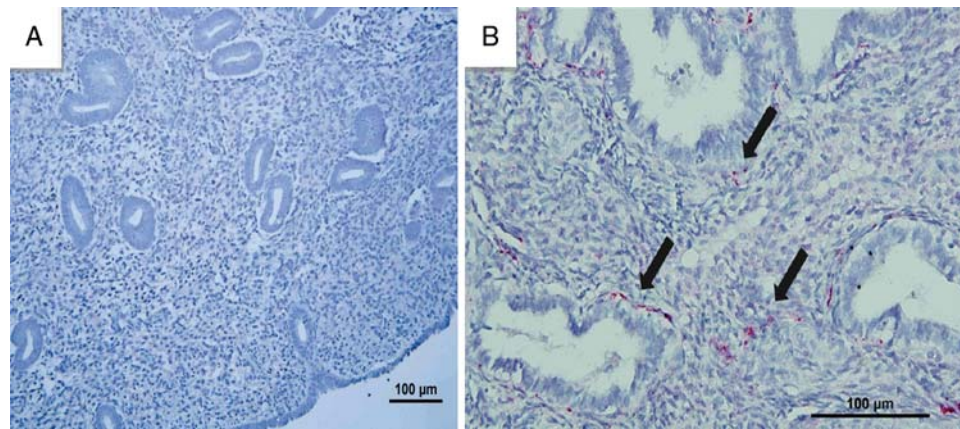
K–W  $\chi^2$ : Kruskal–Wallis chi-square test.

In women with laparoscopically confirmed endometriosis, mean ( $\pm$ SD) nerve fibre density of the Endosampler endometrial biopsy was 2.7 ( $\pm$ 3.4) per mm<sup>2</sup>, (range 0–24), compared with 3.1 ( $\pm$ 1.7) for women without endometriosis ( $P < 0.001$ , Table II, Fig. 2B). On average, nerve fibres were detected in 50–60% of high power fields; however, these nerve fibres were not distributed homogeneously throughout the endometrium. We also formally calculated specificity as 83%, sensitivity as 98%, positive predictive value (PPV) as 91%, negative predictive value (NPV) as 96% and prevalence of endometriosis in this referred patient population was 64% (Table III).

In women who were found not to have endometriosis at laparoscopy [ $N = 35$ ; uterine fibroids  $n = 4$ , adhesions not related to endometriosis  $n = 2$ , functional cysts  $n = 3$ , adenomyosis  $n = 1$  (ultrasound diagnosis), polycystic ovaries  $n = 5$ , none  $n = 20$ ] no nerve fibres were found in the functional layer of endometrium in 29 of the cases (Fig. 2A). In six cases the endometrial biopsy was positive (with mean nerve fibre density 3.1 per mm<sup>2</sup>) but there was no definite evidence of endometriosis at laparoscopy; three of these cases had severe dysmenorrhoea and dyspareunia with history of

infertility. It is possible that some had endometriosis which was not visible at laparoscopy. One case had a single spot of adhesions on the Pouch of Douglas which was not considered convincing for endometriosis. A further case underwent laparoscopy for secondary infertility (previous tubal ligation) and no evidence of endometriosis was found at laparoscopy. The last case had endometriosis diagnosed and removed at laparoscopy 7 years previously but no evidence of active endometriosis was found at recent laparoscopy (yet nerve fibres were found in the functional layer). We found only one case with no nerve fibres in the functional layer but with clear evidence of endometriosis at laparoscopy. This case (age 43 years) had severe pain symptoms and no previous history of endometriosis. At laparoscopy she had stage IV endometriosis involving bowel and both ovaries with adhesions.

Significant pain symptoms were recognized in 62.5% of the 64 cases with proven endometriosis, with dysmenorrhoea the most common symptom in 48.4% (Table I). Women with a history of previous endometriosis ( $n = 20$ ) had a higher density of nerve fibres (3.5 nerve fibre per mm<sup>2</sup>) when compared with women without previous history of



**Figure 2** (A) Absence of nerve fibres in the functional layer of endometrium in a woman with adenomyosis but no endometriosis ( $\times 20$  magnification). (B) Small unmyelinated C nerve fibres (black arrows) in the functional layer of endometrium in a woman with peritoneal endometriosis (immunohistochemical staining with protein gene product 9.5 and fast red chromogen,  $\times 40$  magnification).

**Table III** Presence of nerve fibres in endometrial biopsy in women with and without endometriosis, as diagnosed at laparoscopy

	Endometriosis diagnosis at laparoscopy		Total
	Yes	No	
Endometrial Nerve fibres present	Yes 63	6	69
	No 1	29	30
Total	64	35	99
Specificity*	83% (66–93%)		
Sensitivity*	98% (90–99%)		
PPV*	91% (81–96%)		
NPV*	96% (81–99%)		

\*95% Confidence interval.

PPV, positive predictive value; NPV, negative predictive value.

endometriosis ( $n = 79$ , with density of nerve fibres = 1.6 nerve fibre per  $\text{mm}^2$ ,  $P = 0.01$ ; Table II). No significant differences were found for density of nerve fibres related to stage of disease, quality of specimens and different menstrual phases.

## Discussion

This double blind study introduces the concept of using micro-anatomical endometrial markers in the diagnosis of endometriosis and suggests that immunohistochemical analysis of a carefully taken endometrial biopsy may be a reliable means of making a diagnosis of endometriosis in women who are not currently on any hormonal treatment. It has been reported that these endometrial nerve fibres may no longer be detectable in most women on hormonal therapy (Tokushige *et al.*, 2007). We also investigated the presence of endometrial nerve fibres in the functional layer in women with adenomyosis (Fig. 1A), endometrial hyperplasia, chronic endometritis, leiomyomas

and women with endometrial polyps, and failed to find any nerve fibres in the functional layer in these pathologies, which again emphasizes the unique presence of small C-nerve fibres in the functional layer in women with endometriosis (Al-Jefout, *et al.*, unpublished data). Although we have previously shown a higher prevalence of nerve fibres in the secretory phase (Tokushige *et al.*, 2006a, b, 2007), in the present study, with a larger sample, we found no difference in nerve fibre density according to phase of the menstrual cycle.

Although laparoscopy is still the gold standard, it has some limitations in terms of false negative findings, such as mistaking lesions for corpus luteum cyst, or missing a peritoneal or deep lesion in difficult locations. So the sensitivity is not always reliable. Other issues include the accuracy of confirmation of diagnosis by histopathology of biopsied lesions, as sometimes the lesion is not included in biopsies taken or limited experience of the pathologist in identifying the characteristic features of endometriosis especially in cases of mild disease where glandular elements may not be obvious. The high prevalence of endometriosis in this study (64%) can be explained by the fact that patients were recruited from clinics of tertiary referral for patients with pain and infertility complaints.

It is well established that laparoscopic excision of endometriosis can improve fecundity in couples with infertility (Marcoux *et al.*, 1997). Thus carefully planned laparoscopic surgery has the potential to help women with infertility as a result of endometriosis. However, the fertility of those women who are found not to have endometriosis is not usually helped by laparoscopy. At best these women suffer the cost and discomfort of surgery, and a delay in progressing to assisted reproduction technology. At worst these women suffer a complication of their laparoscopy. The use of endometrial biopsy for the diagnosis of endometriosis would allow gynaecologists to triage fertility patients (with no other indication for surgery) and plan for a potentially valuable laparoscopy or no laparoscopy.

Our results also indicate that a negative endometrial biopsy result would miss endometriosis in only 4% of women. Performing a planned laparoscopy only on a woman with a positive endometrial biopsy would result in endometriosis being confirmed in 80–90% of

these women. Thus using the PGP9.5 diagnostic test in an infertility workup would significantly reduce the number of laparoscopies performed without reducing the number of women whose endometriosis is diagnosed and surgically treated. A further benefit for patients is that most endometrial biopsies can be performed without local or general anaesthesia, and good biopsies can easily be obtained without the use of an anaesthetic.

Meticulous attention to biopsy technique is of great importance in providing a good quality tissue for this evaluation. With trial and error we improved our biopsy technique to provide a narrow, solid sample of endometrial tissue. It is valuable to initially rotate the Endo-sampler 180° against the curve of the uterus so as to ensure firm pressure backward against the endometrium and to withdraw the biopsy cannula in a straight line (Al-Jefout et al., 2007). Our experience showed that this biopsy technique ensured an adequate endometrial sampling (Fig. 1). Another important issue is the meticulous attention to immunohistochemical staining technique. We fine-tuned the PGP9.5 antibody and immunohistochemical assay conditions several times until we achieved clear discrimination of these small nerve fibres from background staining (Tokushige et al., 2006a).

## Conclusion

This study has confirmed the validity of the detection of endometrial nerve fibres, using immunohistochemical techniques on an endometrial biopsy, as a diagnostic test, which has a high level of sensitivity and specificity. This test probably has equivalent accuracy to surgical assessment, since it is unclear how often endometriosis is overlooked even by experienced gynaecologists. Endometrial biopsy is clearly less invasive than laparoscopy, and this test could help to reduce the current lengthy delay in diagnosis of the condition, as well as allow more effective planning for formal surgical or long-term medical management. It may be particularly helpful in cases of infertility.

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