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The Expression and Cellular Localisation of Neurotrophin and Neural Guidance Molecules in Peritoneal Ectopic Lesions

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Abstract

Endometriosis is a gynaecological disorder characterised by the presence of endometrial-like tissue outside the uterus. It affects 10–15% of women during their reproductive age. The existence of close and complex relationship between chronic pelvic pain and endometriosis are widely recognised. However, the mechanisms of pain generation in women with endometriosis remain poorly understood. Immunohistochemistry was used to assess the density of nerve fibres stained with protein gene product 9.5 (PGP9.5) and the expression of various neurotrophins including glial cell derived neurotrophic factor (GDNF), persephin, neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) and neuronal guidance molecules semaphorin 3E and Slit-2 and their receptors Plexin-D1 and Robo4 in peritoneal ectopic lesions from women with endometriosis and uninvolved peritoneum samples. Neurotrophins and neuronal guidance molecules and their receptors are synthesised in situ within peritoneal ectopic lesion which suggest their role in facilitating and maintaining the growth of nerve fibres. These molecules were found to be overall most highly expressed in the glands of endometriotic peritoneal lesions. In addition, the presence of ectopic lesions within the peritoneal cavity may affect the environment; in turn, the peritoneum altered appeared to play a role in the growth of nerve fibres and their development and maintenance in peritoneal lesions. Through exploring different neuronally active factors in and around ectopic lesions which may be contributing to pain generation, this study provides an insight and better understanding of the pain mechanisms associated with peritoneal endometriosis.

Keywords Neurotrophin · Neural · Guidance · Peritoneal · Endometriotic · Lesions

Introduction

Endometriosis is an oestrogen-dependant gynaecological disorder, defined as the growth of endometrial-like tissue outside the uterus. It affects around 10–15% of women of reproductive age [1, 2]. Pain is the most common consequence of the disease and the existence of a close and complex relationship between chronic pelvic pain and endometriosis is widely recognised. Women with endometriosis suffer from a variety of pain symptoms including dysmenorrhea, noncyclical pelvic pain, dyspareunia and dyschezia [3]. However, the relationship between pain and endometriosis and also the link with the severity of the disease are poorly understood [4].

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The presence of nerve fibres in peritoneal ectopic lesions has been confirmed [5–7]. Tulandi [5] was the first study that investigated the presence of nerve fibres in peritoneal ectopic lesions compared to normal peritoneum in women with endometriosis using an antibody against neurofilament (NF) protein. There were no differences in the density of nerve fibres within peritoneal ectopic lesions compared to normal peritoneum. In contrast, Tokushige [7] showed that peritoneal ectopic lesions contained small unmyelinated nerve fibres and indicated that the density of nerve fibres in peritoneal ectopic lesions stained with protein gene product 9.5 (PGP9.5) was significantly higher than in the normal peritoneum from women without endometriosis. However, the definitive pathways of pain generation in women with endometriosis still remain unclear.

Neurogenesis occurs mainly through the regulation and control of neuronal growth by attractant neurotrophic factors [8] and different repulsive molecules [9]. These molecules are activated through interaction with their specific substrates. Activation of these molecules controls the proliferation, plasticity and sensitivity of the nerve fibres [10,

11]. There is evidence that neurotrophins, their receptors and other molecules involved in neurogenesis are expressed in ectopic lesions. Neurotrophin-3 (NT-3), nerve growth factor (NGF) and receptors, tropomyosin receptor kinase A (TrkA) also known as high affinity nerve growth factor receptor and low-affinity nerve growth factor receptor (p75 neurotrophin receptor), are expressed in endometrial glands and the stroma of peritoneal lesions, ovarian and deep infiltrating endometrial (DIE) lesions [7, 12, 13]. Other families of neuronal generation and guidance molecules such as semaphorin 3C and semaphorin 3F have also been proposed to be upregulated in peritoneal endometriosis [14]. However, to date, only few of these molecules have been fully examined.

This study investigated the expression of various neurotrophins including glial cell derived neurotrophic factor (GDNF), persephin, NT-3 and NT-4 and neuronal guidance molecules semaphorin 3E and Slit-2 and their receptors Plexin-D1 and Robo4 in peritoneal ectopic lesions. Through exploring different neuronally active factors in and around ectopic lesions which may be contributing to pain generation, this study will provide an insight and better understanding of the pain mechanisms associated with peritoneal endometriosis.

Method

Archival tissue samples of peritoneal ectopic lesions ($n = 30$; mean age 37; range 20–45 years) and normal uninvolved peritoneum, which are peritoneum biopsies collected away from ectopic peritoneal lesions ($n = 14$; mean age 36; range 19–42 years), from women with endometriosis were collected from the Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital (RPAH), Sydney, Australia. Samples were collected from women with endometriosis who had presented with pain symptoms and/or infertility; however, none had received any hormonal treatment within the 3 months prior to tissue sampling.

This study was approved by the Ethics Review Committee (Royal Prince Alfred Hospital Zone) of the Sydney Local Health District (Protocol No. X12-0129 & HREC/12/RPAH/216).

Tissue samples had previously been fixed in 10% neutral buffered formalin (Table 1) for 18–24 h then processed and embedded in paraffin wax according to a standard protocol (Table 2). Sections were cut at 5 μm and mounted onto glass slides (SuperFrost Ultra Plus, Menzel Glaser, Braunschweig, Germany) and dried at 60 °C for 1 h and routinely stained with haematoxylin and eosin (H&E). Prior to immunohistochemical staining, the tissue sections were deparaffinised and rehydrated.

Immunohistochemistry

Immunohistochemical (IHC) analysis was conducted to examine the expression of neuronal guidance and neurotrophic factors and their receptors. The Dako Autostainer Plus (DakoCytomation, Carpinteria, CA, USA) was utilised for all staining. Briefly, antigen retrieval was performed with

Table 1 Details of primary antibody, species and clonicity, supplier, antigen retrival, background blocking reagent, dilution and incubation times, secondary incubation times, Dako detection system and control tissue

Primary antibody	Species and clonicity	Supplier	Antigen retrieval	Background blocking reagent	Dilution and incubation time	Secondry antibody incubation	Dako detection system	Control tissue
GDNF	Polyclonal rabbit	Abcam, Cambridge, UK	None	Protien block, 5 min	1:200 60 min	None	Real	Rat brain
Persephin	Polyclonal rabbit	BIOSS, Woburn, MA, USA	pH 9 20 min	None	1:400 60 min	None	Real	Rat brain
NT-3	Polyclonal rabbit	Abcam, Cambridge, UK	None	None	1:300 60 min	Rabbit anti-goat 1:250, 30 min	Envision	Rat brain
NT-4	Monoclonal rabbit 2E8	Abcam, Cambridge, UK	pH 6 20 min	None	1:1600 60 min	None	Real	Liver
Semaphorin 3E	Polyclonal goat	Abcam, Cambridge, UK	pH 6 20 min	None	1:1400 60 min	Rabbit anti-goat 1:250, 30 min	Real	Prostate
Plexin-D1	Polyclonal goat	Abcam, Cambridge, UK	pH 6 20 min	None	1:800 60 min	Rabbit anti-goat 1:250, 30 min	Envision	Liver
Slit-2	Monoclonal rabbit EPR2771	Abcam, Cambridge, UK	pH 6 20 min	None	1:1600 60 min	None	Real	Fetal kidney
Robo4	Polyclonal rabbit	Abcam, Cambridge, UK	pH 6 20 min	None	1:100 60 min	None	Real	Prostate carcinoma
PGP9.5	Polyclonal rabbit	Dako, Glostrup, Denmark	pH 6 20 min	None	1:400 30 min	None	Real	Colon

Table 2 Correlations between the expression of neurotrophic factors and neuronal guidance molecules and receptors, and nerve fibre density

Region	Correlated Markers	*r* value	*p* value
Gland	Persephin, NT-3	0.62	0.001
	NT-3, NT-4	0.41	0.007
	Slit-2, Robo-4	0.41	0.032
Stroma	Persephin, NT-3	0.59	0.007
	GDNF, NT-4	0.47	0.021
	NT-3, semaphorin 3E	0.46	0.002
Surrounding peritoneum	Persephin, NT-3	0.59	0.002
	GDNF, persephin	0.56	0.003
	GDNF, NT4	0.64	0.001
	Persephin, NT4	0.46	0.019
	NT-4, semaphorin 3E	0.48	0.014
	NT-4, Plexin-D1	0.53	0.05
	Semaphorin 3E, Plexin-D1	0.55	0.003
	Plexin-D1, Slit-2	0.42	0.030
	Plexin-D1, Robo-4	0.48	0.008
	NT-4, PGP9.5	0.41	0.038
	Semaphorin 3E, PGP9.5	0.41	0.034
Uninvolved peritoneum	Plexin-D1, Slit-2	0.56	0.037

EnVision FLEX Target Retrieval Solutions (pH 6 or pH 9; Dakocytomation, Golstrup, Denmark) as required (Table 1). Slides were then incubated with Flex H_2O_2 (Dakocytomation, Golstrup, Denmark) to reduce background staining caused by endogenous peroxidase, pseudoperoxidase and alkaline phosphatase activity in the tissue. Protein block (Dakocytomation, Golstrup, Denmark) was applied as required (Table 1). Primary and secondary antibodies were applied according to optimised protocols (Table 1) to demonstrate the presence of nerve fibres and neuronal activity. Then, EnVision Flex+/HRP (Dakocytomation, Golstrup, Denmark) or Dako REAL™ streptavidin peroxidase (HRP; Dakocytomation, Golstrup, Denmark) was used for detection according to what was optimal with each primary antibody. Finally, all slides were treated with 3,3′ diaminobenzidine (DAB) chromogen (Dakocytomation, Golstrup, Denmark), for visualisation. Slides were counterstained with Mayer's Hematoxylin (Fronine, Australia), mounted in DPX ultramount (Fronine, Australia) and coverslipped.

Positive control tissue (Table 1) were used in every run and stained with the same protocol as the study sample slides. Negative controls (Table 1), which were prepared by the omission of the primary antibody, were also included in each staining run. Positive tissue controls always showed positive staining and negative controls were always negative.

Microscopy and Analysis

Images of all ectopic lesion areas in the tissue and normal uninvolved peritoneum were captured with an Olympus microscope BX51 under × 100 magnification using a DP70 digital camera (Olympus, Tokyo, Japan).

Tissue Regions

Using microscopy automation and image analysis software (MetaMorph®) (Molecular Device, PA, USA), four free-hand regions were drawn around each peritoneal ectopic lesion area: (1) immediately inside the glandular epithelium,

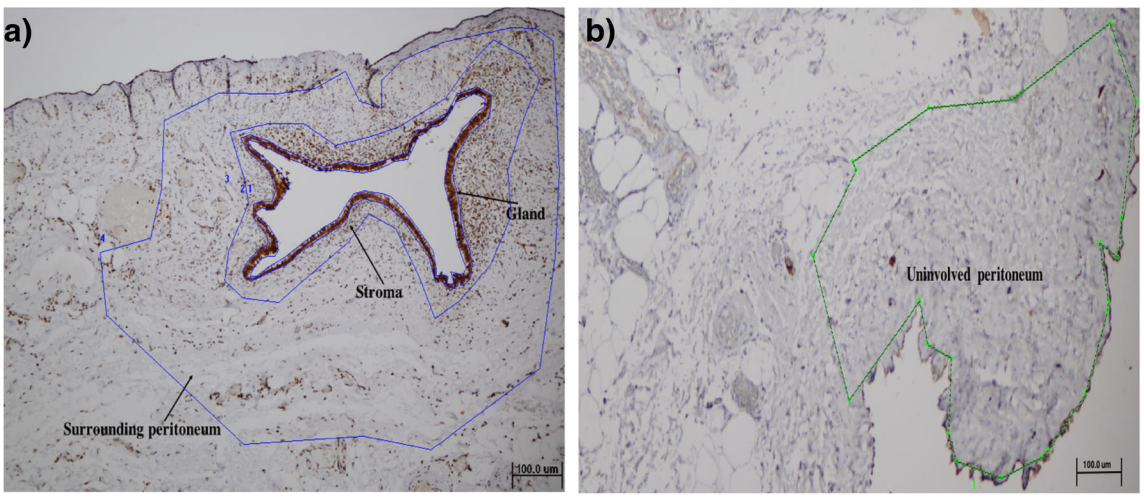


Fig. 1 **a** Region areas drawn by software (MetaMorph). The first region area drawn immediately inside the glandular epithelium was used for 'masking'. Then, region areas were drawn outside glandular epithelium (glandular epithelium region), at the edge of the endometriotic stroma (stromal region), 250 μm away from the edge of the stroma (surrounding peritoneum region) of peritoneal endometriotic lesion and **b** region area was drawn within the uninvolved peritoneum tissue (roundabout guidance receptor 4 (Robo4) staining; image captured under × 10 magnification)

which was used for 'masking' the area within glands; (2) immediately outside the glandular epithelium (gland region); (3) at the edge of the endometriotic stroma (stromal region); and (4) approximately 250 μm from the edge of the stroma (surrounding region) as shown in (Fig. 1a). In addition, one free-hand region of approximately 250 μm dimension was drawn in each uninvolved peritoneum sample (Fig. 1b).

Thresholding, Measurement and Scoring of Staining Intensity

Positive staining (brown) thresholds were manually created for each marker using the 'Set Colour Threshold' function of MetaMorph®. This was prepared using the 'Set by Example' option and clicking on positive (brown) pixels until all shades of brown staining were included in the threshold. Thresholds were tested on multiple images (at least five) for every marker to ensure all positive staining was included within the threshold. After that, the Integrated Morphometry Analysis function of MetaMorph® was used to measure the staining within the pre-drawn image regions. Measurements were directly logged to Microsoft® Excel. For each region, the following measurements were obtained:

(i) Integrated optical density (OD): the sum of the optical densities of all pixels detected by the threshold within the region. Optical density was calculated as the inverse logarithm of the grayscale transmittance, where the transmittance at a given pixel was considered to be its grayscale value divided by the maximum possible number of grayscale levels [15]. This measurement was used to determine the intensity of marker expression.
(ii) Pixel area of all positive staining and
(iii) Total region area.

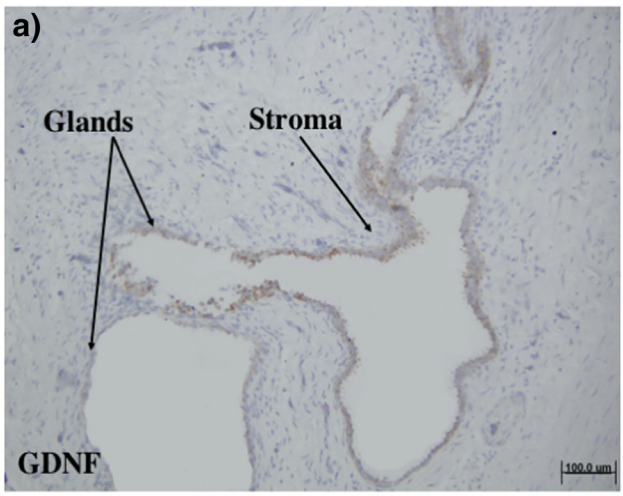


Fig. 2 **a** GDNF and **b** persephin localisation and expression pattern in peritoneal endometriotic lesions stained brown with 3,3′-diaminobenzidine (DAB^+) chromagen (× 200 magnification)

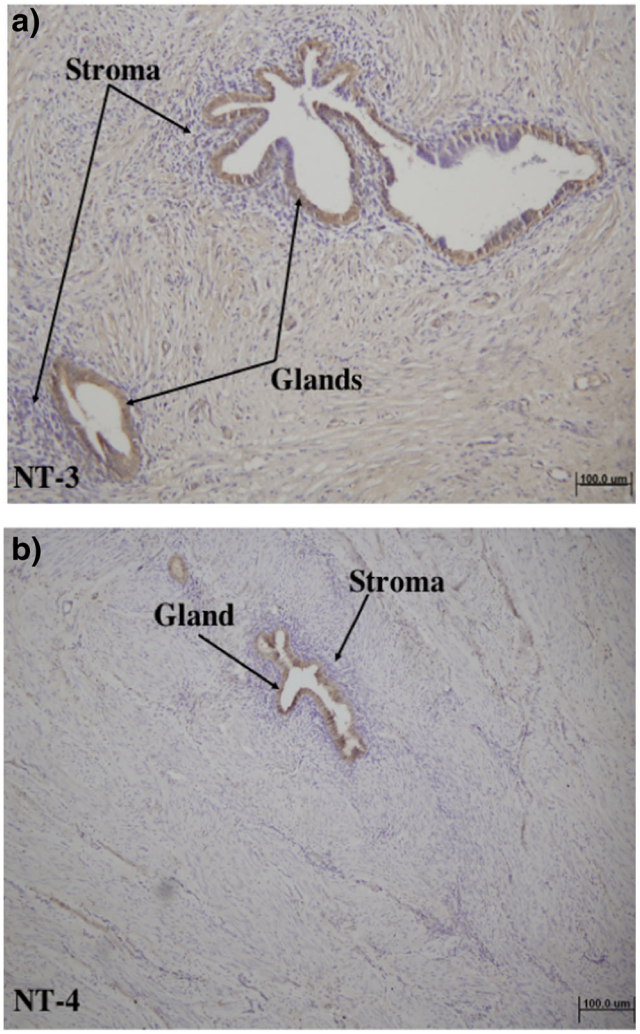


Fig. 3 **a** Neurotrophin-3 (NT-3) and **b** neurotrophin-4 (NT-4) localisation and expression pattern in peritoneal endometriotic lesions visualised brown with 3,3′-diaminobenzidine (DAB^+) chromagen (× 200 magnification)

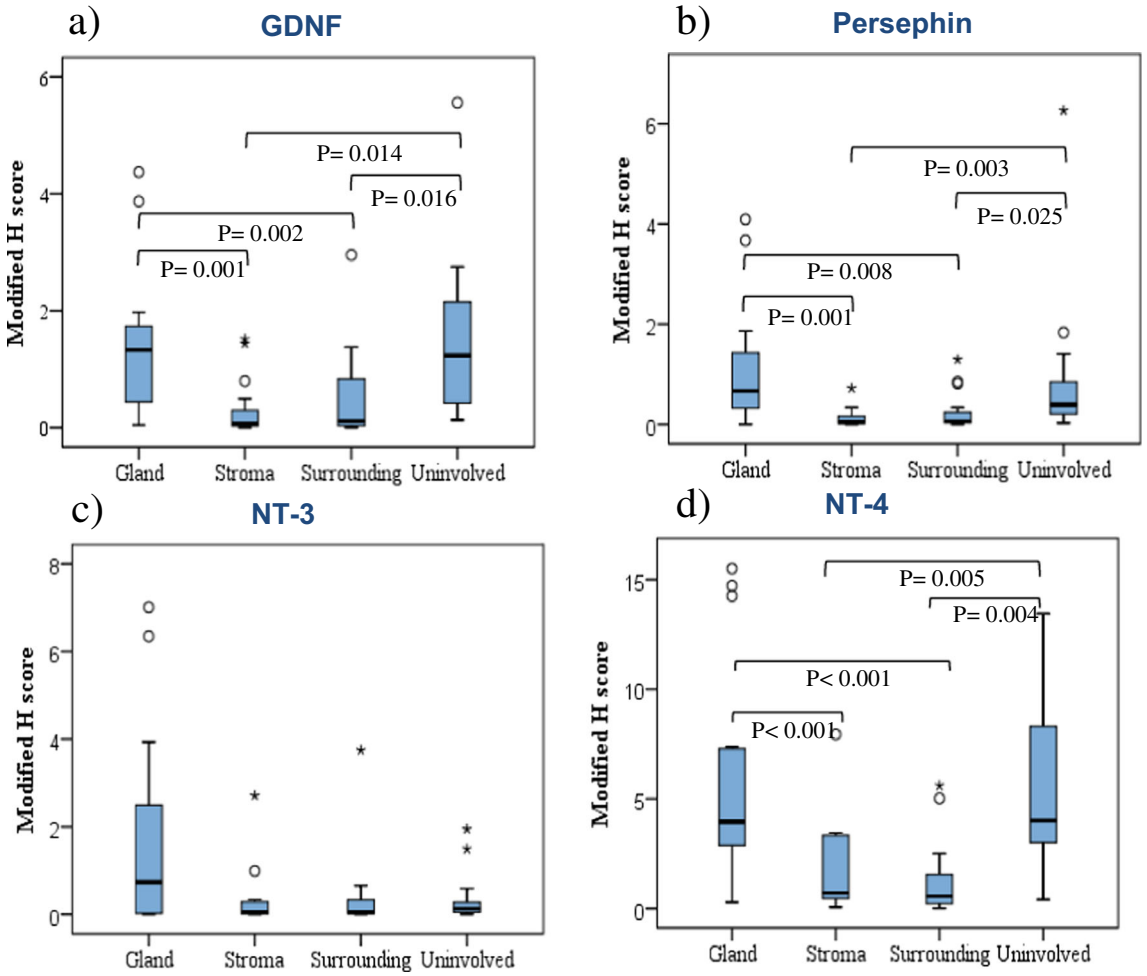


Fig. 4 Box plots showing modified *H* scores for the expression of the neurotropic factors. **a** Glial cell derived neurotrophic factor (GDNF), **b** persephin, **c** neurotrophin-3 (NT-3) and **d** neurotrophin-4 NT-4 in peritoneal endometriotic lesion glands and stroma, and surrounding and uninvolved peritoneum. Circular signs represent outlier values and single asterisk represents extreme outlier values

Raw measurements included the total optical density and total area within entire regions (including smaller regions contained within) so that exact measurements for endometriotic gland, stroma and surrounding tissue areas alone were obtained by subtracting smaller embedded region measurements. Area measurements from MetaMorph® were provided in pixels and converted to mm^2 using the following formula (derived as 250 μm = 450 pixels):

$$\text{Area mm}^2 = \frac{\text{Pixel area}}{3240000}$$

As many peritoneal ectopic lesion samples contained more than one area of lesion on the stained slides, the total optical density, total positively stained area and total area were next summed within each region type over all lesion areas per sample. A modified *H* score was chosen as the parameter to compare between regions of interest of each marker as it took into consideration the staining intensity in conjunction with the percentage positive area *H* score consisted of a sum of the percentages of positively stained cells multiplied by a weighted intensity of staining [16]. To calculate the modified *H* score:

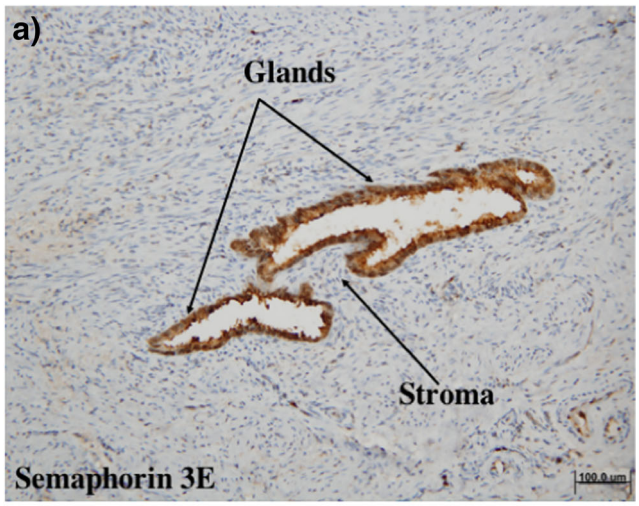
$$\text{Modified } H \text{ score} = \%\text{positive area} \times \text{intensity score}$$

$$\text{where } \%\text{positive area} = \frac{\text{positively stained area}}{\text{total area}}$$

$$\text{and area intensity score} = \frac{\text{integrated OD}}{\text{positively stained area}}.$$

Nerve Fibre Counting

Identification and counting of longitudinally and transversely sectioned nerve fibres and small nerve trunks were considered as positive by their labelled brown appearance visualised with 3,3′-diaminobenzidine (DAB) chromogen undertaken in the stroma and surrounding regions manually by two independent researchers experienced in nerve fibre identification. Less



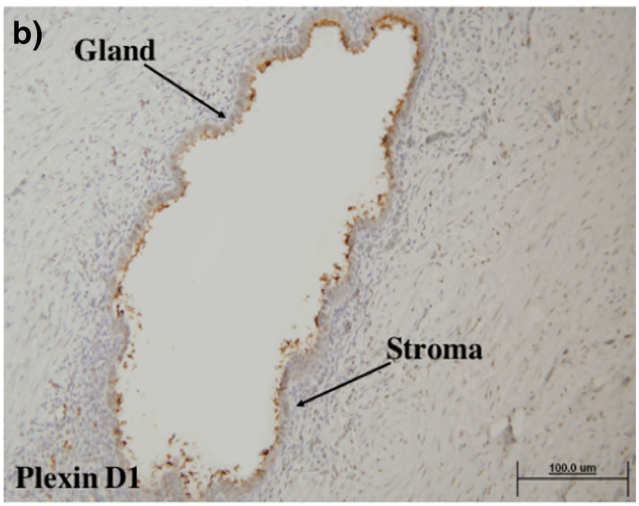
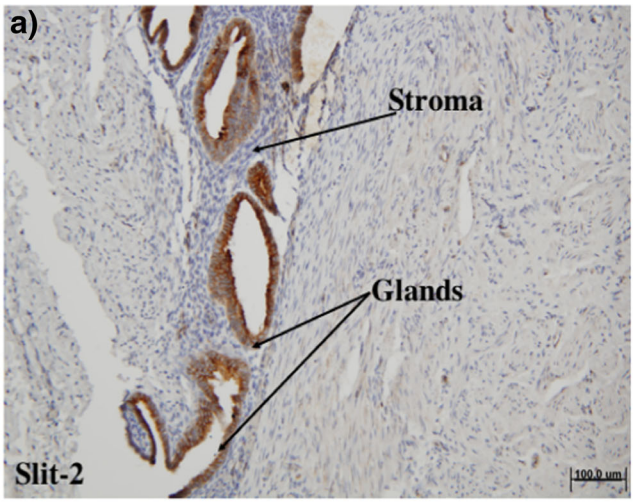


Fig. 5 Neuronal guidance molecule **a** Semaphorin 3E and receptor **b** Plexin-D1 localisation and expression patterns in peritoneal endometriotic lesions visualised brown with 3,3′-diaminobenzidine (DAB^+) chromagen (× 200 magnification)



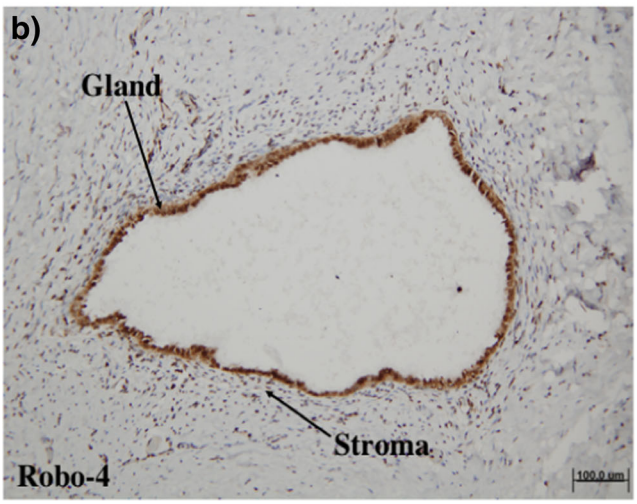


Fig. 6 Neuronal guidance molecule **a** Slit-2 and receptor **b** Roundabout Guidance Receptor 4 (Robo-4) localisation and expression patterns in peritoneal endometriotic lesions visualised brown with 3,3′-diaminobenzidine (DAB^+) chromagen (× 200 magnification)

intensely positively stained tissue components which were cellular, which contained a clear nucleus, were not counted. Blinded counting gave close correlation between the two individuals with an interclass correlation coefficient (ICC) of 0.999 (95% confidence interval 0.998–0.999, $p < 0.001$). The density of $PGP9.5^+$ nerve fibres per square millimetre in the stroma and surrounding regions were also calculated.

Statistical Analyses

Statistical analyses were performed using IBM Statistical Package for the Social Sciences (SPSS) version 24.0 (SPSS Inc., Chicago, IL, USA). As the data was not normally distributed and the modified *H* scores were calculated using proportion measurements, non-parametric tests were applied to compare the levels of marker antibody expression and nerve fibre densities between different regions of interest. To compare the levels of marker antibody expression and nerve fibre densities in glandular epithelium versus stroma versus surrounding tissue, the Kruskal-Wallis chi-square was used. Correlations between intensities of marker antibodies expression and nerve fibre densities within glandular epithelium, stroma or surrounding tissue were examined using the non-parametric Spearman correlation coefficient (r). Results were considered to be statistically significant with p values < 0.05.

Results

Neurotrophic Factors

Each of the four neurotropic factor markers showed different localisations within the sample tissues (Figs. 2 and 3). GDNF

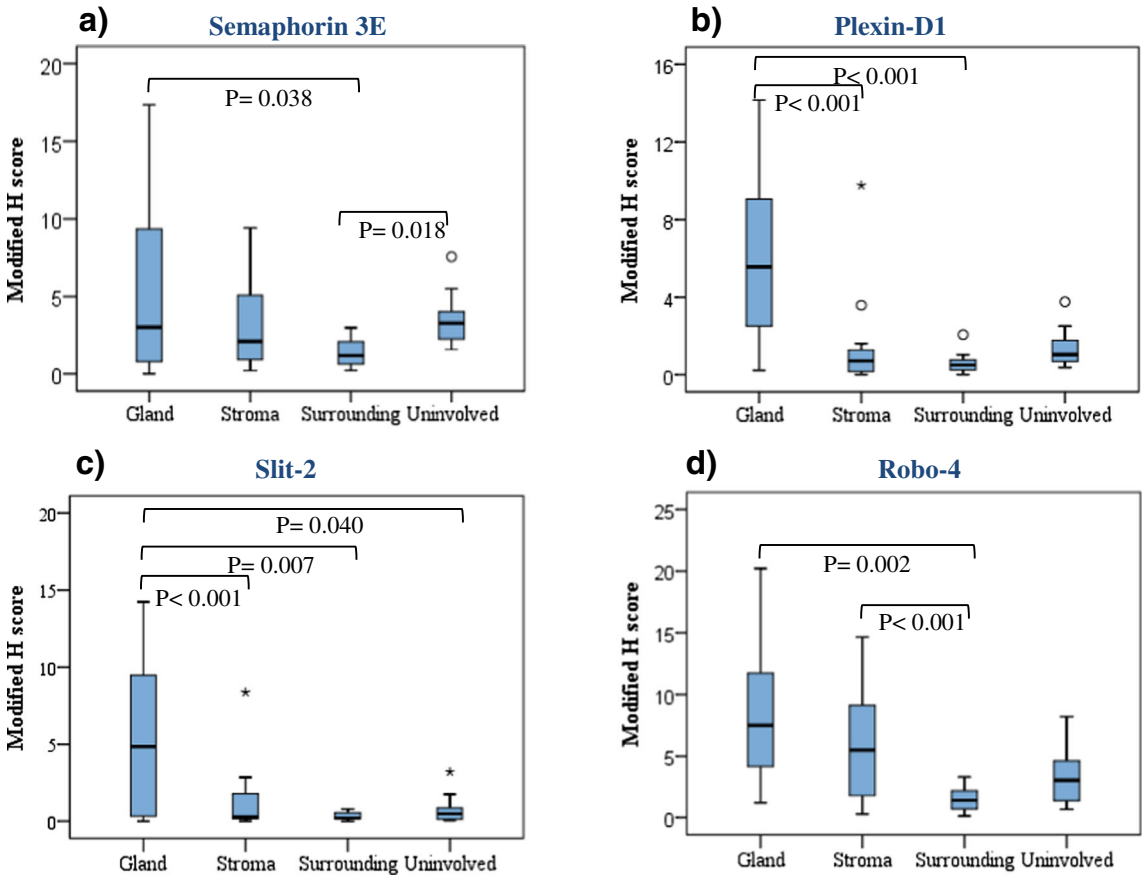


Fig. 7 Box plots showing *H* scores for the expression of **a** semaphorin 3E, **b** Plexin-D1, **c** Slit-2 and **d** roundabout guidance receptor-4 (Robo-4) in peritoneal endometriotic lesion glands and stroma, surrounding peritoneum and uninvolved peritoneum. Circular signs represent outlier values and single asterisk represents extreme outlier values

expression was membranous in glandular epithelium and cytoplasmic in stromal cells, with patchy variation in the stroma (Fig. 2a). Persephin, NT-3 and NT-4 each showed variable and diffuse localisation in the gland, the cytoplasm of stroma and peritoneum around lesions and the uninvolved peritoneum (Figs. 2b and 3).

Each marker demonstrated significant differences in the levels of expression between the four regions of interest (gland, stroma, surrounding and uninvolved peritoneum; Fig. 4a–d). The expression of GDNF in glandular epithelium was significantly higher compared to the stroma ($p = 0.001$) and surrounding peritoneum ($p = 0.002$; Fig. 4a). The level of expression of GDNF in stroma was significantly lower compared to uninvolved peritoneum ($p = 0.016$; Figs. 4a). In addition, GDNF expression in surrounding peritoneum was significantly lower compared to uninvolved peritoneum ($p = 0.014$; Fig. 4a). The expression of GDNF did not significantly differ between the stroma and surrounding peritoneum (Fig. 4a).

Persephin expression in glands was significantly higher compared to the stroma ($p = 0.001$) and surrounding peritoneum ($p = 0.008$; Fig. 4b). Persephin expression in the stroma was significantly lower compared to uninvolved peritoneum ($p = 0.003$; Fig. 4b). Persephin expression in surrounding peritoneum was significantly lower than uninvolved peritoneum ($p = 0.025$; Fig. 4b). The expression of persephin did not significantly differ between the stroma and surrounding peritoneum (Fig. 4b).

There were no significant differences in the level of expression of NT-3 throughout the four regions of interest (Fig. 4c). The expression of NT-4 in glands was significantly higher compared to the stroma ($p < 0.001$) and surrounding peritoneum ($p < 0.001$; Fig. 4d). The level of expression of NT-4 in the stroma was significantly lower compared to uninvolved peritoneum ($p = 0.005$; Fig. 4d).

NT-4 expression in the surrounding peritoneum was significantly lower compared to uninvolved peritoneum ($p = 0.004$; Fig. 4d). The expression of NT-4 did not significantly differ between the stroma and surrounding peritoneum (Fig. 4d).

In glandular epithelium, the expression of NT-3 was significantly positively correlated with persephin and NT-4

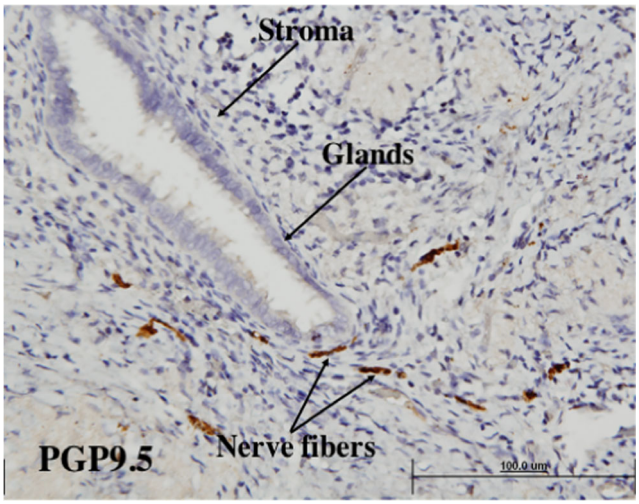


Fig. 8 Protein Gene Product 9.5 (PGP9.5) positive nerve fibres in a peritoneal endometriotic lesion visualised brown with 3,3′-diaminobenzidine (DAB^+) chromogen (× 400 magnification)

(Table 2). In the stroma, persephin expression was significantly correlated with NT-3, and GDNF was significantly correlated with NT-4 (Table 2). In the surrounding peritoneum, persephin expression was significantly correlated with GDNF, NT-3 and NT-4 and NT-4 was significantly correlated with GDNF (Table 2).

Neuronal Guidance Molecules and Their Receptors

Semaphorin 3E, Slit-2 and Robo-4 were localised to the cytoplasm of glandular cells, stromal and peritoneal nuclei, endothelial cells and nerve fibres (Figs. 5 and 6). Plexin-D1 was primarily localised to the luminal edge of glandular epithelium (Fig. 5b).

Each marker demonstrated some significant differences in the levels of expression between the four regions of interest (gland, stroma, surrounding and uninvolved peritoneum; Fig. 3a–d). The expression of semaphorin 3E in glands was significantly higher compared to the surrounding peritoneum ($p = 0.038$, Fig. 7a). The expression of semaphorin 3E in the surrounding peritoneum was significantly lower compared to the uninvolved peritoneum ($p = 0.018$; Fig. 7a).

Plexin-D1 showed a significantly higher expression in the glands compared to the stroma ($p < 0.001$) and surrounding peritoneum ($p < 0.001$; Fig. 7b).

The expression of Slit-2 in glands was significantly higher compared to the stroma ($p = 0.007$), surrounding peritoneum ($p = 0.040$) and uninvolved peritoneum ($p < 0.001$; Fig. 7c). Robo-4 expression in the glands was significantly higher compared to surrounding peritoneum ($p = 0.002$) (Fig. 7d). Robo-4 expression in the stroma was also significantly higher compared to the surrounding peritoneum ($p < 0.001$, Fig. 7c, d).

There was a significant positive correlation between Slit-2 and Robo-4 expression in glands. There were significant correlations between semahoprin 3E and persephin expression in the stroma (Table 2). In the surrounding peritoneum, there were significant correlations between NT-4 and semaphorin 3E between and NT-4 and Plexin-D1 expression. There was also a significant correlation between Pelxin-D1 and Slit-2 expression in the uninvolved peritoneum (Table 2).

Nerve Fibres

Nerve fibres were present in the stroma, surrounding and most of the uninvolved peritoneum of all peritoneal endometriotic samples (Fig. 8). Whilst the density of nerve fibres tended to be slightly higher in lesion stroma compared to uninvolved peritoneum, there were no significant differences in nerve fibre density between the three regions of interest (Fig. 9). The density of nerve fibres was significantly positively correlated with the expression of NT-4 and semaphorin 3E in the surrounding peritoneum (Table 2).

Discussion

In our study, we have demonstrated an increased density of sensory nerve fibres stained with PGP9.5 in the stroma of peritoneal ectopic lesions, compared to the surrounding and uninvolved peritoneum. Like all classes of peripheral neurons, sensory neurons require to be connected with their peripheral target tissues, a source of neurotrophins and other neuronal factors to survive during development [17]. In this study,

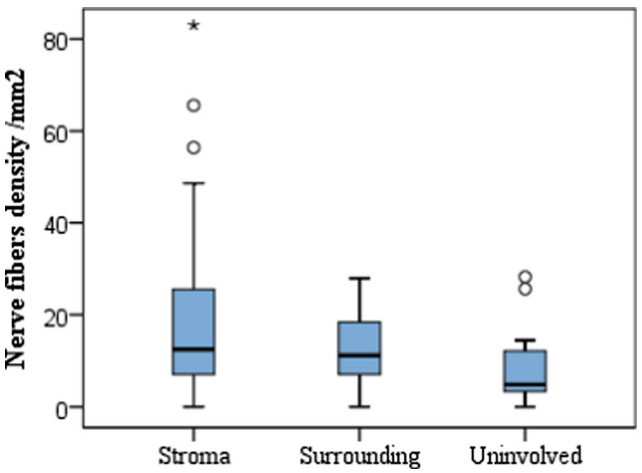


Fig. 9 Box plot illustrating nerve fibre densities in peritoneal endometriotic lesion stroma, surrounding peritoneum and uninvolved peritoneum. Circular signs represent outlier values and single asterisk represents extreme outlier values

neurotrophic factors, persephin, GDNF and NT-4, demonstrated the same pattern of expression. The expression of these molecules was significantly higher in the glandular epithelium of the peritoneal ectopic lesions and uninvolved peritoneum compared to the stroma and surrounding peritoneum of peritoneal ectopic lesions. NT-3 was also expressed in the glands, stroma, surrounding peritoneum of peritoneal ectopic lesions and uninvolved peritoneum of women with endometriosis, although there were no significant differences observed between regions. Also, previous studies have investigated neurotrophic factors such as NGF and NT-3 which were found to be overexpressed, whilst BDNF showed no significant differences in the peritoneal fluid (PF) from women with endometriosis compared with the PF from women with adenomyosis, adhesions or asymptomatic controls [18, 19].

In addition, Tokushige [7] showed intense NGF immunoreactivity near endometriotic glands. BDNF and NT-4/5 in the endometrium of women with endometriosis have been reported to be significantly higher in women with endometriosis compared to women without the disease [20]. Wessels [21] also revealed that women with endometriosis showed a significantly higher BDNF in the plasma of women with endometriosis in comparison to women without the disease. However, to date, there have been no prior studies that have investigated persephin, GDNF and NT-3 in the peritoneal ectopic lesions. Our results suggest that there are local neurotrophic activities in peritoneal lesions that facilitate the growth and maintenance of nerve fibres. In complementary, the presence of ectopic lesions may affect the environment of the peritoneal cavity and, in turn, the peritoneum altered seems to play a role in the nerve fibre growth, development and maintenance in peritoneal lesions.

Neuronal guidance molecules and their receptors, which can either attract or repel axons and signalling for branching or sensitisation, are all important factors for neuronal development. The neuronal guidance molecules investigated in this study and their receptors were mostly significantly overexpressed in the glandular epithelium of peritoneal lesions. Semaphorin 3E showed a similar expression pattern to neurotrophins. It was significantly highly expressed in the gland and in the uninvolved peritoneum compared to the surrounding peritoneum. Semaphorin 3E receptor and Plexin-D1 were significantly highly expressed in the gland and surrounding peritoneum. Class III semaphorins including semaphorin 3E have been shown to be chemorepulsive molecules, which are able to stimulate growth cone collapse when applied intensely and consistently and to direct axons away when presented as a point source, although some semaphorins have also been reported to act as chemoattractants, reviewed in [22, 23]. Other members of the semaphorin class III have been investigated in women with endometriosis. Women with endometriosis have shown a significantly higher semaphorin 3A in endometrium, semaphorin 3C in the endometrium and peritoneal ectopic lesions and semaphorin 3F in peritoneal ectopic lesions [14, 24, 25].

Our study also revealed that Slit-2 was significantly expressed in the gland compared to the stroma, surrounding and uninvolved peritoneum. Slit-2 receptor and Robo-4 were significantly highly expressed in the gland and stroma of peritoneal ectopic lesions. Slits are secreted proteins that bind to Robo receptors and act as neuronal repellents and prevent ipsilateral axon crossing [26, 27]. Shen [28] reported a higher expression of Slit and Robo1 proteins, as well as an increased microvascular density, in cases of endometrioma recurrence. However, this previous study did not link the high expression with nerve development within the ovarian endometrioma. In our study, we have hypothesised that the expression of neuronal guidance molecules and their receptors in glands, stroma and the surrounding peritoneum of peritoneal ectopic lesions and uninvolved peritoneum of women with endometriosis facilitates the ingrowth and development of nerve fibres.

To conclude, neurotrophins and neuronal guidance molecules and their receptors are synthesised in situ within peritoneal ectopic lesions. These molecules were found to be overall most highly expressed in the glands of endometriotic peritoneal lesions. In addition, the presence of ectopic lesions within the peritoneal cavity may affect the environment, and in turn, the peritoneum altered appeared to play a role in the growth of nerve fibres and their development and maintenance in peritoneal lesions.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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