

Peripheral changes in endometriosis-associated pain

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Submitted on February 1, 2014; resubmitted on April 12, 2014; accepted on April 22, 2014

TABLE OF CONTENTS

- Introduction
- Methods
- Endometriosis background
- Nerve fibres in endometriosis: human studies
 - Peritoneal endometriosis
 - Effects of peritoneal fluid
 - Uterosacral and rectovaginal endometriosis
 - Intestinal endometriosis
 - Ovarian endometriosis
 - Nerves in endometrium and myometrium
 - Adenomyosis
- Association between nerve and vascular growth
 - Molecular guidance cues for developing nerves and blood vessels
 - VEGF and NGF: guidance cues for vessels and nerves?
- Peripheral mechanisms generating and modulating pain in endometriosis
 - Inflammation, neuroangiogenesis and peripheral sensitization
 - A role for the autonomic nervous system?
 - Pelvic and lower abdominal cross-organ sensitization
 - Role of estrogens
- Therapeutic implications
- Conclusions

BACKGROUND: Pain remains the cardinal symptom of endometriosis. However, to date, the underlying mechanisms are still only poorly understood. Increasing evidence points towards a close interaction between peripheral nerves, the peritoneal environment and the central nervous system in pain generation and processing. Recently, studies demonstrating nerve fibres and neurotrophic and angiogenic factors in endometriotic lesions and their vicinity have led to increased interest in peripheral changes in endometriosis-associated pain. This review focuses on the origin and function of these nerves and factors as well as possible peripheral mechanisms that may contribute to the generation and modulation of pain in women with endometriosis.

METHODS: We conducted a systematic search using several databases (PubMed, MEDLINE, EMBASE and CINAHL) of publications from January 1977 to October 2013 to evaluate the possible roles of the peripheral nervous system in endometriosis pathophysiology and how it can contribute to endometriosis-associated pain.

RESULTS: Endometriotic lesions and peritoneal fluid from women with endometriosis had pronounced neuroangiogenic properties with increased expression of new nerve fibres, a shift in the distribution of sensory and autonomic fibres in some locations, and up-regulation of several neurotrophins. In women suffering from deep infiltrating endometriosis and bowel endometriosis, in which the anatomical distribution of lesions is generally more closely related to pelvic pain symptoms, endometriotic lesions and surrounding tissues present higher nerve fibre densities compared with peritoneal lesions and endometriomas. More data are needed to fully confirm a direct correlation between fibre density in these locations and the amount of perceived pain. A better correlation between the presence of nerve fibres and pain symptoms seems to exist for eutopic endometrium. However, this appears not to be exclusive to endometriosis. No correlation between elevated neurotrophin levels and pain severity appears to exist, suggesting the involvement of other mediators in the modulation of pain.

CONCLUSIONS: The increased expression of neurotrophic factors and nerve fibres in endometriotic lesions, eutopic endometrium and the peritoneum imply a role of such peripheral changes in the pathogenesis of endometriosis-associated pain. However, a clear link between these findings and pain in patients with endometriosis has so far not been demonstrated.

Key words: Endometriosis / peripheral changes / nerve fibres / neuroangiogenesis / neurotrophins

Introduction

Endometriosis is an estrogen-dependent inflammatory disease estimated to affect ~10% of women of reproductive age (Giudice and Kao, 2004). Many unanswered questions remain about the pathophysiology of endometriosis. Not least of these, is how endometriosis generates one of its predominant clinical features: pain.

Pain may be nociceptive, inflammatory, neuropathic or a mixture of these (Fig. 1). It is likely that endometriosis gives rise to all three types of pain, though the extent to which any one type predominates may vary between patients. Furthermore, given the widespread distribution of endometriotic deposits on both pelvic viscera and parietal peritoneum, the pain associated with endometriosis can be both visceral and somatic in origin.

Endometriosis-associated pain is as complex as the disease itself (Fig. 2). It is well accepted that no correlation exists between the extent of endometriosis seen at laparoscopy and the degree of pain symptoms (Fauconnier and Chapron, 2005).

The experience of pain is complex involving many mechanisms and interactions between the periphery and the central nervous system (CNS) (Stratton and Berkley, 2011). Recent work has shown alterations in both the peripheral and CNS of women with endometriosis-associated pain (Stratton and Berkley, 2011; As-Sanie et al., 2012) in addition to demonstrating direct innervation of endometriotic deposits (Berkley et al., 2005).

In this review, we focus on possible peripheral mechanisms by which endometriosis may engage and alter the nervous system to generate and perpetuate endometriosis-associated pain. In particular, we will summarize the existing data on nerve fibres in endometriotic and endometrial tissue, the novel concept of neuroangiogenesis in endometriosis and their clinical implications. Finally, we discuss potential therapeutic targets that may evolve from these processes. While a related review will focus on the central changes in endometriosis and pelvic pain (Brawn et al., 2014).

Methods

We conducted a primary computerized search for all publications in PubMed, MEDLINE, EMBASE and CINAHL from January 1977 to October 2013 that related to the role of the peripheral nervous system in the pathophysiology of

endometriosis-related pain. We searched using the following MeSH or key word terms: endometriosis AND nervous system OR nerve OR nerve fibres OR nerve fibers OR nerve tissue OR nerve endings OR nerve growth factor OR denervation OR neurotransmitter agents OR pain OR chronic pain OR pelvic pain OR visceral pain OR nociceptive pain OR hyperalgesia OR pain measurement OR dysmenorrhea OR dyspareunia OR dyschezia AND humans.

One author (M.M.) independently identified relevant abstracts, and the full texts were then obtained. The bibliographies of the retrieved articles and reviews were then searched and any additional relevant articles included. Only English language publications were included. Studies were evaluated according to specific criteria (Table I).

Endometriosis background

Endometriosis is a clinically important and highly prevalent, but only poorly understood disease. The true prevalence of endometriosis in the general female population is difficult to quantify due to the 'unlucky triad' of the virtually exclusive intra-abdominal location of the disease in combination with unspecific symptoms and a lack of sufficiently sensitive and specific non-invasive diagnostic methods including biomarkers (May et al., 2010). Estimates based on population-based prevalences of pain and infertility, and endometriosis diagnostic rates reported in symptomatic patient groups seen in clinics, suggest that ~6–10% of women of reproductive age suffer from the disease (Eskenza and Warner, 1997; Giudice and Kao, 2004). While different theories about the origin of endometriotic lesions exist, most experts in the field believe that Sampson's theory of retrograde menstruation accounts for most of the origin of peritoneal, and possibly also rectovaginal disease (Sampson, 1927). Many potential factors have been described that promote the establishment and growth of the endometrial deposits (Bulun, 2009). For example, it is well established that patients suffering from the disease have an impaired immune system (Burney and Giudice, 2012). Dysfunctional macrophages and other immune cells appear to play a central role, possibly either as an underlying cause of the disease or resulting from its presence with the concomitant aberrant expression of many cytokines, enzymes and growth factors (Young et al., 2013). Furthermore, angiogenesis (the growth of blood vessels) is an essential step in the pathogenesis of the disease (Becker D'Amato, 2007; Taylor et al., 2009). Endometriotic lesions only grow if sufficiently supplied by nutrients and oxygen and many *in vitro* and *in vivo* studies using

Pain	An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.
Neuropathic pain	Pain caused by a lesion or disease of the somatosensory nervous system. Neuropathic pain is a clinical description (and not a diagnosis) which requires a demonstrable lesion or a disease that satisfies established neurological diagnostic criteria.
Nociceptive pain	Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors. Nociceptive pain may also be divided into "visceral", "deep somatic" and "superficial somatic" pain depending on the localisation of nociceptors.
Inflammatory pain	Pain associated with active inflammation. Inflammatory pain falls into the category of nociceptive pain, however, it is recognized that the nociceptive system may be altered in chronic inflammatory pain.
Allodynia	Pain due to a stimulus that does not normally provoke pain.
Hyperalgesia	Increased pain from a stimulus that normally provokes pain.

Adapted from IASP Taxonomy (Merskey and Bogduk, 1994) and the Kyoto Protocol of Basic Pain Terminology (Loeser and Treede, 2008).

Figure 1 Pain taxonomy.

different animal models have shown the efficacy of antiangiogenic therapy in endometriosis (Nap *et al.*, 2004; Becker *et al.*, 2005; Laschke and Menger, 2012).

The parallel growth of blood vessels and nerve fibres (neuroangiogenesis) is an established dogma in embryology and development. More recently, this concept has also been described in pathological processes such as cancer and endometriosis (Carmeliet and Tessier-Lavigne, 2005; Asante and Taylor, 2011). Interestingly, increasing evidence exists for the presence of nerve fibres in ectopic and eutopic endometrium suggesting a possible role of these in endometriosis-associated pain (Tokushige *et al.*, 2006a, b; Mechsner *et al.*, 2007, 2009). Below we will summarize the currently available data, examine possible mechanisms and critically discuss these findings with respect to endometriosis-associated pain.

Nerve fibres in endometriosis: human studies

Peritoneal endometriosis

The first study that explored the presence of the nerve fibres in healthy peritoneum and peritoneal lesions in women with endometriosis demonstrated no differences in the amount of nerve fibres (rare, few and many) between endometriosis and non-endometriosis patients (Tulandi *et al.*, 2001). Using an antibody against neurofilament (NF) protein, 79.6% (39/49) of specimens in the lateral pelvic wall peritoneum

stained positive in women with endometriosis. Interestingly, the distance between the nerve fibres and endometrial glands tended to be lower in women with pelvic pain than in those without pain suggesting a functional role of such nerve structures. Tokushige *et al.* demonstrated the presence of multiple, small unmyelinated nerve fibres in peritoneal endometriotic lesions in 40 women with confirmed peritoneal endometriosis (Tokushige *et al.*, 2006a), and showed that the density of nerve fibres in endometriotic lesions stained with protein gene product 9.5 (PGP9.5), a specific pan-neuronal marker, was greater than in normal peritoneum in women without endometriosis. Furthermore, these nerve fibres were more frequently seen near endometriotic glands and blood vessels than elsewhere in the stroma ($P < 0.001$). Confirmation by specific markers, such as substance P (SP), calcitonin gene-related peptide (CGRP), acetylcholine (ACh) and tyrosine hydroxylase (TH), demonstrated that these nerve fibres contained a mixture of sensory A δ , sensory C, cholinergic and adrenergic nerve fibres, that often were co-localized (Tokushige *et al.*, 2006a) (Table II).

The results were confirmed by Mechsner *et al.* who found nerve fibres, stained with NF and SP, in direct contact with endometriotic lesions in 74.5% (79/106) of the specimens (Mechsner *et al.*, 2007). However, in agreement with Tulandi *et al.* (2001), they did not detect any difference in total mean nerve scores when comparing peritoneum from patients with and without endometriosis. Interestingly, inside or near the endometriotic implants, nerve fibres stained with glycoprotein component-43 (GP-43), a marker for newly grown nerve fibres, were co-localized with immature blood vessels. In contrast, mature nerve fibres were accompanied by mature blood vessels and could not be detected in contact with endometriotic glandular and stromal cells, suggesting that the fibres encountered in the lesions were newly formed possibly through neuroangiogenesis (see below). In another study the same group found significantly more NF and PGP9.5 positive nerve fibres in peritoneum of women with higher pain scores for dysmenorrhoea and pelvic pain compared with peritoneum from women with lower pain scores, but no correlation existed with the presence of dyspareunia, dyschezia or dysuria (Mechsner *et al.*, 2009). No differences were found between the presence of endometriosis-associated nerve fibres and the activity of peritoneal endometriosis (as assessed by red, black, white lesions).

Different results were reported in studies investigating the presence of nerve fibres in women undergoing hormonal treatment. Fraser's group showed a decreased density of nerve fibres stained with PGP9.5 in peritoneal endometriotic lesions of hormone-treated women (combined oral contraceptive or progestin) compared with untreated women (Tokushige *et al.*, 2009). Unfortunately, the severity of pain was not systematically assessed; therefore, it is unclear whether the histological findings had a clinical effect. In another study, no differences in the density of nerve fibres, stained with NF or PG 9.5, were seen at different stages of the menstrual cycle (Wang *et al.*, 2011).

Interestingly, a recent study showed qualitative changes of endometriosis-related nerve fibres, demonstrating an imbalance between sympathetic and sensory innervation in peritoneal lesions (Arnold *et al.*, 2012). Using antibodies against the pan-neuronal marker PGP9.5, the sensory neurotransmitter SP and TH as marker for adrenergic nerve fibres, the authors found a lower density of sympathetic nerve fibres in endometriotic lesions compared with healthy peritoneum ($P < 0.001$), whilst the opposite was true for sensory nerve fibres ($P < 0.001$): for both sensory and sympathetic markers, no correlations with the menstrual

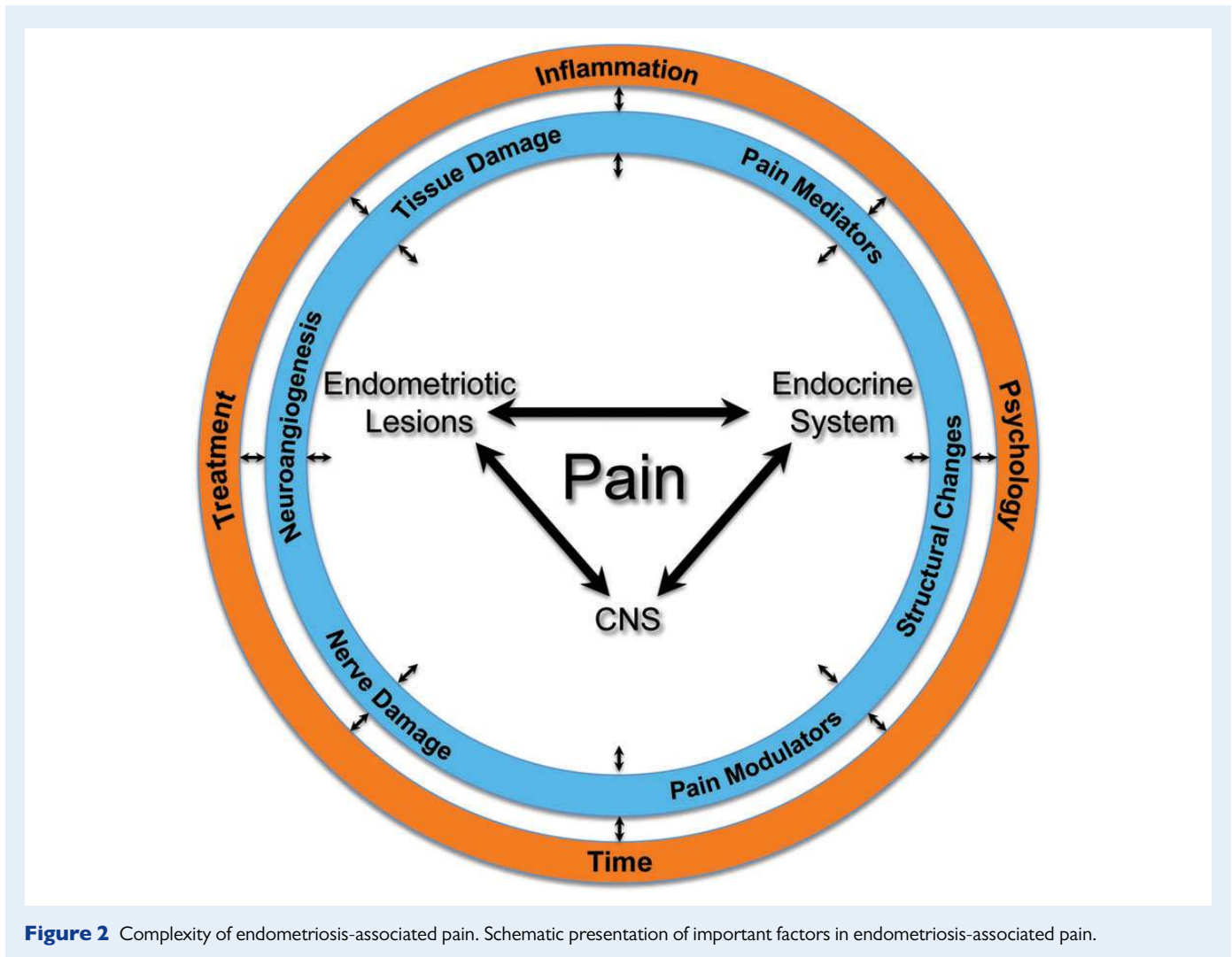


Figure 2 Complexity of endometriosis-associated pain. Schematic presentation of important factors in endometriosis-associated pain.

Table 1 Inclusion and exclusion criteria for studies on the role of the peripheral nervous system in the pathophysiology of endometriosis-related pain.

Inclusion criteria	Exclusion criteria
Visual and/or histological confirmation of endometriosis, defined as the presence of peritoneal endometriotic lesions, endometrioma and/or DIE	Anecdotal reports, editorials, letters to the editor, conference abstracts, duplicate papers, reviews without original data, surgical technique description or surgical trials, surgical or diagnostic case reports.
Patients and controls clearly shown to have, or not to have, endometriosis (all participants to have undergone either laparoscopy or laparotomy to confirm presence or absence of disease)	Surgical verification of participants not performed or unclear (including 'hysterectomy' unless clearly laparoscopic/laparoscopically assisted or transabdominal)
English language publication	

DIE, deep infiltrating endometriosis.

cycle or disease stage (as per the American Society for Reproductive Medicine) were seen. Remarkably, higher expression of nerve growth factor (NGF) and interleukin (IL)-1 β from endometriotic epithelial and stromal cells was associated with a higher ratio of sensory to sympathetic nerve fibres ($P < 0.001$) supporting NGFs role in the development, survival and maintenance of sensory nerve fibres. In another study using identical markers, the same group also demonstrated a reduction in the density of sympathetic nerve fibres in unaffected, distant peritoneum of patients with endometriosis compared with healthy peritoneum of non-endometriosis patients whilst sensory nerve fibres remained the same (Arnold et al., 2013). However, the density of distant sympathetic fibres in women with endometriosis was higher in comparison to the density within the lesions, suggesting a possible restructuring of sympathetic innervation in peri-lesional endometriotic implants compared with distant sites, as seen in other chronic inflammatory disease (Pongratz and Straub, 2013).

Recently, Mechsner's group demonstrated the presence of calcium-binding proteins (calbindin, parvalbumin and calretinin), involved in calcium homeostasis, an important modulator of neuronal activity, and sensitization in neurons in peritoneal endometriosis-associated nerve

Table II Biological molecule functions and most used antibody in detecting nerve fibres.

	Nerve fibres detected	Most used antibodies
Acetylcholine (ACh)	Cholinergic nerve fibres	Polyclonal rabbit anti-ACh
Calcitonin gene-related peptide (CGRP)	Sensory Ad and C fibres	Monoclonal rabbit anti-CGRP
Neuropeptide Y (NPY)	Sensory Ad and C fibres; adrenergic fibres (sympathetic)	Polyclonal rabbit anti-human NPY
Neurofilament (NF)	Myelinated nerve fibres	Monoclonal mouse anti-human NF
Neuron-specific enolase (NSE)	Pan-neuronal marker	Polyclonal rabbit anti-NSE
Protein gene product 9.5 (PGP9.5)	Pan-neuronal marker against myelinated and unmyelinated fibres	Polyclonal rabbit anti-PGP9.5
Substance P (SP)	Sensory Ad and C fibres	Polyclonal rabbit anti-SP
Protein S100	Myelinated nerve fibres	Monoclonal mouse anti-S100
Vasoactive intestinal polypeptide (VIP)	Sensory Ad and C fibres; cholinergic fibres (parasympathetic)	Polyclonal rabbit anti-human VIP
Tyrosine hydroxylase (TH)	Adrenergic nerve fibres	Monoclonal anti-TH

fibres (Barcena de Arellano *et al.*, 2013b). The amount of calbindin, calretinin and parvalbumin associated-nerve fibres was significantly increased in proximal and distal areas of peritoneal endometriotic lesions compared with the healthy peritoneum. Additionally, the amount of calbindin, but not calretinin and parvalbumin-positive nerve fibres, was increased in the distal areas of peritoneal endometriotic lesions when compared with the central areas. Among the calcium-binding proteins, only calretinin, involved in nociceptive pathways (Ren *et al.*, 1993), levels were higher in the peritoneal fluid (PF) of women with symptomatic endometriosis when compared with asymptomatic women. These proteins, known to have a role in central and peripheral sensitization, might represent one of the links between the development of new nerve fibres and the pain modulation in women with endometriosis and represent an interesting field to be further investigated.

Finally, Tamburro *et al.* demonstrated that a greater staining intensity of TGF- β 1 in nerve fibres was related to a higher dysmenorrhoea score and to the appearance of red peritoneal endometriotic lesions (Tamburro *et al.*, 2003). TGF beta plays a key role in the immune response, angiogenesis and endometrial stromal cell proliferation in endometriosis (Zhang *et al.*, 2012). Many cell types such as macrophages secrete TGF beta especially after inflammatory stimulation. Remarkably, a higher macrophage density correlated positively with the amount of nerve fibres both in the peritoneal endometriotic lesions and in the healthy peritoneum of women with endometriosis. In general,

macrophage density was significantly higher in endometriotic patients compared with women without endometriosis (Tran *et al.*, 2009). The increased density of immune cells near the nerve fibres suggests a potentially bi-directional role of the immune system and nerve fibres in endometriosis-related pain, which we will discuss below.

Effects of peritoneal fluid

To test the potential effect of the intra-abdominal environment on nerve growth Mechsner's group then investigated the effect of PF of endometriosis patients on inducing neurite outgrowth of sensory nerve fibres in an *in vitro* model (Barcena de Arellano *et al.*, 2011a). Cultured chicken dorsal root ganglia (DRG) incubated with PF from these women showed increased neurite growth compared with DRG incubated with the PF from patients without endometriosis ($P < 0.01$), and neurite outgrowth of ganglia treated with PF from endometriosis patients was significantly reduced by two NGF inhibitors (anti-NGF and K252a) suggesting a NGF-dependent pathway of nerve growth in this disease. NGF belongs to a family of proteins (neurotrophins) that promote development and survival of neurons (Huang and Reichardt, 2001). Interestingly, neurite outgrowth was similar when PF from women with and without hormonal therapy was used. Together with the data from Tokushige *et al.* (2009) mentioned above, this suggests that the potential effect of hormonal treatment on peritoneal nerve fibres is unlikely mediated directly via NGF. In addition, no differences in the assay were seen between endometriosis patients with different pain scores (Barcena de Arellano *et al.*, 2011b). Finally, exposure of DRG and sensory ganglia to PF from endometriosis patients and controls *in vitro* also showed increased sensory versus sympathetic neurite outgrowth in the endometriosis group (Arnold *et al.*, 2012). Taken together, these studies indicate that NGF may play an important role in endometriosis-associated nerve growth, but that other factors seem to be involved in endometriosis-related pain.

The presence of neurotrophins (NT), such as NGF, in PF then prompted investigations to test their correlation with clinical data including preoperative pain scores. Including different groups of women with peritoneal endometriosis, adenomyosis, adhesions or asymptomatic controls, Mechsner's group found elevated levels of NGF and neurotrophin-3 (NT-3), but not of brain-derived neurotrophic factor (BDNF) in the PF of women with peritoneal endometriosis compared with the other groups (Barcena de Arellano *et al.*, 2013a). However, the concentrations of these neurotrophins did not correlate with the pain symptoms in any of the groups, again suggesting other mechanisms may also be involved in pain generation/modulation. A recent publication showed contrasting results, with a significant association between elevated NGF levels and the severity of dysmenorrhoea and moderate or severe dyspareunia in a dataset of women with peritoneal or ovarian endometriosis (Kajitani *et al.*, 2013). These contrasting results are difficult to explain as the pain evaluation scales were similar and in similar patient datasets. However, the NGF-PF levels were very different in the two studies (2000 pg/ml in the first one versus 60 pg/ml in the second one (Barcena de Arellano *et al.*, 2013a; Kajitani *et al.*, 2013), supporting the need for standardization of protocols for patient recruitment, sample collection and processing as recently developed as part of the international EPHeCT study (Endometriosis Phenome and Biobanking Harmonisation Project) (papers in review).

Uterosacral and rectovaginal endometriosis

Endometriotic lesions involving the rectovaginal septum and the uterosacral ligaments are often deep infiltrating (>5 mm) and show histological signs of fibrosis. They are strongly associated with pelvic pain (Cornillie et al., 1990; Vercellini et al., 2007) and dysmenorrhoea (Fauconnier et al., 2002; Chapron et al., 2003), but the mechanisms remain unclear. Anaf et al. were the first to describe the presence of myelinated nerve fibres, stained with S100, in 28 women with rectovaginal endometriotic nodules (Anaf et al., 2000). Patients with more severe preoperative pelvic pain, dysmenorrhoea and dyspareunia (visual analogue scale >7) demonstrated a significantly higher proportion of nerve fibres within the endometriotic nodules and a close histological relationship between the nerve fibres, endometriotic nodules and fibrosis. Nodules from these women also showed more intra- and peri-neural infiltration. A high concentration of nerve fibres in deep infiltrating endometriosis (DIE), in particular uterosacral ligaments (stained with S100 and PGP9.5) were also described subsequently in two case reports (Quinn and Armstrong, 2004; Quinn and Kirk, 2004). Interestingly, specimens from women with DIE (uterosacral ligaments, pouch of Douglas and rectosigmoid endometriosis) seemed to contain more chymase-positive and trypsinase-positive (activated) mast cells than biopsy specimen from women with peritoneal and ovarian endometriosis, and the mast cells appeared to be in a closer relationship with nerves (<25 µm) compared with other sites (Anaf et al., 2006). These data further support a potential interaction between the immune system and the presence of nerve fibres and suggest a possible cellular and molecular mechanism, and not just mechanical (fibrosis/nerve compression) explanation, for the higher rate of pain in DIE compared with other variants. Different from peritoneal lesions, Kelm and co-workers reported an increased presence of sympathetic (positive for neuropeptide Y: NPY) and parasympathetic (positive for vasoactive intestinal peptide: VIP) nerve fibres in the uterosacral ligament and adjacent connective tissue in women with DIE, compared with women without endometriosis (Kelm et al., 2008).

However, the different antibodies used (NPY versus TH, the rate-limiting enzyme of catecholamine biosynthesis) as well as the different inclusion criteria might account for these differences.

An increased density of nerve fibres stained with PGP9.5 and NF in DIE (uterosacral ligaments, $n = 10$; pouch of Douglas, $n = 10$; peritoneal sidewall, $n = 7$ and rectum, $n = 7$) compared with peritoneal endometriotic lesions ($P < 0.001$) was subsequently demonstrated (Wang et al., 2009a). In particular, the densities of nerve fibres stained with PGP9.5 and NF were higher in rectal lesions than other sites. NGF and NGF high-affinity receptor (TrkA-A) were strongly expressed in endometriotic glands and stroma while NGF low-affinity receptor (NGFRp75) was only strongly expressed in endometriotic DIE glands. The DIE nerve fibres expressed TH, VIP, vesicular acetylcholine transporter (VACHT), SP, NPY and CGRP, without differences in their densities, indicating a mixture of sensory and sympathetic fibres (Wang et al., 2009b). Similar results of a higher presence of nerve fibres in DIE compared with other sites were further confirmed in a more recent study (McKinnon et al., 2012).

Several studies investigated the influence of hormones on the development of nerve fibres in DIE. Signorile et al. showed a direct correlation among the expression of estrogen and progesterone receptors, S100-positive nerve fibres and CD34 staining in rectovaginal endometriotic nodules, suggesting a role of hormones in maintaining not only

the presence of endometriotic implants but also the nerve fibres (Signorile et al., 2009). In a small study, the expression of Wilms' tumour gene 1 (WT1) was demonstrated in 5–70% of the nerves in seven patients and 100% in two patients with DIE nodules (under hormonal treatment) (Coosemans et al., 2009). WT1 has been implicated with neuronal development and apoptosis (Wagner et al., 2002, 2005; Lovell et al., 2003). Coosemans et al. (2009) speculated that these findings might support a potential role of estrogens in maintaining and promoting endometriosis-associated pain. In fact, estrogens positively regulate Src and mitogen-activated protein kinase, which is activated when NGF binds TrkA, promoting the NGF nociceptive pathway. They therefore proposed that in patients under hormonal treatment a down-regulation of the NGF-TrkA-Ras/Src pathway fails, with a consequent rise of WT1 expression, thereby inhibiting NGF-associated nociceptive function.

DIE showed a higher presence of endometriosis-associated nerve fibres compared with other pelvic sites (Table III). However, the reason for this particular nerve fibre density in DIE remains unclear. It is conceivable that it represents a further step in a similar pathophysiological process seen in other locations. Another possibility is that DIE may show a higher potential for promoting nerve growth compared with other sites. DIE often develops in richly innervated anatomical sites, so it is not clear if the presence of the new nerves fibres is partially due to a higher presence of nerve fibres in the normal tissue (uterosacral ligaments, bowel), where the proinflammatory reaction of the endometriotic implants may lead to more aggressive neuroangiogenesis than in other sites. This is typically seen in many cancers that grow in highly vascularized areas, above all the pancreatic carcinoma (Liu and Lu, 2002). However, a direct correlation between a higher density of nerve fibres in endometriotic lesions (defined as any nerve fibre within 1.5 mm from the lesion) and the pain perceived was only seen in peritoneal lesions but it is less clear in the rectovaginal lesions (McKinnon et al., 2012), challenging again the role of these fibres in endometriosis-associated pain.

Intestinal endometriosis

Anaf et al. were also the first group to study the relationship between bowel endometriosis and nerve density in the different layers of the large bowel. S100 immunohistochemistry staining of large bowel sections showed that $53 \pm 15\%$ of endometriotic lesions were either in direct contact with nerves and along the nerve pathways or directly invaded the nerves (Anaf et al., 2004). These findings were confirmed by Ferrero et al. who compared bowel specimens obtained from 11 women with colorectal endometriosis with those from eight colon adenocarcinoma control women (Ferrero et al., 2010). The density of sympathetic nerve fibres stained with TH was significantly lower in the mucosal and muscular layers near the endometriotic lesions compared with areas far from the lesions while the density of sensory nerve fibres was unaltered in all of the analysed areas near the endometriotic lesions compared with areas far from the lesions.

The authors did not investigate whether these TH+ fibres were extrinsic sympathetic fibres (with the main neurotransmitter norepinephrine) or intrinsic dopaminergic nerve fibres (present in the enteric nervous system and TH and dopamine b-hydroxylase positive); the preponderance of SP+ fibres compared with sympathetic nerve fibres in the inflamed area surrounding endometriotic lesions highlights the hypothetical role of this autonomic/sensorial imbalance during chronic inflammation.

Table III Nerve fibre density in different endometriotic lesions.

	NF (mean \pm SD/mm ²)	PGP9.5 (mean \pm SD/mm ²)	Sympathetic (mean \pm SD/mm ²)	Parasympathetic anti-VIP (mean \pm SD/mm ²)	Anti SP (mean \pm SD/mm ²)
Peritoneal	6.7 \pm 3.7 (Tokushige <i>et al.</i> , 2006a) 5.2 \pm 4.7 and 5.4 \pm 5.2 in the proliferative and secretory phase, respectively) (Wang <i>et al.</i> , 2011)	8.0 \pm 8.5 with low macrophage density and 24.6 \pm 17.5 with high macrophage density) (Tran <i>et al.</i> , 2009) 16.3 \pm 10.0 (Tokushige <i>et al.</i> , 2006a) 14.9 \pm 11.2 and 15.9 \pm 10.2 in the proliferative and secretory phase, respectively) (Wang <i>et al.</i> , 2011)	Anti-TH 0.67 \pm 1.15 (Arnold <i>et al.</i> , 2013) Anti-TH 1.2 \pm 2.6 (Arnold <i>et al.</i> , 2012)		6.56 \pm 2.78 (Arnold <i>et al.</i> , 2013) 4.0 \pm 3.2 (Arnold <i>et al.</i> , 2012)
DIE	17.5 \pm 12.8 (Wang <i>et al.</i> , 2009b)	67.6 \pm 65.1 (Wang <i>et al.</i> , 2009b)	Anti-TH 8.4 \pm 4.8 (Wang <i>et al.</i> , 2009b)	9.5 \pm 5.2 (Wang <i>et al.</i> , 2009b)	6.7 \pm 4.1 (Wang <i>et al.</i> , 2009b)
Bowel	51.9 \pm 28.1 (Wang <i>et al.</i> , 2009a)	174.8 \pm 91.7 (Wang <i>et al.</i> , 2009a)	Anti-NPY 28.8 \pm 15.0 (Wang <i>et al.</i> , 2009a)	26.8 \pm 15.6 (Wang <i>et al.</i> , 2009a)	21.6 \pm 11.0 (Wang <i>et al.</i> , 2009a)
Rectal	49.0 \pm 19.2 (Wang <i>et al.</i> , 2009a)	171.0 \pm 97.6 (Wang <i>et al.</i> , 2009a)	Anti-NPY 24.6 \pm 16.7 (Wang <i>et al.</i> , 2009a)	23.8 \pm 13.6 (Wang <i>et al.</i> , 2009a)	25.3 \pm 19.2 (Wang <i>et al.</i> , 2009a)
Ovarian	5.1 \pm 0.9 (Tokushige <i>et al.</i> , 2010)	55.6 \pm 11.8 (Tokushige <i>et al.</i> , 2010) 0.72 \pm 1.36 (Zhang <i>et al.</i> , 2010a)	Anti-NPY 31.0 \pm 3.5 (Tokushige <i>et al.</i> , 2010)	7.8 \pm 2.2 (Tokushige <i>et al.</i> , 2010)	15.1 \pm 2.7 (Tokushige <i>et al.</i> , 2010)

Wang *et al.* demonstrated a significantly higher amount of nerve fibres stained with PGP9.5 and NF (myelinated) in intestinal DIE compared with non-intestinal DIE (subperitoneal and uterosacral ligaments) and normal colon. NGF and TrkA-A were strongly expressed in the endometriotic stroma of intestinal DIE. Nerve fibres strongly expressed GP-43, SP, VIP and NPY, suggesting also in bowel endometriosis the presence of a mixture of sensory A δ , sensory C, cholinergic and adrenergic nerve fibres. There was no difference in the density of these distinct nerve fibres between different intestinal sites (Wang *et al.*, 2009b).

Unfortunately, although intestinal endometriosis is often associated with significant morbidity, none of the authors evaluated clinical symptoms in their cohorts.

Ovarian endometriosis

A preliminary study on ovarian endometriosis found no nerve fibres in endometriomas removed by cystectomy or oophorectomy stained with NF (Al-Fozan *et al.*, 2004). Other groups contradicted these results (Anaf *et al.*, 2002, 2006; Odagiri *et al.*, 2009; Tokushige *et al.*, 2010; Zhang *et al.*, 2010b). In fact, two studies demonstrated that ovarian endometriomas had more intense staining with neural cell adhesion molecule and NGF than peritoneal endometriosis (Anaf *et al.*, 2002; Odagiri *et al.*, 2009) and presented a higher rate of mast cells than peritoneal lesions (Anaf *et al.*, 2006).

Nerve fibres stained for NF were present at significantly higher density in ovarian endometriomas compared with normal ovaries from women with ovarian endometriomas and normal ovaries from women without endometriosis (Tokushige *et al.*, 2010). Similar to other anatomical sites, nerve fibres in ovarian endometriosis were found to be a mixture of sympathetic (NPY, 31.0/mm²),

sensory (SP, 15.1/mm²) and parasympathetic (VIP, 7.8/mm²) fibres (Tokushige *et al.*, 2010), but without the imbalance between sensory versus sympathetic seen in peritoneal and bowel endometriosis, suggesting also differential qualitative neuroangiogenic properties of differential endometriosis location. Zhang *et al.* detected nerve fibres in only 31.1% (19/61) of women with ovarian endometriosis using PGP9.5. Significantly more nerve fibres were seen in patients with endometriomas and pain (40.0%) compared with those without pain (19.2%) whilst no differences were seen between women who had ovarian endometriosis alone and the women who had ovarian and other extra-ovarian endometriotic implants (Zhang *et al.*, 2010a). Finally, Liu *et al.* demonstrated the immunoreactivity of TRPV1 (transient receptor potential cation subfamily VI) positive nerve fibres in and/or surrounding the endometriotic ovarian lesions and in eutopic endometrium in women with endometriosis. TRPV1 is a nociceptor, which predominantly transmits heat and pain sensation, and may play a role in the interaction between the inflammatory environment and pain as well as hyperalgesia (Koerber *et al.*, 2010). The TRPV1 nerve density in ectopic endometrium was significantly higher than that in the eutopic endometrium in both epithelial and stromal components and correlated positively with the severity of dysmenorrhoea (Liu *et al.*, 2012).

Nerves in endometrium and myometrium

Ever since Sampson's theory of retrograde menstruation was published (Sampson, 1927), eutopic endometrium has been a focus of interest in endometriosis research. Due to its accessibility via a semi-invasive procedure (endometrial biopsy) it also has the potential to be used in a biomarker assay (May *et al.*, 2011). A preliminary study on hysterectomy

specimens showed an increase of nerve fibres, stained with anti-S100, in the myometrium of the lower half of the uterus in patients with endometriosis or those with chronic pelvic pain (CPP) without endometriosis compared with controls (with painless gynaecologic indications or uterine fibroids) (Atwal et al., 2005). A subsequent study described the presence of nerve fibres both in the basal and in the functional layer of the endometrium in all endometriosis patients ($n = 35$), while no fibres were seen in the functional layer of the endometrium in women without endometriosis ($n = 82$) (Tokushige et al., 2006b). In another study the same authors confirmed some of these findings and demonstrated that the nerve fibres in the functional endometrial layer stained for VIP, NPY, SP and CGRP suggesting a mixture of sensory,

adrenergic and cholinergic fibres (Tokushige et al., 2007). In the myometrium the presence of nerve fibres was seen in both in patients with and without endometriosis. These endometrial fibres seem to be hormone dependent as their presence was reduced in the functional and basal endometrial layers of women undergoing hormonal treatment compared with untreated women with endometriosis (Tokushige et al., 2008) (Table IV).

The results of these studies supported the potential use of endometrial nerve fibres as a biomarker for the disease (May et al., 2011). The first study to test this hypothesis using PGP9.5 staining demonstrated a specificity and sensitivity of 100% (Al-Jefout et al., 2007). A second study showed that the combined analysis of neural markers PGP9.5, VIP and

Table IV Nerve fibres in endometrium and myometrium of women with endometriosis.

	Number of patients	Results	Site	Number of fibres (total nerve fibres density)
Atwal et al. (2005)	48	Sixteen uteri of women with EM (group 1); 15 uteri of women without EM but CPP (group 2) and 17 controls uteri (group 3)	Myometrium	Myometrium stained with anti-S-100 (median, range): Group 1: 31.5 (17–53) Group 2: 22 (14–66) Group 3: 12 (6–29)
Tokushige et al. (2006b)	117	Endometrial curettage ($n = 25$) and hysterectomies ($n = 10$) performed on women with EM (group 1) and without EM ($n = 47$ and $n = 35$, respectively) (group 2)	Endometrium	PGP9.5 staining (mean \pm SD/mm ²): curettage (group 1): 10 ± 7 curettage (group 2): 0 hysterectomy (group 1): 11 ± 5 hysterectomy (group 2): 0
Al-Jefout et al. (2007)	37	20 with EM (group 1) and 17 without EM (group 2)	Endometrium	PGP9.5 staining (mean \pm SD/mm ²): 20.4 ± 33.1 versus 0 in group 1 and 2, respectively
Tokushige et al. (2007)	45	Ten women (group 1) and 35 women (group 2) with and without EM	Endometrium and myometrium	PGP9.5 staining (mean \pm SD/mm ²): Endometrium: functional and basal layer (11 ± 5 versus 0 and 18 ± 8 versus 0 in group 1 and 2, respectively) Myometrium: 3.3 ± 1.2 versus 0.9 ± 0.8 in group 1 and 2, respectively
Tokushige et al. (2008)	36	18 uteri and 8 curettage from women with EM under hormonal treatment (group 1) and 10 women with untreated EM (group 2)	Endometrium and myometrium	PGP9.5 staining (mean \pm SD/mm ²): Endometrium: functional layer 0.4 ± 0.9 versus 11 ± 5 Basal layer: 0.9 ± 1.3 versus 18 ± 8 Myometrium: 1.5 ± 0.8 versus 3 ± 1
Zhang et al. (2009)	90	18 women with EM (group 1); 33 EM women with AM (group 2); 26 women with AM (group 3) and 13 with uterine fibroids (group 4)	Endometrium (functional layer)	PGP9.5 staining (range/mm ²): Group 1: 1.5 (0–3.0) Group 2: 0.6 (0–2.8) Group 3: 0.5 (0–7.0) Group 4: 0.6 (0–5.8)
Al-Jefout et al. (2009)	99	64 with EM (group 1) and 35 without EM (group 2)	Endometrium (functional layer)	PGP9.5 staining (mean \pm SD/mm ²): 2.71 ± 3.4 (group 1, 63/64 patients) versus 3.1 ± 1.7 (group 2, 6/35 patients)
Bokor et al. (2009)	40	20 women with EM (group 1) and 20 women without EM (group 2)	Endometrium	(mean \pm SD/mm ²) group 1 and 2: PGP9.5: 2.62 ± 2.19 and 0.21 ± 0.28 ; NPY: 2.52 ± 3.91 and 0.15 ± 0.23 ; SP: 2.29 ± 2.2 and 0.1 ± 0.2
Aghaey Meibody et al. (2011)	27	12 women with EM (group 1) and 15 without EM (group 2)	Endometrium	PGP9.5 staining (mean \pm SD/mm ²): 13.1 ± 3.3 versus 2.2 ± 4.7 in group 1 and 2, respectively

AM, adenomyosis; CPP, chronic pelvic pain; EM, endometriosis.

SP could predict the presence of minimal–mild endometriosis with a 100% specificity and 95% sensitivity (Bokor *et al.*, 2009), showing a nerve density 14 times higher in women with minimal–mild endometriosis than in controls with a laparoscopically confirmed absence of endometriosis and normal pelvis. Another group presented similar findings (Aghaey Meibody *et al.*, 2011). A further study evaluated efficacy of nerve fibre detection with PGP9.5 in endometrial biopsies in a double-blind comparison with laparoscopically verified endometriosis. The specificity and sensitivity were 83 and 98%, respectively, positive predictive value was 91% and negative predictive value was 96%. The authors found that women with endometriosis and pain symptoms had a significantly higher nerve fibre density in comparison to women without pain, but found no differences regarding phase of the menstrual cycle (Al-Jefout *et al.*, 2009).

These data were quite encouraging; however, Zhang *et al.* found that PGP9.5-immunoreactive nerve fibres in the functional layer of the endometrium were present in women with symptomatic endometriosis, but also in women with adenomyosis or uterine fibroids and pain symptoms. There was no statistical difference in the nerve fibre density among the different diseases (Zhang *et al.*, 2009) and the same results were achieved evaluating endometrial nerve fibres with PGP9.5 and NF in women with adenomyosis or uterine fibroids (Zhang *et al.*, 2010b). These results were further confirmed by a recent study showing no correlation between the presence of nerve fibres in the functional layer and the presenting symptoms, endometrial histology, or current hormonal therapy in women with peritoneal endometriosis (Leslie *et al.*, 2013). Another recent study confirmed that the protein expression levels of neuronal or neurotrophic markers, such as PGP9.5, NGFp75 or TPVRI, are independent of the cycle phase with no differences between patients with or without endometriosis (Newman *et al.*, 2013). It remains unclear why recent studies have contradicted earlier results. The heterogeneity of the endometriosis phenotype, different tissue collection and processing protocols may be only a few of the possible explanations. However, by standardization of these factors in large RCTs with particular emphasis on minimal and mild endometriosis we hopefully will get an answer to the use of the presence of nerve fibres in eutopic endometrium as a biomarker for endometriosis (d'Hooghe *et al.*, 2006; Fassbender *et al.*, 2013).

Despite this controversy, most studies mentioned above have shown nerve fibres in eutopic endometrium; however it is unclear what promotes their growth. Mechsner's group recently addressed this issue by investigating the potential role of neurotrophins in eutopic endometrium. Using western blot, they found that NGF, BDNF and NT-3 are expressed in uterine fluid and endometrial biopsies, but no difference between endometriosis patients and controls could be detected (Barcena de Arellano *et al.*, 2012a). Also no or minimal neurite outgrowth occurred in the neuronal outgrowth assay using conditioned endometrial cell culture media and uterine fluids from women with endometriosis and controls. However, another recent paper using a proteomic approach found that the levels of NT-4/5 and BDNF were greater in endometriosis cases compared with controls while NGF levels did not differ between the two study groups (Browne *et al.*, 2012). The authors postulated that these high local concentrations of NT-4/5 and BDNF, but not NGF, in women with endometriosis might stimulate a differential nerve fibre growth pattern in these patients, potentially contributing to the higher levels of pain perceived by this

population. Again, larger studies under standardized conditions are necessary to shed light onto this issue. In summary, these studies seem to show that nerve fibres in the functional layer of eutopic endometrium may only be associated with the presence of pain symptoms, but be independent of the underlying pathology. Indeed, these nerve fibres might be important in generation of pain; however, until data from larger prospective studies are available the use of such fibres as a diagnostic tool for endometriosis remains to be confirmed (May *et al.*, 2011).

Adenomyosis

Adenomyosis, which describes the presence of endometrial stroma and glands in the myometrium, is typically diagnosed by imaging (predominantly magnetic resonance imaging, sometimes by ultrasound) or histologically in hysterectomy specimen. A common pathogenesis for adenomyosis and endometriosis has been hypothesized (Leyendecker *et al.*, 1998), but they are generally regarded as separate entities. As dysmenorrhoea is the cardinal symptom of adenomyosis, several studies investigated the presence of nerve fibres in adenomyosis specimens. Counter intuitively however, the first study demonstrated the absence of nerve fibres in the neurovascular bundles of the endometrial–myometrial interface (PGP9.5 staining) in uteri containing adenomyosis compared with controls (Quinn, 2007). In fact no nerves were found in 95% of adenomyotic nodules tested (22/23 uteri) and only focal proliferation of small diameter nerve fibres was observed at the margins of adenomyosis. Zhang and colleagues found fewer nerve fibres in the functional layers of the endometrium in women with symptomatic adenomyosis compared with women with symptomatic endometriosis or fibroids (PG9.5 and NF) but this did not reach statistical significance (Zhang *et al.*, 2009).

Patients with adenomyosis-associated dysmenorrhoea are often successfully treated by a levonorgestrel-releasing intrauterine system (LNG-IUS). Therefore, Choi *et al.* tested the expression of NGF and its receptors NGFR p75 and TrkA in the uteri of affected women (Choi *et al.*, 2010) and found reduced expression levels in the glandular epithelium, stroma, and myometrial cells compared with control women. Barcena de Arellano *et al.* (2013c) found no overall significant difference in nerve fibre density (stained with S100, PGP9.5 and p75NTR) and the expression of NGF, NT-3 and their receptors (p75NTR and TrkA) in all endometrial layers between patients with adenomyosis and the control group (uterine fibroids). Their findings suggested that the myometrium of women with adenomyosis or with adenomyosis and endometriosis had a tendency to show fewer nerve fibres compared with those without the disease (Barcena de Arellano *et al.*, 2012b, 2013c). In particular the myometrium of these patients seems to be depleted of noradrenergic nerves in an estrogen-dependent way, as the authors found a shift in the estrogen receptor (ER) α /Er β ratio, with higher ER α expression in the adenomyosis group compared with the control group (Barcena de Arellano *et al.*, 2013c).

In summary, these studies suggest a reduced nerve fibre density in adenomyotic tissue compared with endometriotic lesions. It is possible that qualitative nerve fibre remodelling takes place in the myometrium of women with adenomyosis with a depletion, especially of noradrenergic fibres, via an ER α -dependent mechanism, an interesting concept that deserves to be further investigated.

Association between nerve and vascular growth

To improve our understanding of the potential implications of nerve fibres in eutopic and ectopic endometrium and their potential role in pain it is crucial to study possible mechanisms of nerve growth. As not much is yet known about such mechanisms in endometriosis we will summarize data from other systems and present relevant endometriosis data where available.

It is now well accepted that vasculogenesis and angiogenesis, the growth of blood vessels *de novo* or from existing blood vessels, respectively, play an integral part in the establishment and growth of endometriotic lesions (May and Becker, 2008; Laschke et al., 2011). In the adult, blood vessels form mostly during wound healing, skeletal growth and the menstrual cycle as well as in some pathological conditions such as cancer and endometriosis (Becker and D'Amato, 2007). The growth of both blood vessels and nerve fibres are closely integrated processes, linked by common pathways and molecules (Carmeliet and Tessier-Lavigne, 2005; le Noble et al., 2008).

Molecular guidance cues for developing nerves and blood vessels

Alignment of blood vessels and nerves is commonly observed in adult tissues and probably reflects the mutual requirement of one for the other, as larger nerves require vascularization to ensure nutrient and oxygen supply, whereas blood vessels need innervation to control vasodilation or constriction (Arese et al., 2011). In fact, endothelial cells and pericytes produce signals, such as artemin and NT, that attract axons to the pioneer vessel (Honma et al., 2002; Kuruvilla et al., 2004).

Conversely, nerves may also produce signals such as vascular endothelial growth factor (VEGF) to guide blood vessels (Mukouyama et al., 2002).

The development of the nervous system shares some patterns with the formation of blood vessels (Carmeliet and Tessier-Lavigne, 2005). Neurons can project axons over long distances in order to reach their final targets, a mechanism facilitated by the growth cone: a highly mobile, sensory structure that, through filopodial cyclic patterns of extensions and retraction, selects a trajectory from several possible routes (Dickson, 2002; Huber et al., 2003). Similar to the endothelial tip cell, which is essential for angiogenesis (see below), the growth cone detects and responds to environmental cues, which can be attractive or repulsive signals, and guides the neurons to their appropriate targets (Mortimer et al., 2008). Common signalling molecules that play a role in the process of neuronal development and vascular morphogenesis are listed in Table V (Eichmann et al., 2005; Nico et al., 2008; Gelfand et al., 2009; Adams and Eichmann, 2010; Melani and Weinstein, 2010). These bi-functional guidance cues depend on a variety of factors including the intracellular state of the growth cone, differential expression of receptor complexes, crosstalk between intracellular signalling cascades and a myriad of axon guidance signalling events (Larrivée et al., 2009) (Fig. 3).

VEGF and NGF: guidance cues for vessels and nerves?

VEGF and NGF are key mediators of both angiogenesis and neurogenesis and are highly expressed in both eutopic and ectopic endometrial tissue of women with endometriosis. VEGF-A is the predominant member of a family of growth factors promoting blood vessel growth and endothelial cell survival and permeability (Dvorak et al., 1995). Its role in angiogenesis and vasculogenesis in endometriosis is well established (McLaren et al.,

Table V Roles of guidance cues in nerves and blood vessels development.

		Nerve fibres	Blood vessels	Endometriosis
Netrins	Family of evolutionarily conserved secreted molecules which regulate axon guidance. Attraction to Netrin-1 is mediated via activation of receptors of the DCC family or UNC5 family	Dual activity to attract or repel axons depending on the receptor type to which the netrins bind	Netrin-1 seem to have a repulsive role for navigating vessels	No
Semaphorins (SEMA)	Large family of cell-associated and secreted proteins. Membrane-associated SEMA bind to Plexins, whereas secreted SEMA bind to Neuropilins, which function as co-receptors for Plexin signalling	Dual activity in attracting or repelling axons depending on the receptor bind and on the crosstalk between SEMA receptors and other pathways	The PlexinD1/Sema3E interaction seems to mediate repulsive vessel guidance	Yes (Kao et al., 2003)
Slits	Large secreted glycoproteins that bind to trans-membrane receptors of the Roundabout family. They have a role in commissural midline crossing and other axon guidance processes, as differentiation and migration of diverse neuronal cell populations	Predominant repulsive role	Vascular Slit may act as an attractive or a repulsive cue, depending on the receptor to which it binds.	Yes (Shen et al., 2009; Nie et al., 2011; Guo et al., 2013)
Ephrins (Eph)	Cell surface proteins that are either attached via a glycosylphosphatidylinositol anchor (Eph-A subclass) or a trans-membrane region (Eph-B ligands). They bind to Eph receptor tyrosine kinases, a large family of trans-membrane proteins.	Eph signalling interactions usually generate repulsive signals. However the response seem to be related to the levels of Eph/Eph receptors interactions.	Dual activity depending on the receptor type to which the Eph bind. In particular the B-class ligands and receptors, seem to selectively mark the endothelium of arteries and veins.	Yes (Kao et al., 2003)

DCC, deleted in colorectal cancer; SEMA, semaphorins.

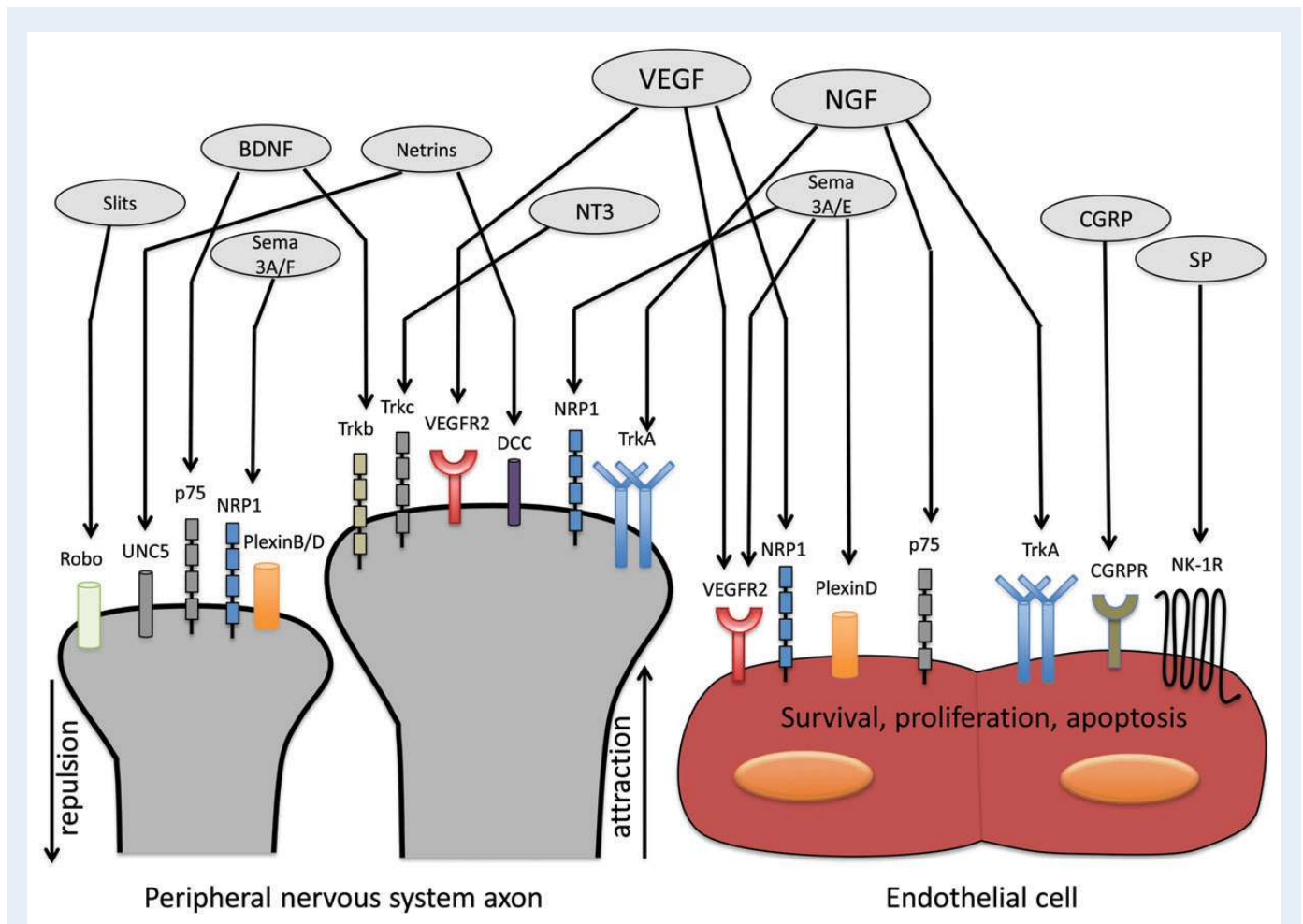


Figure 3 Common cues of nerve and blood vessels wiring. Abbreviations: BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; CGRPR, calcitonin gene-related peptide type receptor; DCC, deleted in colorectal carcinoma; NGF, β -nerve growth factor; NK-1R, neurokinin-1 receptor; NRP1, Neuropilin-1; NT3, neurotrophin 3; p75, low-affinity nerve growth factor receptor; Robo, Roundabouts; SP, substance P; Sema, semaphorin; Trk-A, high-affinity nerve growth factor receptor; Trk-B, BDNF/NT3 growth factors receptor; Trk-C, NT3 growth factor receptor; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2. Schematic representation of the different molecules involved in nerve and blood vessel remodelling. In general, VEGF and NGF stimulate neuronal migration, axonal growth and blood vessels growth. However, the filopodia of both endothelial cells and axons express several receptors that may account for the different roles of several molecules in nerve and blood vessel remodelling. The VEGF¹⁶⁴ throughout Nrp1 attracts the filopodia whereas Sema3A/F binding to plexins/Nrp1 repels the filopodia (Atwal *et al.*, 2003). In blood vessels Sema3E might bind to Plexins-d directly, independently of Npn1, promoting angiogenesis, whereas Sema3A stimulation of VEGFR/Nrp-1 inhibits endothelial cell migration. Conversely, binding of NGF to p75 on endothelial cells might inhibit endothelial survival (by inducing apoptosis) and encourage vascular regression. BDNF-p75 signalling shows a repulsive role while BDNF-TrkB signalling accounts for survival of 30–35% of sensory neurons (Jones *et al.*, 1994). The attractive effects of Netrins in nerves are mediated by receptors of the DCC family, whereas members of the UNC5 family mediate the repulsive effects. Slits are implicated in axonal repulsion through Robo receptors. Other molecules, such as substance P and CGRP which are more important in inflammation, can also have an angiogenic role.

1996; Becker *et al.*, 2011; Laschke and Menger, 2012). Interestingly, VEGF-A appears to play multiple roles in the adult nervous system development by acting independently both on blood vessels and neurons (Mackenzie and Ruhrberg, 2012). It is also a mitogen for astroglia and Schwann cells *in vitro* (Krum *et al.*, 2002; Mani *et al.*, 2005). Studies have shown that exogenous VEGF-A, with the modulation of semaphorin3A, increases sympathetic neurite outgrowth from sympathetic ganglion explants (Long *et al.*, 2009) and promotes sympathetic axon growth in surgically denervated adult rats (Marko and Damon, 2008). Semaphorin 3A is a secreted protein, which has both chemorepulsive

and chemoattractive properties (Klagsbrun and Eichmann, 2005). In the CNS, VEGF-A promotes the commissural axons guidance through the optic chiasm and spinal cord (Erskine *et al.*, 2011; Ruiz de Almodovar *et al.*, 2011). Both studies demonstrate that VEGF-A promotes axon guidance independently of its vascular roles through two receptors, either VEGFR2 (Ruiz de Almodovar *et al.*, 2011) or neuropilin-1 (NRP1) (Erskine *et al.*, 2011). NRP1 and NRP2, originally discovered as neuronal receptors for semaphorins, bind the most common splice-variant of VEGF-A (VEGF₁₆₅) and form a complex with VEGFR-2 and VEGFR-1 to regulate their signalling (Fuh *et al.*, 2000). Recent evidence

suggests that NRP1 in endothelial cells can also transmit VEGF signals independently of VEGFR-2 (Wang et al., 2003, 2007), and loss of NRP1 or NRP2 is associated with distinct vascular and neuronal defects (Geretti et al., 2008). Similarly, NGF acts both at a neuronal and a vascular level. However, it is still unclear if these processes occur directly via NGF-induced angiogenesis or indirectly, via the induction of classical angiogenic factors such as VEGF (Hansen-Algenstaedt et al., 2006).

NGF has been shown to stimulate proliferation of human umbilical vein, choroidal and dermal microvascular endothelial cells (Raychaudhuri et al., 2001; Cantarella et al., 2002; Steinle and Granger, 2003). Two studies showed that repeated subcutaneous injections of NGF in the ischaemic hind limb accelerated the process of revascularization, increasing the number of arterioles (Emanueli et al., 2002; Turrini et al., 2002). Indeed it seems that NGF can exert effects on endothelial cells as well as neurons, and VEGF can exert effects on neurons as well as blood vessels (Lazarovici et al., 2006).

Interestingly, these two pleiotropic factors (VEGF and NGF) as well as other neurotrophins (NT-3, NT-4/5 and BDNF) are expressed by ectopic endometrial cells or present in PF of women with endometriosis. Recently, *in vitro* and *in vivo* studies have provided the first clues about such mechanisms in endometriosis. In adult female rats with surgical induction of endometriosis, dynamic estrous changes in both VEGF and NGF in endometriotic cysts occurred in parallel with changes in the cysts' innervation and vascularization, suggesting that the developing innervation in an endometriosis model occurs through a cooperation between perivascular fibres accompanying the sprouting blood vessels that vascularize the cysts (Zhang et al., 2008). As mentioned above, in an *in vitro* assay neuronal growth of DRG incubated with PF from women with endometriosis was higher than from women without endometriosis, and treatment with NGF inhibitors significantly reduced neurite outgrowth (Barcena de Arellano et al., 2011b). Furthermore small interfering RNA knockdown of NGF was able to reduce both sensory and sympathetic nerve growth in a xenograft ectopic endometriotic implant (Chen et al., 2014).

These results suggest that NGF is an important neurotropic factor for mediating nerve growth in endometriosis; however, its role in pain generation needs to be clarified and considered together with other peripheral mechanisms.

Peripheral mechanisms generating and modulating pain in endometriosis

Increasing evidence suggests a role of new sensory fibres innervating endometriotic implants in maintaining the inflammatory phenotype through the release of proinflammatory molecules. Conversely, nerve fibres themselves may become sensitized through the proinflammatory milieu (Stratton and Berkley, 2011). The impaired immune response to endometrial cells and tissue in endometriosis patients is well established and has been implicated in peritoneal attachment, implantation, angiogenesis and growth of endometrial cells (May and Becker, 2008; Burney and Giudice, 2012). The bi-directional association of inflammation and peripheral nerves is mediated through various mechanisms involving multiple molecules such as growth factors and cytokines.

Inflammation, neuroangiogenesis and peripheral sensitization

The endometrial cells in the peritoneal cavity appear to promote the recruitment and activation of macrophages that synthesize and secrete different cytokines into the PF including IL-1, IL-6, IL-8, IL-10, tumour necrosis factor- α (TNF- α), TGF- β , VEGF and cyclo-oxygenase-2 (COX-2) (Herington et al., 2011; Capobianco and Rovere-Querini, 2013). Molecules secreted directly from endometriotic lesions include prostaglandins (Sacco et al., 2012), IL-1, IL-6 and TNF- α (Bergqvist et al., 2001) and NGF (Anaf et al., 2002). NGF, induced in part by IL-1 and TNF- α secretion (Bandtlow et al., 1990), may promote a positive proinflammatory feedback stimulating macrophage chemotaxis and the release of neuroactive cytokines or inflammatory mediators (Samah et al., 2008) as well as the activation of mast cells (Horigome et al., 1994; Kawamoto et al., 2002). Therefore, macrophages and their products can directly stimulate the synthesis of NGF (Bandtlow et al., 1990; Vigé et al., 1991) as well as the synthesis of BDNF and NT-3 (Asami et al., 2006) which play a crucial role for the survival, development and function of neurons in both the CNS and peripheral nervous system (Skaper, 2008). Interestingly, the presence of inflammatory cells near the new peri-endometriotic nerves seems to be more pronounced in patients with more severe symptoms (Mechsner et al., 2009; McKinnon et al., 2012). These new nerve fibres appear to be surrounded by mast cells that have also been associated with other pathological conditions in which pain is a predominant symptom and that can share a high comorbidity rate with endometriosis, such as interstitial cystitis/painful bladder syndrome and irritable bowel syndrome (O'Sullivan et al., 2000; Sant et al., 2007).

Both sympathetic and sensory nerve fibres may maintain a proinflammatory condition through the release of several peptides (Straub, 2007). SP plays a proinflammatory role, as it stimulates almost all immune cells (Harrison and Geppetti, 2001) and norepinephrine is able both to inhibit and enhance the activity of immune cells (Nance and Sanders, 2007). Perivascular release of CGRP might induce vasodilation and dural mast cell degranulation (Raddant and Russo, 2011).

As the tissue innervation is responsible for the amount of neurotransmitters (Straub, 2007), the density of nerve fibres (sympathetic and sensory nerve fibres) might strongly be involved in the local inflammation in endometriosis as well. *In vivo* experiments in a rodent model of endometriosis demonstrated that sensory fibres innervating ectopic implants express CGRP (Berkley et al., 2004), indicating the presence of C-fibre nociceptors in these lesions (Snider and McMahon, 1998). During inflammation, these fibres could theoretically convey information to the CNS and at the same time release several peptides into the local environment, and once activated may display an on-going electrical activity even after the inflammation resolves (Zhang et al., 2008).

Interestingly, all of these molecules involved in complex interactions promoting and maintaining the endometriotic implants can also participate in the sensitization and activation of peripheral nerve fibres thus creating a link between the endometriosis microenvironment and the generation of pain (Fig. 4).

A role for the autonomic nervous system?

The regulatory function of the autonomic nervous system (ANS) includes monitoring and influencing immune homeostasis. The activation of this system and the hypothalamic-pituitary-axis (HPA) configures the body to fight foreign antigens, and leads to liberation of fatty acids, amino

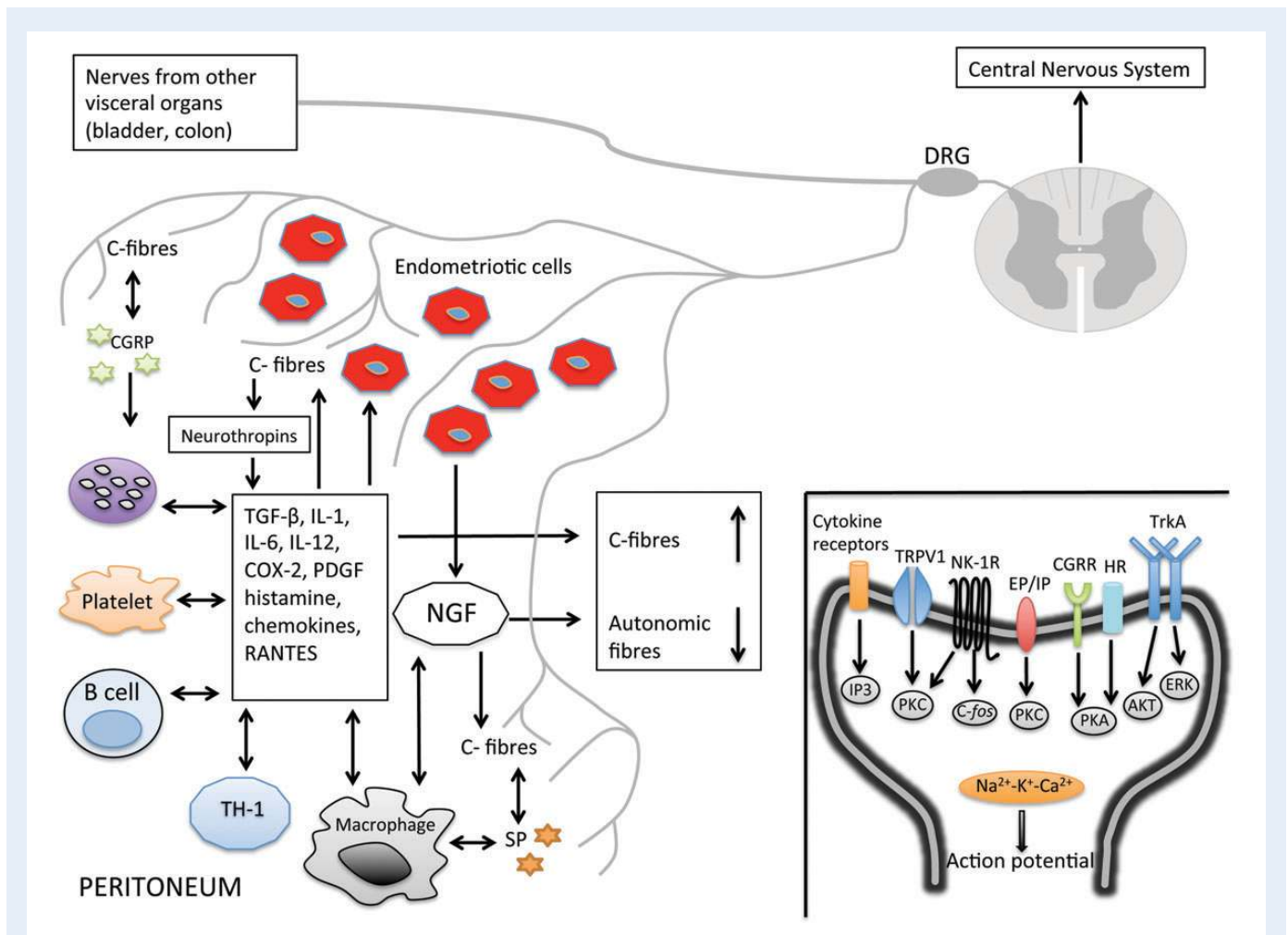


Figure 4 Possible pathophysiologic alterations in the peritoneal cavity of women with endometriosis. Abbreviations: AKT, protein kinase B; COX-2, cyclooxygenase-2; DRG, dorsal root ganglia; EP/IP, prostaglandins receptors; CGRP, calcitonin gene-related peptide; ERK, extracellular signal-regulated kinases; HR, histamine receptor; IL, interleukin; IP3, inositol trisphosphate; PDGF, platelet-derived growth factor; PK, protein kinase; RANTES, regulated on activation, normal T cell expressed and secreted; SP, substance P; TGF- β , transforming growth factor beta; TRPV1, transient receptor potential cation channel subfamily V member 1. Several immune mediators (cytokines, interleukins, growth factors) are up-regulated in peritoneal fluid of women with endometriosis. All these molecules can be secreted by different immune cells and mediate the release of each other, thus maintaining a vicious circle. NGF is markedly up-regulated in nerve fibres associated with the inflamed area. NGF is secreted by inflammatory and endometriotic cells and can stimulate the synthesis of SP and CGRP (which activate mast cells to release histamine). NGF and these molecules also sensitize (lower the threshold) or excite the terminals of sensory nerve fibres. Other neurotrophins also play a critical role in the bidirectional signaling mechanisms between immune cells and the neurosensory network structures in the peritoneal cavity. Hypothetical mechanisms of peripherally mediated cross-organ sensitization through the innervation of the visceral organs and endometriosis implants by a single sensory neuron. Schematic drawing of a sensory nociceptor ending in the tissue. This schematic depicts neuropeptides and their receptors on a sensory afferent nerve fibre ending. The list of receptors and their associated neuropeptides is not complete, but those that are most relevant to inflammation and most researched are shown. These receptors act on different second messenger systems, which can act on voltage-gated ion channels, responsible for the generation of action potentials.

acids and glucose, and to mobilization of immune cells in order to redistribute the energy resources towards the immune system (Kariagina *et al.*, 2004). It is widely accepted that SP has a proinflammatory role whilst the sympathetic nervous system transmitters (such as noradrenaline and adenosine) have anti-inflammatory effects when local concentrations are high (via β -adrenoceptors, A2 adenosine receptors and μ -opioid receptors). However, catecholamines might exert proinflammatory effects at low doses and this phenomenon could explain the different roles for ANS at different stages of inflammation (Eskandari *et al.*, 2003). During initial phases of inflammation, the sympathetic nervous

system provides a proinflammatory milieu facilitating chemoattraction (Straub *et al.*, 2000) and sensitizing sensory nerve endings (Chen *et al.*, 1996). In the later phase of inflammation the sympathetic tone is not decreased on a systemic level, but a shift in the balance of proinflammatory nerve fibres (high density of sensory nerve fibres) and anti-inflammatory nerve fibres (low density of sympathetic nerve fibres) in chronic inflamed tissue has been demonstrated in rheumatoid arthritis and Crohn's disease (Lorton *et al.*, 2005; Weidler *et al.*, 2005; Boissé *et al.*, 2009). Pongratz and Straub postulated that this qualitative imbalance of nerve fibres seen in chronic inflamed tissues, i.e. a local increase

of sensory nerve fibres and a concomitant loss of sympathetic nerve fibres, might maintain a local proinflammatory milieu. In addition, the preponderance of sympathetic fibres in the vicinity of the disease could be responsible for the increase of systemic sympathetic activity, which in turn might act synergistically with the HPA axis in neuroimmunomodulation of the inflammatory process (Pongratz and Straub, 2013).

Similar dysregulations were seen in peritoneal and bowel endometriosis specimens (Ferrero et al., 2010; Arnold et al., 2012). Interestingly, loss of sympathetic nerve fibres was showed only in the area adjacent to the endometriotic intestinal lesions and not in the healthy tissue collected at the margin of the bowel resection (mean distance from the endometriotic nodule ~6 cm) (Ferrero et al., 2010), while recently in non-affected peritoneum of women with peritoneal endometriosis a shift towards an increased sympathetic innervation was demonstrated, supporting a more peripheral generalized role of ANS in the pathophysiology of endometriosis (Arnold et al., 2013). However, the mechanism underlying the differential loss of sympathetic nerve fibres is still not clear, although some authors demonstrated in patients with rheumatoid arthritis that the loss of sympathetic nerve fibres was accompanied by an increased expression of semaphorin 3C and neuropilin-2, which are known repellents of sympathetic nerve fibres (Miller et al., 2004; Fassold et al., 2009). This autonomic dysregulation in endometriosis, similar to other chronic inflammatory diseases, needs to be further investigated in order to determine how it contributes to the development, persistence and exacerbation of the disease.

Pelvic and lower abdominal cross-organ sensitization

Another role for the nervous system in modulating endometriosis-related pain is the so-called pelvic-lower abdominal cross-organ sensitization between the gastrointestinal, urinary and gynaecological organs (Malykhina, 2007). This phenomenon may be a partial explanation for CPP in women with endometriosis and its common comorbidities such as interstitial cystitis/painful bladder syndrome or irritable bowel syndrome.

Several animal studies have demonstrated the cross-organ modulations among the lower urinary tract, colon and gynaecologic structures, partly via the hypogastric nerve (Winnard et al., 2006). This mechanism may partially be explained by so-called dichotomizing fibres (sensory endings of a single neuron innervating two different tissues). Although the number of dichotomizing sensory neurons in DRG varies between animals and studies (from 0.1 to 21% of all traced neurons) (Taylor et al., 1983; Dawson et al., 1992), their identification suggests an anatomical and physiological basis for the occurrence of referred pain.

Cross-sensitization between pelvic organs has been demonstrated following acute colonic and bladder inflammation in rats, producing an increased sensitivity to both colonic and bladder distension (Pezzone et al., 2005; Ustinova et al., 2010). These changes in bladder or colon sensory neurons could also result in alterations in the sensitivity of their nerve terminals in the target organ. In fact, acute (Ustinova et al., 2006) or chronic (Ustinova et al., 2007) colon irritation revealed sensitization of bladder afferents to both mechanical (innocuous and noxious bladder distension) and chemical (capsaicin, bradykinin and SP) stimuli (Ustinova et al., 2006) in rat models. These effects were abolished by systemic capsaicin pretreatment (Ustinova et al., 2007), suggesting a role for TRPV1 sensory neurons in the cross-organ sensitization.

Interestingly, in humans endometriotic-related peritoneal nerve fibres from women with CPP, ovarian endometriosis and adenomyosis, over-express TRPV-1 (Poli-Neto et al., 2009; Nie et al., 2010; Rocha et al., 2011; Liu et al., 2012). TRPV1 may play a role in neurogenic inflammation through a vicious cycle, i.e. inflammatory mediators stimulate peripheral nociceptors including TRPV1 and the stimulation of TRPV1-positive nerves triggers the release of neurotransmitters in the peripheral tissues, which in turn increases the local neurogenic inflammatory response (Fernandes et al., 2012).

Experimental endometriosis in female rats influenced the development of vaginal hyperalgesia (Cason et al., 2003; Berkley et al., 2007; McAllister et al., 2009) and induced urinary bladder hypersensitivity and bladder plasma extravasation (Morrison et al., 2006). Similarly, uterine inflammation produces signs of inflammation in an otherwise healthy bladder (Winnard et al., 2006). Clinically, it has been demonstrated that women with endometriosis and urinary calculosis have significantly more and intense urinary colic events and lower lumbar thresholds compared with women without endometriosis (Giamberardino et al., 2001, 2010). Taken together, there is evidence that cross-organ sensitization plays a role in modulation of endometriosis-associated pain possibly via activation of receptors such as TRPV1 leading to comorbidity of pelvic disorders that is frequently observed in the clinical setting.

Role of estrogens

Endometriosis is an estrogen-dependent inflammatory disease. Estrogens have been shown to support the growth and inflammation processes in endometriotic lesions (Bulun et al., 2012). It has also been reported that estrogens modulate nociceptive responses in functional pain syndromes (as reviewed in Craft, 2007); however, they might also have a role in the peripheral nervous system.

Estrogens enhance the differentiation of neural stem cells into endothelial stem cells both *in vitro* and *in vivo*, and the biological activities, such as differentiation, proliferation and migration, stimulated by estrogens can be blocked by a non-selective ER antagonist (Sekiguchi et al., 2013), supporting a direct link between estrogens and neuroangiogenesis. It has been demonstrated that sympathetic innervation of the rat uterus undergoes profound remodelling influenced by estrogen action with sympathetic axon degeneration with high estrogens levels (Zoubina et al., 2001).

Estrogens significantly up-regulate rat uterine Semaphorin 3F (Sema3F) mRNA expression, a known chemorepellent for sympathetic nerves (Richeri et al., 2011). Similarly, an increased immunostaining for Semaphorin3A has been demonstrated in the human myometrium during pregnancy (Marzioni et al., 2004). Interestingly, Sema3F can antagonize NGF-stimulated TrkA signalling in sympathetic neurons (Atwal et al., 2003). Conversely, estrogens can up-regulate NGF, VEGF and BDNF and modulate neurotrophin receptors such as p75 (Bjorling et al., 2002; Krizsan-Agbas et al., 2003; Richeri et al., 2005) that are necessary for Sema3A and Sema3F mediated growth cone collapse in sympathetic neurons (Naska et al., 2010). These mechanisms suggest an interesting direct or, through semaphorin signalling, indirect role of estrogens in nerve fibre modulation, in particular in sympathetic nerve sprouting. Therefore, hormonal therapies may substantially reduce nerve fibre density in eutopic endometrium and myometrium in women with endometriosis and in peritoneal endometriotic implants (Tokushige et al., 2008, 2009). A recent study showed an up-regulation of calcium-binding proteins in endometriosis-associated nerve fibres

(Barcena de Arellano *et al.*, 2013a). It has been reported that genomic expression of these proteins is up-regulated after estrogen treatment (Yang *et al.*, 2011). These new data on estrogen-induced peripheral nerve modulation, in particular sympathetic nerves, might represent a new area for understanding the pronounced neuroangiogenic properties of estrogen-related inflammatory disease such as endometriosis and need to be further investigated.

Therapeutic implications

As proposed by Stratton and Berkley (2011) endometriosis has not yet been widely recognized as a disease associated with neural dysfunction and thus, to our knowledge, therapies directed at the nervous system have not yet been studied in clinical trials of endometriosis-associated pain. Although classically used for the treatment of neuropathic pain, antidepressant and anticonvulsant drugs, such as amitriptyline and gabapentin, that modulate neuropathic and/or central components of pain can be used in patients with endometriosis as adjuncts in the treatment of CPP. Surgery still plays a central role in the management of CPP although results vary. Laparoscopic uterine nerve ablation (LUNA) for CPP and endometriosis-associated pain has not been demonstrated as useful in controlling the pain in RCTs and is therefore not recommended as an addition to conservative surgery (Proctor *et al.*, 2005; Daniels *et al.*, 2010; Dunselman *et al.*, 2014).

New treatments focusing on pathophysiological mechanisms, such as the use of direct-acting antiangiogenic agents, e.g. VEGF blockers and receptor tyrosine kinase inhibitors, have been effectively tested *in vitro* and *in vivo* with the aim of reducing endometriotic implantation and growth. Dual inhibition of angiogenesis and nerve growth may be an attractive therapeutic strategy for patients with endometriosis. An antiangiogenic treatment, such as cabergoline, that promotes endocytosis of VEGFR2, was able to diminish the immature blood vessels as well as nerve fibre density in experimental endometriosis lesions compared with controls in mice: the authors also found diminished levels of macrophages and mast cell density and postulated that the interruption of the inflammation pathway might be responsible for the antineuroangiogenic effect (Novella-Maestre *et al.*, 2012).

The selective inhibition of NGF as a key regulator of chronic pain conditions showed promising results with potentially tolerable adverse effects in a rheumatologic setting; however, its association with rapid and unexpected joint destruction or progressive disease in a minority of participants receiving the active drug (Lane *et al.*, 2010) posed the question of targeting molecules with pleiotropic effects, such as NGF. Indeed, it is difficult to propose antineuroangiogenic drugs for the treatment of endometriosis because the mechanism of nerve and vascular growth in this disease remains to be better understood. As such, the role of these drugs in the clinical setting still needs to be clarified especially in a patient population of reproductive age (Becker *et al.*, 2005; Soares *et al.*, 2012).

The bi-directional interaction between inflammatory mediators and nerve growth and sensitization opens novel potential therapeutic approaches. Pre-clinical models have generated promising results. For example, the administration of nuclear factor- κ B andrographolide in a rat model of endometriosis results in reduced lesion size, improved sensitivity to a noxious thermal stimulus and decreased immunoreactivity to NGF, p-p50, p-p65 and COX-2 in ectopic implants (Zheng *et al.*, 2012). In another study, L1 cell adhesion molecule interference as a therapeutic

strategy has been tested *in vitro* and *in vivo*. The antibody treatment resulted in reduced numbers of endometriotic implants, intraperitoneal adhesions at implantation sites and a reduced nerve density compared with controls (Agic *et al.*, 2010; Silveira *et al.*, 2013).

As mentioned above, activated and degranulating mast cells are found in close proximity to nerves in peritoneal and ovarian sites and DIE (Anaf *et al.*, 2006). Mast cells play an integral role in the cellular immune response and are abundant in inflammatory tissue. Controlling mast cell activation and degranulation could therefore have a beneficiary effect on endometriosis-associated pain. A preliminary clinical study has shown that palmitoylethanolamide-polydatin combination therapy (which acts on degranulation of mast cells) is effective in controlling pain in patients with DIE. However the limited size of the cohort (four patients) was too small to draw any conclusions (Indraco and Barbieri, 2010). Furthermore, a recent RCT evaluated the intrauterine instillation of lignocaine, a local anaesthetic drug that has anti-inflammatory properties and exerts effects on nerve endings and intraperitoneal macrophages (Butterworth and Strichartz, 1990; Hollman and Durieux, 2000), in the treatment of dysmenorrhoea in patients with endometriosis. In the intention-to-treat analysis, the success rate of lignocaine treatment was 41.7% (10 of 24) compared with 16.7% (3 of 18) in the placebo group ($P = 0.100$) while in the per protocol analysis, 9 of 20 (45.0%) compared with 1 of 14 (7.1%) had a decrease of $\geq 50\%$ on the visual analogue scale ($P = 0.024$) (Wickström *et al.*, 2012).

Conclusions

Over the last decade, evidence has accumulated of nerve fibres in and around ectopic and eutopic endometrium as well as elevated levels of neurotrophic factors in the PF of patients with endometriosis, suggesting their potential role in the pain process. Yet no clear correlation between these findings and pain intensity or type has been shown so far. While the presented findings are highly interesting, the exact role of the observed peripheral changes discussed here remains elusive. However, given that most phenomena in nature have a meaning, it is possible that the high number of nerve fibres and the expression of neurotrophic and angiogenic factors are related to endometriosis-associated pain. The contradicting results between some studies could be explained by differences in patient selection and tissue and data collection. Therefore, more research and clinical studies using standardized methods are needed to improve our understanding and treatment of endometriosis.

Authors' roles

M.M. performed the bibliographic search, screening and data extraction. M.M., K.V., J.B. and C.M.B. interpreted the results and wrote the manuscript. C.M.B. and K.T.Z. supervised the whole study procedure including design of the study and interpretation of results. All authors revised the manuscript and approved the final version.

Funding

This work was supported by the Oxford Partnership Comprehensive Biomedical Research Centre with funding from the Department of Health's NIHR Biomedical Research Centres Scheme.

Conflict of interest

K.T.Z. is a member of the scientific advisory boards of AbbVie, Inc., Bayer Healthcare and Roche Diagnostic.

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