Original Article

Pain Mechanisms in Peritoneal Diseases Might Be Partially Regulated by Estrogen

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Abstract

To identify factors influencing the differential pain pathogenesis in peritoneal endometriosis (pEM) and peritoneal carcinomatosis in ovarian cancer (pOC), we undertook an experimental study. Tissue samples of 18 patients with pEM, 15 patients with pOC, and 15 unaffected peritoneums as controls were collected during laparoscopy or laparotomy. Immunohistochemical stainings were conducted to identify nerve fibers and neurotrophins in the tissue samples. Additionally, 23 pEM fluids, 25 pOC ascites fluids, and 20 peritoneal fluids of patients with myoma uteri as controls were collected. In these fluids, the expression of neurotrophins was evaluated. The effects of peritoneal fluids and ascites on the neurite outgrowth of chicken sensory ganglia were estimated by using a neuronal growth assay. An electrochemiluminescence immunoassay was carried out to determine the expression of estrogen in the peritoneal fluids and ascites. The total and sensory nerve fiber density was significantly higher in pEM than in pOC ($P < .001$ and $P < .01$). All neurotrophins tested were present in tissue and fluid samples of pEM and pOC. Furthermore, the neurotrophic properties of pEM and pOC fluids were demonstrated, leading to sensory nerve fiber outgrowth. Estrogen concentration in the peritoneal fluids of pEM was significantly higher compared to ascites of pOC ($P < .001$). The total and sensory nerve fiber density in the tissue samples as well as the estrogen expression in the peritoneal fluid of pEM was considerably higher than that in pOC, representing the most notable difference found in both diseases. This might explain the differential pain perception in pEM and pOC. Therefore, estrogen might be a key factor in influencing the genesis of pain in endometriosis.

Keywords

endometriosis, peritoneal carcinomatosis in ovarian cancer, genesis of pain, estrogen, neurotrophins

Introduction

The peritoneum is the largest serous membrane in the human body. Its inner visceral and outer parietal layers form the peritoneal cavity. Both layers are innervated by the autonomic nervous system and the somatosensory system. Accordingly, the transmission and perception of pain differ between both layers. Visceral pain is described as diffuse, appearing intermittently and is associated with vegetative symptoms, whereas somatic pain is clearly located, sharp, and persistent.[1] The peritoneum transudates the peritoneal fluid that circulates throughout the peritoneal cavity to reduce friction. Besides water, electrolytes, proteins, and solubilized substances, it contains immune cells from the innate and adaptive system.[2]

Peritoneal endometriosis (pEM) and peritoneal carcinomatosis in ovarian cancer (pOC) are 2 gynecological diseases of special interest.

Endometriosis affects around 10% of women of reproductive age[3] and is characterized by the presence of ectopic endometrial-like tissue, commonly in the direct environment of the uterus and mostly present in the peritoneum. The predominant symptom, besides infertility and cycle disorders, is pain which manifests itself in several qualities and quantities. Surprisingly, the subjective perceived pain shows no correlation to the extent of endometriosis.[4,5]

Unlike endometriosis, ovarian cancer often remains unrecognized because of scarcely presented clinical symptoms and nonspecific characteristics.[6-8] As a consequence, ovarian cancer is usually diagnosed at an advanced stage when tumor cells spread throughout the peritoneal cavity and cause peritoneal carcinomatosis.[9]

The first studies on the pain pathophysiology of endometriosis showed an increased innervation with sensory nerve fibers in the proximity of endometriotic lesions, which might be responsible for pain perception, immunomodulation, and neurogenic inflammation.[10-13] The functional clarification of the mechanism, which guides neurites or causes de novo

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innervation, is still unclear. To define the underlying pathologic pain mechanisms, attempts have been made to transfer neurobiological concepts into the disease model. Interestingly, studies have revealed that nerve growth factor (NGF) levels are enhanced in endometriosis.[10,11,14] Moreover, it is suggested that NGF is involved in inflammatory processes and thereby leads to sensory hypersensitivity.[15-18] Currently, an increasing amount of evidence is describing endometriosis as a chronic inflammatory disease. This is validated by an elevated number of macrophages in the peritoneal fluid and affected tissue sites of endometriosis patients.[19-23] As a part of the immune system, macrophages secrete inflammatory mediators, which may directly activate and sensitize sensory nerve fibers.[24-26] It has been proposed that inflammatory components in the peritoneal fluid, such as interleukin (IL) 1β, IL-8, tumor necrosis factor α, and interferon γ, are elevated in endometriosis and even tend to be enhanced in accordance with the severity of pain experienced by endometriosis patients.[27] This hints toward inflammatory characteristics correlating with pain perception and nociceptor sensitivity. Furthermore, a disturbed hormone homeostasis could explain the differences in pain perception, as suggested by nociceptor sensitization as well as the innervation phenomena in rodents.[28-30]

Peritoneal endometriosis and pOC affect the peritoneum to very different extents, while showing an inverse discrepancy of clinical symptoms. This discrepancy sets the framework for this study. Herein, we aim to shed light on the innervation and expression of key proteins in the pain pathology, related to both peritoneal diseases. We intend to describe the histological and molecular conditions underlying the differential perception of pain in pEM and pOC. Therefore, the total and sensory nerve fiber density in affected peritoneums was analyzed. In addition, we quantified the expression of neurotrophins and analyzed growth characteristics and estrogen levels in peritoneal fluids and ascites.

Methods

Ethical Approval

This study was in agreement with the Charité ethics committee (EA 4/036/12) and patients gave their informed consent.

Patients and Material

Tissue samples of 18 patients with pEM (22-48 years, mean: 33 years) and 15 patients with pOC (29-58 years, mean: 52 years) were collected during laparoscopy or laparotomy. Diagnoses are proven by the surgeon's observation and/or the histological report in retrospective. Fifteen healthy peritoneums of patients undergoing surgery for diagnostic reasons were used as controls. The samples were immediately transferred into 4% formaldehyde and incubated for 24 to 48 hours and embedded in paraffin.

A total of 23 peritoneal fluids from patients with pEM (18-47 years, mean: 32 years), 25 ascites fluids from patients with pOC (44-88 years, mean: 69 years), and 20 peritoneal fluids of patients with myoma uteri (control) were collected. Peritoneal or ascites fluids were transported on ice and centrifuged at 3000 rpm (Laborfuge 400 R, Heraeus, Hanau, Germany). Supernatants were stored at −80°C.

Immunohistochemistry

Paraffin-embedded tissues were stained immunohistochemically. Tissues were cut in 5 μm serial sections. Antigen retrieval was performed in an acidic citrate buffer for protein gene product 9.5 (PGP9.5) and substance P (SP) or an alkaline target retrieval buffer for NGF, neurotrophin 3 (NT-3), and brain-derived neurotrophic factor (BDNF). Primary and biotinylated secondary antibodies (Table 1) and streptavidin-AP (Roche Diagnostics, Rotkreuz, Switzerland) were diluted in antibody diluent (DakoCytomation, Glostrup, Denmark). The antibodies were incubated at room temperature with individual dilution factors and incubation times ranging from 40 to 75 minutes or overnight at 4°C. Antibody–antigen detection was performed using the Fast Red Substrate System (Thermo Fisher Scientific, Waltham, USA). Sections were counterstained with hematoxylin (Merck KGaA, Darmstadt, Germany) and mounted with Eukitt (Sigma-Aldrich, St. Louis, USA).

Nerve Fiber Density and Neurotrophin Expression

Stained sections were microscopically analyzed (Axiophot 40 CFL; Carl Zeiss AG, Jena, Germany), and the images were captured by using the Powershot G5 (Canon, Tokio, Japan).

The total and sensory nerve fiber densities were evaluated on immunostainings against PGP9.5 and SP. In each section, the area of 1 mm^2, which contained the most positive nerve fibers, was determined, and the nerve fiber density was quantified as described previously.[31] The nerve fiber density was assessed in a distance of 1 mm surrounding the epithelium of the endometriotic lesion, the tumor cells, or randomly in the connective tissue layer of healthy peritoneums. If possible, nerve fibers were counted on a peripheral site with a distance of at least 4 mm.

Neurotrophin expression was analyzed in stainings against NGF, NT-3, and BDNF by evaluating the relative number of positive cells. In sections from patients with pEM, the stroma and the periphery, 4 mm distal to the lesion, were quantified. In sections of pOC, cells in a close proximity of 1 mm to tumor cells and the periphery at a distance of 4 mm were analyzed. In healthy peritoneums, neurotrophin expression levels were ascertained in the connective tissue layer.

Western Blot and Immunodetection

For the detection of proteins, Western blot and immunodetection were performed in peritoneal and ascites fluids following the polyacrylamide gel electrophoresis (PAGE) separation.

Total protein concentrations were photometrically analyzed using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific; and Multiscan FC Microplate Photometer, Thermo Fisher Scientific, Waltham, USA). Prior to loading, each fluid was diluted with phosphate-buffered

Table 1. Primary and Secondary Antibodies Used for Immunohistochemistry.

	Dilution	Product No.	Company
Primary antibodies			
Polyclonal rabbit anti-PGP9.5	1:300	Z-5116	Dako Corporation, United States
Monoclonal rat anti-SP	1:100	sc-21715	Santa Cruz Biotechnology, United States
Polyclonal rabbit anti-NGF	1:100	sc-548	Santa Cruz Biotechnology, United States
Polyclonal goat anti-NT-3	1:250	sc-13381	Santa Cruz Biotechnology, United States
Monoclonal mouse anti-BDNF	1:250	ab10505	Abcam, United Kingdom
Secondary antibodies			
Biotinylated mouse anti-rabbit	1:400	211065109	Dianova, Germany
Biotinylated rabbit anti-rat	1:400	E0468	Dako Corporation, United States
Biotinylated rabbit anti-goat	1:400	E0466	Dako Corporation, United States
Biotinylated rabbit anti-mouse	1:400	JAC-315065045	Jackson ImmunoResearch, United States

Abbreviations: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin 3; SP, substance P.

Table 2. Primary and Secondary Antibodies Used for Western Blot Analysis and Immunodetection.

	Dilution	Product No.	Company
Primary antibodies			
Polyclonal rabbit anti-NGF	1:500	sc-548	Santa Cruz Biotechnology, United States
Polyclonal goat anti-NT-3	1:500	sc-13381	Santa Cruz Biotechnology, United States
Monoclonal mouse anti-BDNF	1:500	ab10505	Abcam, United Kingdom
Polyclonal goat anti-actin	1:1000	sc-1616	Santa Cruz Biotechnology, United States
Secondary antibodies			
HRP-conjugated goat anti-rabbit	1:1000	JAC-111036045	Jackson ImmunoResearch, United States
HRP-conjugated rabbit anti-mouse	1:1000	JAC-315065045	Jackson ImmunoResearch, United States
HRP-conjugated rabbit anti-goat	1:1000	JAC-305036003	Jackson ImmunoResearch, United States

Abbreviations: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin 3.

saline to a protein concentration of 0.25μg/μL and denatured for 3 minutes at 96°C (ThermoStat Plus, Eppendorf, Hamburg, Germany). As a positive control, lysates from the breast cancer cell line michigan cancer foundation 7 (MCF-7) were used.

Polyacrylamide gel electrophoresis ran on 12% acrylamide gels in the electrophoresis buffer (Mini-PROTEAN 3 System and PowerPac 200 Power Supply, Bio-Rad, Munich, Germany), followed by an electrophoretical transfer to a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer, Rodgau, Germany; Mini Trans-Blot Cell, Bio-Rad, Munich, Germany). To detect NGF, NT-3, BDNF, and β-actin, a 60-minute incubation of the primary antibody and a 45-minute incubation for the horseradish peroxidase (HRP)-conjugated second antibody (Table 2) were performed on PVDF membranes. Membranes were exposed to X-ray films (HyperfilmTM ECL, Amersham Bioscience, Freiburg, Germany) along with a developer solution. Films were automatically developed (X-OMAT 1000 Processor, Kodak, Rochester, USA) and scanned in a transparency scanner. Pictures were analyzed using ImageJ software (NIH, Bethesda, USA). Signals from proteins of interest were normalized against β-actin.

Neuronal Growth Assay

To investigate the neuronal outgrowth in vitro after treatment with peritoneal fluids or ascites, tissue cultures of chicken sensory ganglia were established. In total, 12 peritoneal fluids of patients with pEM, 12 of the control group and 24 ascites fluids of the patients with pOC were used for this analysis.

Fertilized chicken eggs (Valo SPF eggs, Lohmann Tierzucht, Cuxhaven, Germany) were incubated for 9 days at 37°C (Incubator Model 3000, Jane Schütz GmbH, Hammelburg, Germany). The sensory dorsal root ganglia were dissected from the embryos and transferred to collagen IV-coated plates. Tissue cultures were treated with 50% of patients' peritoneal fluid in Dulbecco's Modified Eagle medium (DMEM, PAA Laboratories GmbH, Pasching, Austria). Nerve growth factor (NGF-2.5 S, Sigma-Aldrich, St. Louis, USA) was diluted in DMEM (1:100) and served as positive control. Negative controls contained DMEM only. Well plates were incubated at 37°C and 5% CO_2 for 48 hours.

Neurite outgrowth was determined microscopically (Axiovert 40, Carl Zeiss AG, Jena, Germany) and pictures were taken (AxioCam MRc, Carl Zeiss AG, Jena, Germany). A scoring system was used that defines 0 for *no outgrowth*, 1 for *little and scattered outgrowth*, 2 for *continuous outgrowth with neurite length over a quarter of ganglion diameter*, and 3 for *continuous outgrowth with neurite length over half the ganglion's diameter*.

Estrogen Concentrations

Estrogen concentration was analyzed in the peritoneal fluid of 21 patients with pEM, 21 ascites fluid of patients with pOC,

and 13 controls and determined by standardized electrochemiluminescence immunoassays (ECLIA; Cobas 6000 analyzer) conducted by the Charité Reference and Diagnostics Laboratory.

Statistical Analysis

Data were analyzed using GraphPad Prism version 4.0 (GraphPad Software Inc, La Jolla, USA) and quoted as mean+standard deviation. Variables were metrical and unpaired. Gaussian distribution was tested in all groups. For significance analysis, P values below .05 were considered as significant (*$P < .05$, **$P < .01$, and ***$P < .001$). Multiple comparisons were carried out with α-level adjustment.

To compare 2 groups, the Mann-Whitney test was used. For more than 2 groups, 1-way analysis of variance (ANOVA) plus Bonferroni correction was performed in normally distributed samples, Kruskal-Wallis test plus Dunn test if distribution was not normal. Two-way ANOVA followed by Bonferroni correction was used to compare different aspects in 2 groups.

Results

Peritoneal Innervation in pEM Is Significantly Enhanced Compared to pOC

Using PGP9.5 as pan neuronal marker, nerve fiber densities were quantified. The direct comparison within the surrounding area of the affected tissue displayed a significantly elevated innervation of PGP9.5-positive nerve fibers in pEM compared to pOC (pEM: 3.83 $\pm$ 2.38/mm^2, n = 18; pOC: 1.07 $\pm$ 1.33/mm^2, n = 15; $P < .001$; Figure 1A, C, and D). The comparison between tissues from both diseases and the control group showed no significant difference (control: 2.73 $\pm$ 1.49/mm^2, n = 15; Figure 1A). The effect observed in the direct surrounding was lowered in the periphery of pEM (1.94 $\pm$ 1.21/mm^2, n = 18; $P < .01$; Figure 1B), while the nerve fiber density in the peritoneal carcinomatosis remained unchanged (pOC: 1.45 $\pm$ 1.21/mm^2, n = 11; Figure 1B).

Sensory Nerve Fiber Density Is Slightly Elevated in pEM Compared to pOC

Substance P was used as a sensory nerve fiber marker. In the direct environment of endometriotic epithelium, significantly more nerve fibers were SP positive than in the 1 mm area around tumor cell groups (pEM: 1.94 $\pm$ 1.73/mm^2, n = 18; pOC: 0.40 $\pm$ 0.63/mm^2, n = 15; $P < .01$; Figure 1E, G, and H). No differences were seen in the sensory nerve fiber density between pEM and pOC compared to the control (control: 0.80 $\pm$ 0.56/mm^2, n = 15; Figure 1E). The sensory innervation of endometriotic lesions was similar to that in the periphery (pEM: 1.72 $\pm$ 2.1/mm^2, n = 18; Figure 1F) as well as the area around the tumor cell groups of pOC compared to the periphery (pOC: 1.00 $\pm$ 1.00/mm^2, n = 11; Figure 1F).

Nerve Growth Factor Expression Is Enhanced in pEM as Well as in pOC

Neurotrophin expression was validated by using antibodies against NGF, NT-3, and BDNF. Nerve growth factor–positive cells appeared in similar amounts within endometriotic stroma and in the direct environment of tumor cell groups (pEM: 42.5% $\pm$ 25.91%, n = 18; pOC: 53.33% $\pm$ 23.20%, n = 15; Figure 2A). In contrast to both pEM and pOC, the control showed significantly less NGF-positive cells (control: 14.13% $\pm$ 20.80%, n = 15; $P < .01$; $P < .001$; Figure 2A). In the stroma or the direct environment of tumor cell groups, NGF expression was clearly increased compared to the periphery (pEM: 14.17% $\pm$ 17.51%, n = 18; pOC: 9.55% $\pm$ 9.07%, n = 11; $P < .001$; Figure 2B).

The number of cells expressing NT-3 was comparable in pEM and pOC (pEM: 59.72% $\pm$ 20.33%, n = 18; pOC: 58.00% $\pm$ 23.05%, n = 15; Figure 2C). The control group showed increased amounts of NT-3-positive cells and was also higher than in the pEM stroma (control: 79.33% $\pm$ 20.08%, n = 15; $P < .05$; Figure 2C). No difference was found in the stroma of endometriotic lesions or in the proximity of tumor cell groups and the periphery (pEM: 60.00% $\pm$ 29.26%, n = 18; pOC: 60.91% $\pm$ 32.08%, n = 11; Figure 2D).

An equal number of cells in the stroma of pEM and the area around tumor cell groups were BDNF positive (pEM: 41.11% $\pm$ 30.08%, n = 18; pOC: 53.33% $\pm$ 24.40%, n = 15; Figure 2E), whereas less cells were positive for BDNF in the control group, achieving significance compared to pOC (control: 18.67% $\pm$ 25.03%, n = 15; $P < .05$; Figure 2E). No difference was found between the stroma and periphery of endometriotic lesions, while BDNF was expressed significantly higher in the direct surroundings of tumor cell groups in contrast to the periphery (pEM: 36.67% $\pm$ 27.87%, n = 18; pOC: 27.27% $\pm$ 28.32%, n = 11; $P < .05$; Figure 2F).

Nerve Growth Factor, NT-3, and BDNF Are Present in Peritoneal Fluids of pEM and Ascites of pOC

Relative concentrations of the neurotrophins NGF, NT-3, and BDNF were assessed. The named neurotrophins were present in all fluids. Quantification showed no differences in the amount of NGF (pEM: 1.01 $\pm$ 0.40, n = 23; pOC: 0.95 $\pm$ 0.40, n = 25; control: 1.00 $\pm$ 0.32, n = 20), NT-3 (pEM: 0.88 $\pm$ 0.30, n = 23; pOC: 0.84 $\pm$ 0.29, n = 25; and control: 1.00 $\pm$ 0.29, n = 20), or BDNF (pEM: 0.89 $\pm$ 0.44, n = 23; pOC: 0.80 $\pm$ 0.17, n = 25; control: 1.00 $\pm$ 0.34, n = 20) in peritoneal fluid and ascites of patients with pEM, pOC, and the control group (Figure 3A-E).

Peritoneal Fluids of pEM and Ascites of pOC Stimulate Outgrowth of Sensory Ganglia

Neurite outgrowth capacity of fluids was analyzed in a neuronal growth assay with sensory dorsal root ganglia of chick

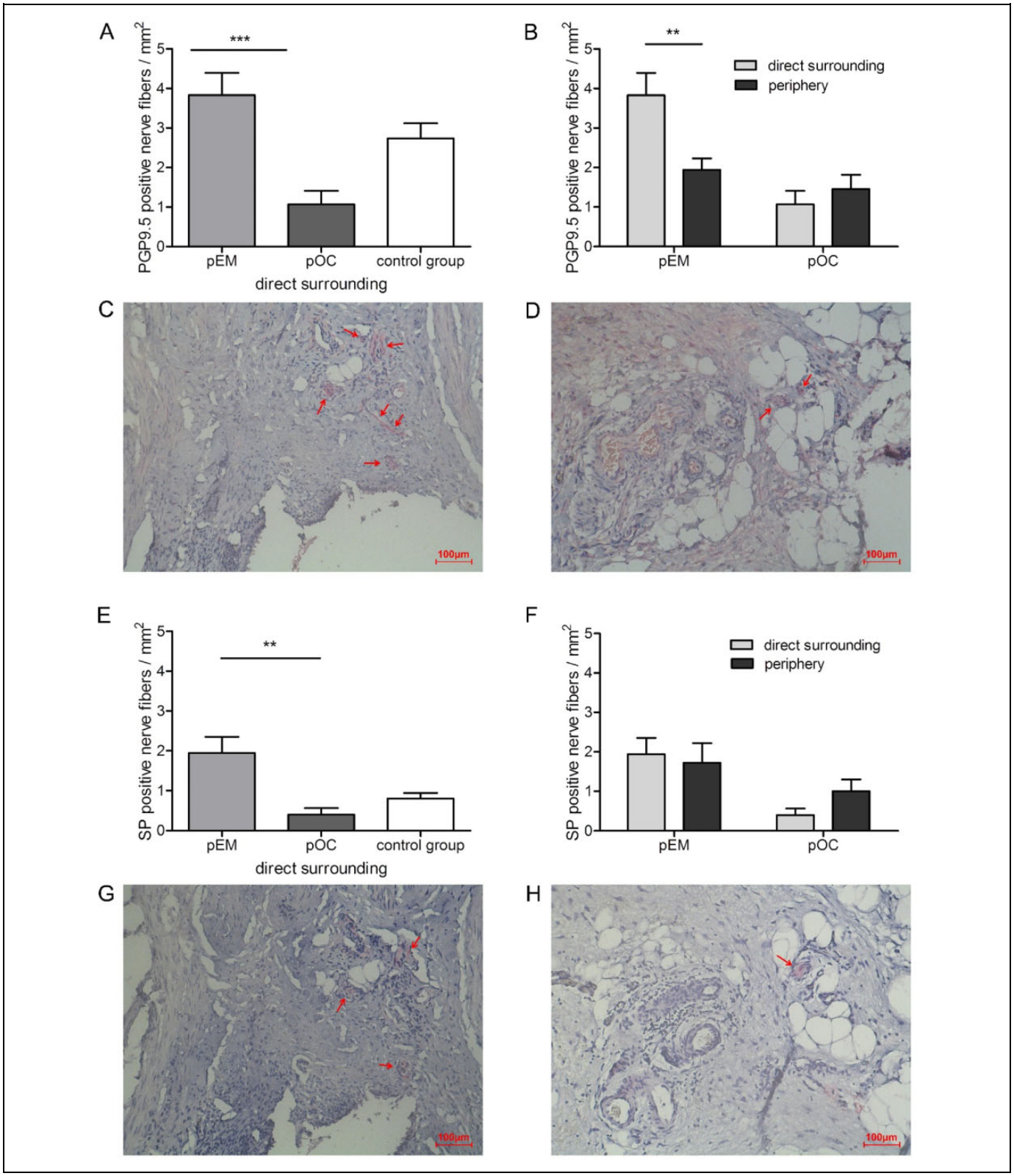

Figure 1. Quantification of protein gene product 9.5 (PGP9.5; total) and substance P (SP; sensory)-positive nerve fibers in tissue sections of peritoneal endometriosis (pEM; n = 18), peritoneal carcinomatosis in ovarian cancer (pOC; n = 15), and healthy peritoneum (n = 15). (A) Total and (E) sensory nerve fiber density in the direct surrounding of endometriotic epithelium or tumor cell groups at a distance of no more than 1 mm and in the connective tissue layer of healthy peritoneum. (B) Total nerve fiber density and (F) sensory nerve fiber density in the direct surrounding of endometriotic epithelium and tumor cell groups compared to the periphery with a distance of at least 4 mm. Exemplary pictures of PGP9.5-positive nerve fibers in direct surrounding to (C) endometriotic epithelium and (D) tumor cell groups. Exemplary pictures of SP-positive nerve fibers in direct surrounding to (G) endometriotic epithelium and (H) tumor cell groups (shown are mean+standard deviation.)

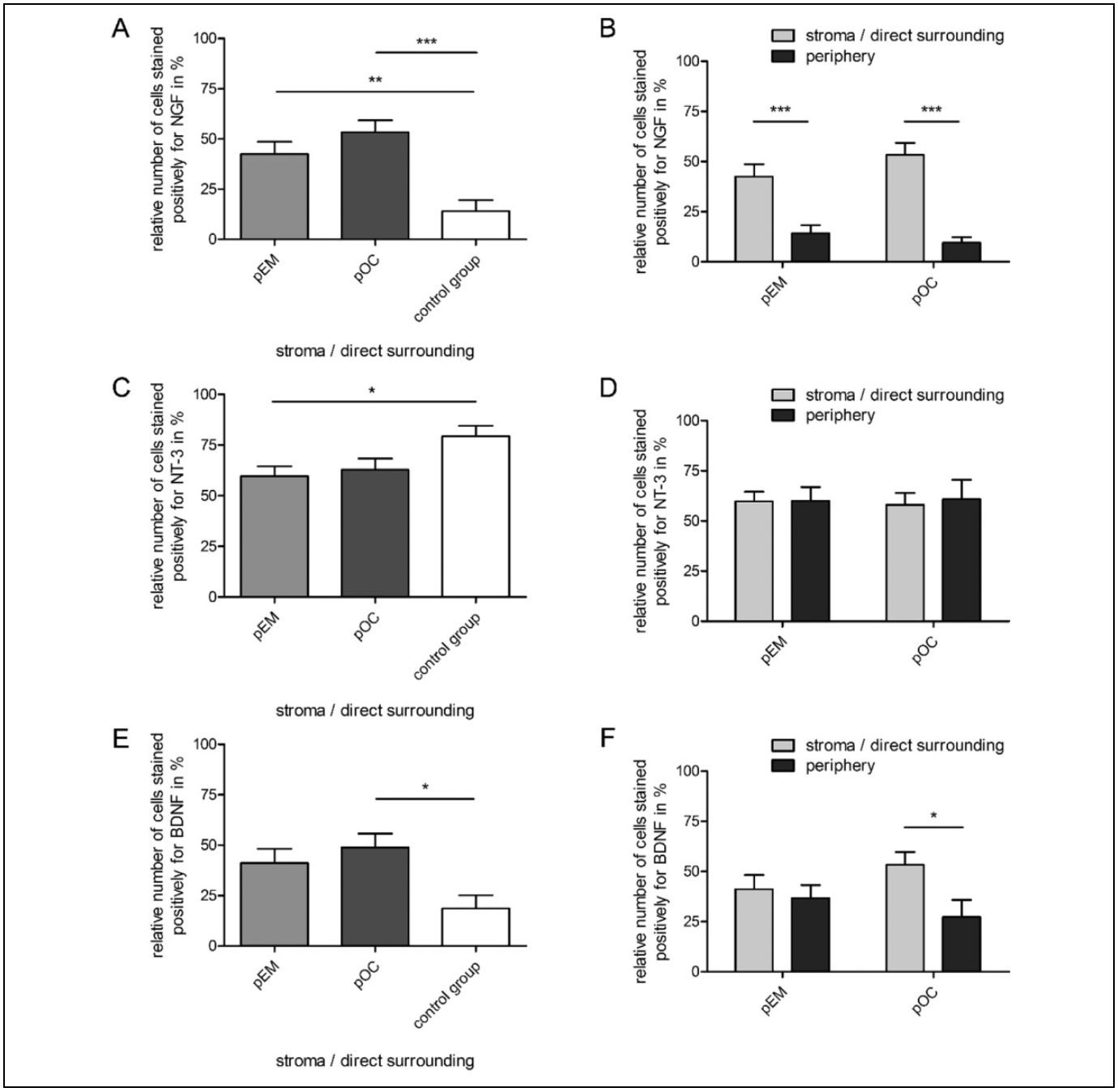

Figure 2. Expression of the neurotrophins nerve growth factor (NGF; A and B), neurotrophin-3 (NT-3; C and D), and brain-derived neurotrophic factor (BDNF; E and F) in formalin-fixed paraffin-embedded sections of peritoneal endometriosis (pEM; n = 18), peritoneal carcinomatosis in ovarian cancer (pOC; n = 15), and healthy peritoneum as control group (n = 15). (A, C, and E) Relative numbers of NGF, NT-3, and BDNF-stained cells in the stroma of endometriotic lesions and direct surrounding of tumor cell groups at a distance of no more than 1 mm and in the connective tissue layer of healthy peritoneum. (B, D, and F) Relative numbers of positive stained cells in the stroma of endometriotic lesions and direct surrounding of tumor cell groups at a distance of no more than 1 mm compared to the periphery with a distance of at least 4 mm (shown are mean+standard deviation).

embryos. Outgrowth in pEM and pOC showed significantly elevated scores compared to the negative control (pEM: 2.54 ± 0.72, n = 12; pOC: 2.46 ± 0.94, n = 24; $P < .01$; Figure 3A). No difference was observed between pEM, pOC, and the control group (control: 1.92 ± 1.17, n = 12; Figure 4A-C).

Estrogen Concentration in Peritoneal Fluids of Patients With pEM Is Much Higher Than in Ascites of Patients With pOC

Estrogen concentration was significantly lower in pOC compared to pEM and control group (pEM: 5185.6 ± 17232.4

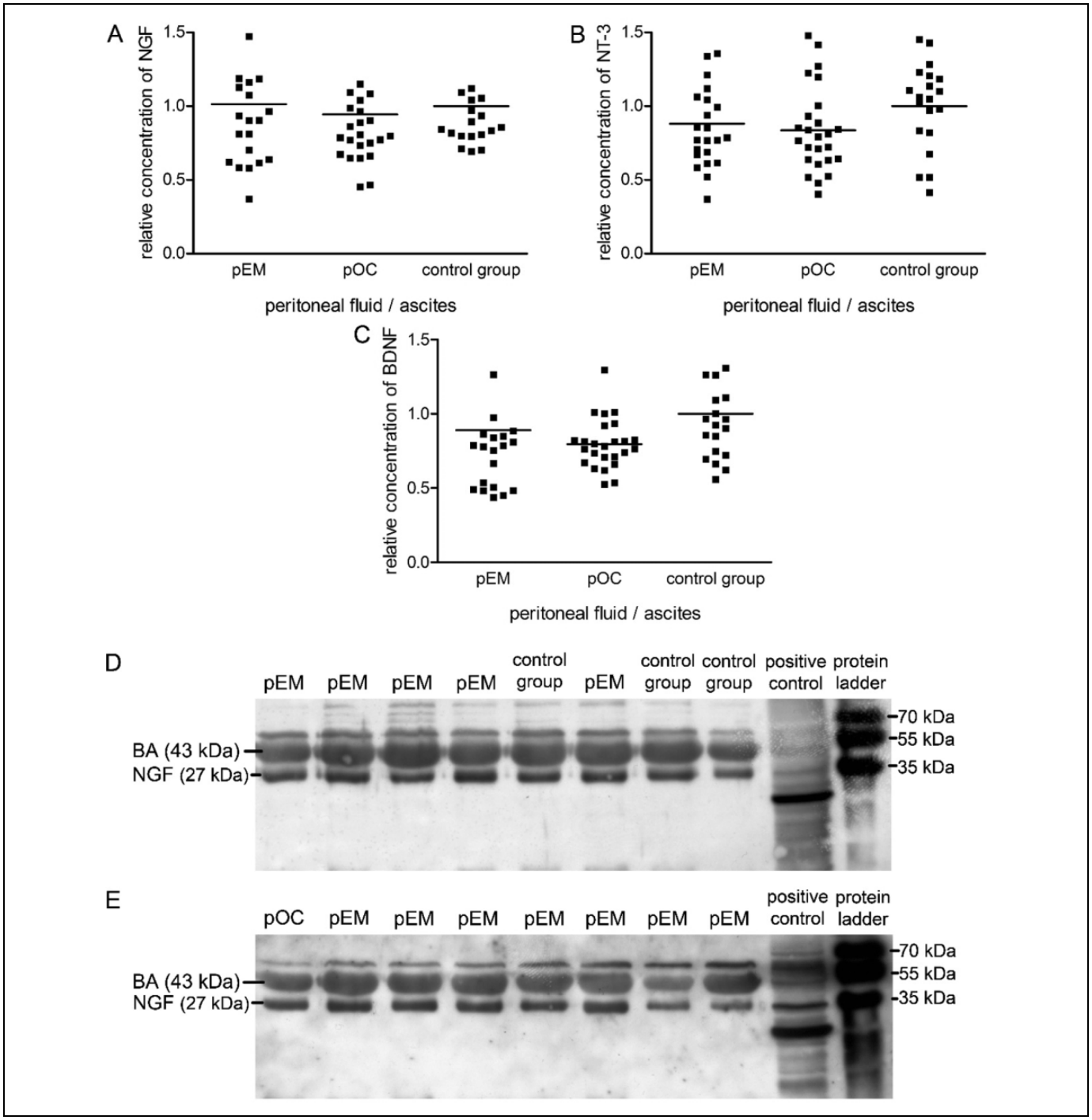

Figure 3. Expression of the neurotrophins nerve growth factor (NGF), neurotrophin 3 (NT-3), and brain-derived neurotrophic factor (BDNF) in peritoneal fluid and ascites. (A-C) Relative concentrations of NGF, NT-3, and BDNF in peritoneal fluid and ascites of peritoneal endometriosis (pEM; n = 23), peritoneal carcinomatosis in ovarian cancer (pOC; n = 25), and a control group (n = 20). (D and E) Exemplary pictures of NGF expression in peritoneal fluid and ascites detected by Western blot (BA, β-actin; shown are mean+standard deviation).

pmol/L, n = 21; pOC: 77.8 ± 32.2 pmol/L, n = 21; control: 1325.2 ± 1866.73 pmol/L, n = 13; $P < .001$; Figure 5). No significant differences could be found between pEM and the control group.

Discussion

These 2 gynecological diseases are of special importance since they affect the peritoneum to different degrees, while showing an inverse extent of pain symptoms. In pEM, pain is one of the

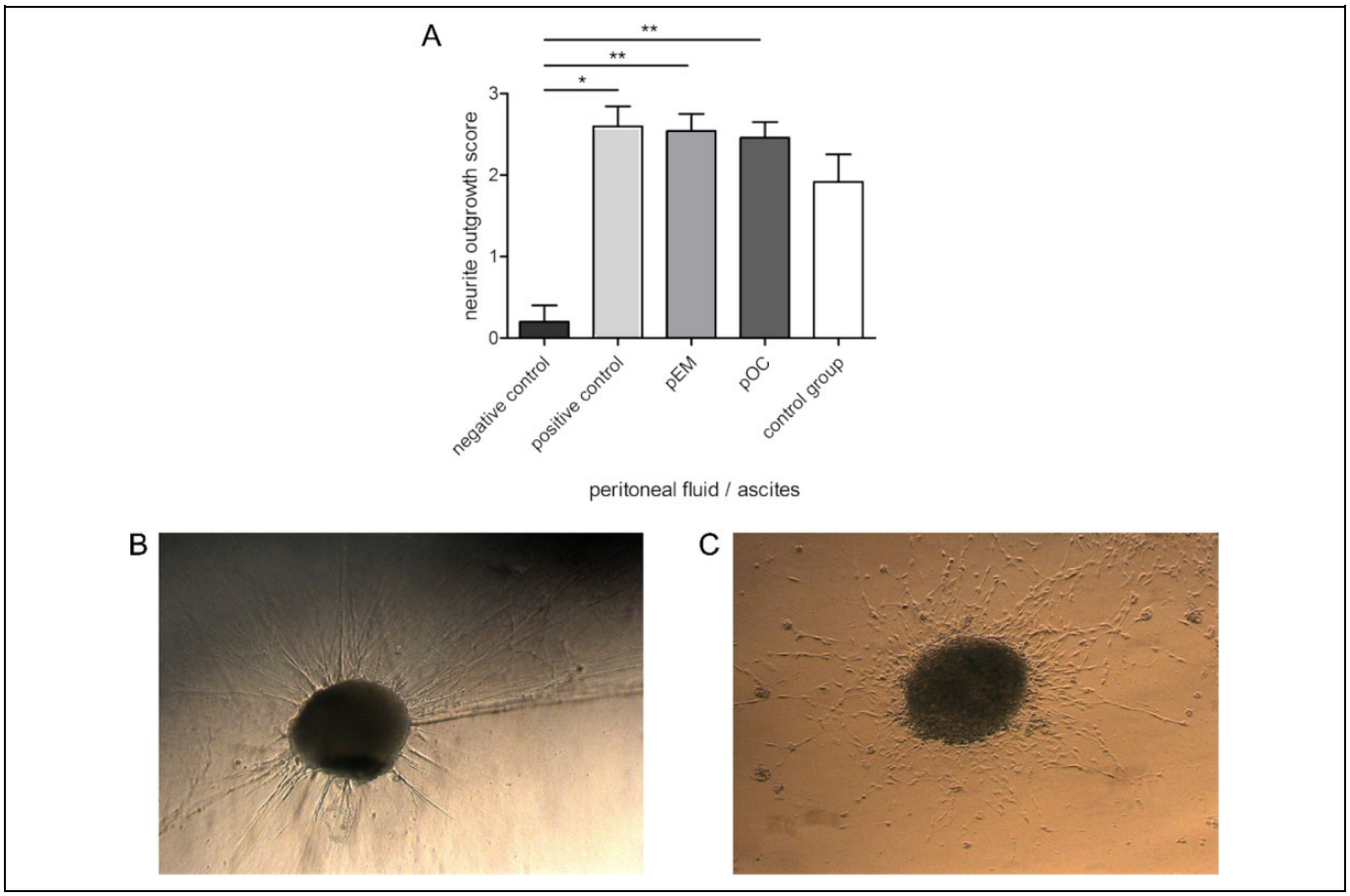

Figure 4. Neurite outgrowth assay. (A) Neurite outgrowth of sensory dorsal root ganglia of the chick embryo incubated in peritoneal fluid or ascites of peritoneal endometriosis (pEM; n = 12), peritoneal carcinomatosis in ovarian cancer (pOC; n = 24), a control group (n = 12), negative and positive controls (nerve growth factor [NGF], 1:100). Exemplary pictures of neurite outgrowth in (B) pEM and (C) pOC (shown are mean+standard deviation).

predominant symptoms and can be caused by small lesions, whereas ovarian cancer scarcely presents clinical symptoms, even though tumor cells spread on the peritoneum in large quantities. It is presumed currently that changes in the nerve fiber density as well as inflammatory parameters and hormones have an impact on the development and maintenance of pain in endometriosis.

Within this study, we aimed to investigate the differential pain perception presented in pEM and the pOC. It is intriguing that small endometriotic lesions are able to induce severe pain, whereas peritoneal metastasis in ovarian cancer is often completely asymptomatic. Therefore, we performed experimental analyses to detect nerve fibers and neurotrophins in these peritoneal diseases to broaden the understanding of pain pathogenesis in the peritoneum. Furthermore, we explored growth characteristics of peritoneal fluids and ascites.

We determined the total nerve fiber density using the pan neuronal marker PGP9.5 and detected a significant elevated nerve fiber density in the close proximity of peritoneal endometriotic lesions compared to the periphery, in accordance with a previous study.[13] According to nociception, sensory nerve fibers transmit sensory stimuli to the central nervous system and from there the pain stimulus reaches the consciousness. Hence, sensory nerve fibers could be a reason for the pain phenotype patients experience in endometriosis. Indeed, we identified SP-positive sensory nerve fibers close to pEM lesions. Nevertheless, no significant difference to the periphery or the control could be demonstrated, which is similar to a previous study.[10] However, other studies have shown significantly more sensory nerve fibers around pEM lesions than in healthy peritonea.[13,32] According to the literature, it is generally accepted that an increased nerve fiber density plays an important role in the pathogenesis of endometriosis. Our data indicated differences between pEM and the control but did not reach significance. Higher sample sizes might generate significant differences and should therefore be analyzed in further studies. Assuming the nerve fibers in pEM are causally involved in the pain pathogenesis, nerve fibers should not be present or elevated in peritoneal carcinomatosis caused by ovarian cancer. In the present study, we could demonstrate a significantly lower total nerve fiber density in close proximity to tumor cells when compared to endometriotic lesions. Similarly, less sensory nerve fibers were found around pOC in contrast to pEM. To the best of our knowledge, no data exist

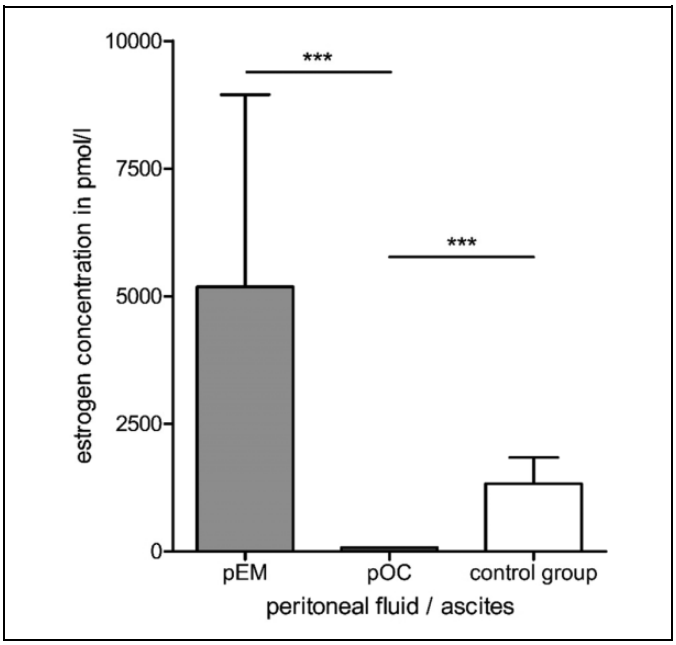

Figure 5. Measurement of estrogen concentration. Estrogen concentration (pmol/L) in peritoneal fluid or ascites of peritoneal endometriosis (pEM; n = 22), peritoneal carcinomatosis in ovarian cancer (pOC; n = 22), and a control group (n = 13) (shown are mean+standard deviation).

investigating the density of nerve fibers, neither in ovarian cancer nor in peritoneal carcinomatosis. The low nerve fiber density matches the scarcely presented clinical symptoms in pOC. Nerve fibers occur rarely around tumor cells of pOC and thereby cannot transmit noxious stimuli from there. In contrast, in pEM the total nerve fiber density is significantly higher and the patients experience more pain. Therefore, tissue nerve fiber densities might be directly related to the pain experienced by the affected persons.

Endometriosis is considered to be a chronic inflammatory disease. Macrophages are a major player in the nonspecific immune system. They are found in endometriosis around lesions and in peritoneal fluids.[19-23] Immune cells and endometriotic lesions themselves are thought to produce proinflammatory mediators.[33-38] These released mediators can sensitize sensory nerve fibers, which in turn undergo a positive feedback loop and prolong inflammation.[24-26] This process is called neurogenic inflammation and could be a reasonable explanation for the elevated pain experience. However, chronic inflammatory changes are also present in ovarian cancer. Thereby, immune suppressive cells composing the tumor microenvironment inhibit the antitumor immunity. Additionally, the present immune cells release various cytokines and chemokines, further enhancing the pathogenesis.[39]

In addition, NGF is expressed in inflammatory tissues and is involved in inflammatory reactions.[40,41] NGF as the main neurotrophin influences outgrowth and signaling state of nerve fibers. Moreover, it is assumed that NGF can activate sensory nerve fibers, thereby causing hyperalgesia.[16,42] Nerve growth factor itself and inflammatory stimuli increase BDNF synthesis and could contribute to the development of pain.[43-46] While NT-3 seems to have pronociceptive as well as antinociceptive effects.[43,47,48] For this reason, we aimed to search for the neurotrophins NGF, NT-3, and BDNF in tissue samples and peritoneal fluids. All neurotrophins were expressed in pEM, pOC, and in the control, in both tissue samples and fluids. In tissue samples, NGF expression was enhanced in close proximity to endometriotic lesions and tumor cells compared to controls. Whereas in peritoneal and ascites fluids, no differences in NGF concentration could be demonstrated between the groups. Previous studies demonstrated NGF expression in the tissue samples of ovarian cancer as well as in peritoneal fluid by means of different experimental methods.[10,49-51] It is suggested that neurotrophins lead to angiogenesis in ovarian cancer to secure survival and the spread of the tumor. Nerve growth factor binds to its receptor tyrosine kinase receptor A (TrkA) and upregulates the expression of vascular endothelial growth factor, which leads to growth and the formation of new vessels in ovarian cancer.[52,53] However, in our study the neurotrophins present did not seem to trigger growth of nerve fibers in pOC. Further studies investigating neurotrophins and their influence on nerve fibers in ovarian cancer are missing.

In an in vitro experiment, we were able to sight the neuronal outgrowth of sensory dorsal ganglia of chick embryos treated with peritoneal or ascites fluids of patients with pEM or pOC. This implies that both peritoneal and ascites fluids are capable of inducing the outgrowth of sensory nerve fibers. Neurotrophins may be responsible for that in endometriosis. A study with a similar test setup showed—vice versa—a reduction in nerve outgrowth through inhibition of NGF.[54] Apparently, the neurotrophins identified in ascites fluids do not lead to neuritogenesis around tumor cells of pOC as shown above. Neurotrophins may have other functions in ovarian cancer as well as the induction of angiogenesis. Therefore, it would seem reasonable that other factors must be involved in nerve outgrowth and peripheral sensitization of nerves in peritoneal lesions. In order to understand the different nerve fiber density in these peritoneal diseases, we studied the estrogen levels in peritoneal fluids and ascites. Interestingly, estrogen concentrations in peritoneal fluids of pEM and controls were significantly higher as compared to levels in ascites of pOC. Of the analyzed patients with pOC samples, 22 were postmenopausal and 3 perimenopausal. The analyzed pEM probes were from women of reproductive age. Nevertheless, the analysis of estrogen levels in the based on the age of the women revealed no significant difference but still may affect the result. It has been proposed that estrogen could stimulate sensory neuritogenesis and could therefore possibly be responsible for pain genesis.[55] The underlying mechanism is still on a suggestive level and needs a broader experimental validation. In further experiments, we aim to analyze the estrogen concentration levels in the peritoneal fluid of healthy postmenopausal patients and ascites from premenopausal patients with pOC as well as the nerve fiber density in peritoneum of postmenopausal women. In addition, further investigations are needed to consolidate the direct impact of estrogen on the pain pathogenesis in endometriosis.

Nonetheless, our results suggest that estrogen might be one of the influencing factors for the elevated nerve fiber density in the peritoneum of pEM but not pOC. Regarding the diverging pain phenotype, it remains unclear if estrogen modulation is a direct pain stimulus, sensitizes nociceptors, or influences the density of sensory neurons. However, estrogens have the potential to modulate the signaling sensitivity toward inflammatory compounds.[56,57] Furthermore, estrogen is known to influence neuritogenesis, which could be related to the perception and integration of nociceptive stimuli.[58-60] The striking differences in peritoneal estrogen concentration potentially influence the peritoneal innervation and may therefore play a role in the differential perception of pain in the presented diseases.

Declaration of Conflicting Interests

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