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Aimee Schickedanz Browne

Possible Role of Neurotrophins in Endometriosis Pathophysiology

By

Aimee Schickedanz Browne, M.D.
Master of Science in Clinical Research

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Robert N. Taylor, M.D., Ph.D.
Advisor

_____

John R. Boring, III, Ph.D.
Committee Member

_____

Henry Blumberg, M.D.
Committee Member

_____

Mitch Klein, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

_____
Date

POSSIBLE ROLE OF NEUROTROPHINS IN ENDOMETRIOSIS PATHOPHYSIOLOGY

By

Aimee Schickedanz Browne
M.D., University of Missouri-Kansas City, 2002

Advisor: Robert N. Taylor, M.D., PhD.

An abstract of
A thesis submitted to the Faculty of the Graduate School of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Clinical Research
2009

ABSTRACT

POSSIBLE ROLE OF NEUROTROPHINS IN ENDOMETRIOSIS PATHOPHYSIOLOGY

By Aimee Schickedanz Browne, M.D.

Purpose: Small nerves have been identified in the functional layer of eutopic endometrium in women with endometriosis, but not in women without endometriosis. The pathophysiology of neuron growth remains unknown, but suggests a possible mechanism to explain the pain associated with endometriosis. Neurotrophins, like nerve growth factor (NGF), regulate nerve growth throughout the body and they contribute to many diseases. The purpose of this study was to evaluate the expression of NGF in the endometrium of women with endometriosis compared to controls.

Null Hypothesis: There is no difference in NGF protein levels between endometriosis patients and controls.

Design: Cross sectional study.

Materials and Methods: We performed endometrial biopsies on women of reproductive age at the time of surgery. Biopsy specimens were split and preserved in RNA-later® or solubilized in protein lysis buffer. Reverse transcription-PCR with sequence-specific primers was performed to detect NGF mRNA transcripts in the human endometrial tissue. Next, we determined if NGF protein concentrations in the biopsy samples differed between endometriosis patients and controls. Western blots confirmed the presence of NGF precursors and mature protein isoforms and a quantitative ELISA was developed to measure NGF protein in biopsy homogenates.

Results: PCR analysis revealed the presence of NGF mRNA in human endometrial tissue. ELISA and Western blots confirmed the expression of NGF protein. A Mann Whitney test was used to compare the mean levels of total NGF protein in endometriosis patients and controls. No differences between the endometriosis patients and controls were noted ($P=0.83$).

Conclusions: NGF mRNA and protein are expressed in eutopic endometrial tissues derived from patients with endometriosis as well as normal controls. The protein concentrations did not differ significantly between the two groups. The findings indicate that the presence of endometrial nerves in endometriosis cases cannot be attributed solely to differential NGF protein expression. Hence, measurement of endometrial NGF cannot be used as a diagnostic test for endometriosis.

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INTRODUCTION

Endometriosis is defined by the histological presence of endometrial glands and stroma outside the uterine cavity. It is a highly prevalent disease affecting 10-15% of reproductive age women. Due to its clinical association with infertility and pelvic pain, it remains a significant public health problem. The etiology of endometriotic implants, as well as the mechanism of the associated pelvic pain, is unknown and generally under-investigated. This study focused on the putative link between endometrial nerve fibers and endometriosis lesions and their possible association with endometriosis related pain.

Recent studies have identified nerve growth in the endometrium and endometriosis lesions and have shown a convincing link between the presence of nerve fibers and the disease (1). Evidence that nerve fibers are present in endometriotic implants and are only seen in the eutopic endometrium of women with endometriosis has now been confirmed by two independent investigative groups. These researchers identified the presence of uncharacteristic nerve fibers in the superficial endometrium, and this finding has led researchers to ask additional questions.

Further research is required to investigate the influence of nerve growth in endometriosis associated pain, as well as to establish modulating factors of nerve growth in these patients. The goal of my study was to explore the relationship between endometriosis and nerve growth by evaluating a widely-

expressed neurotrophin, nerve growth factor, as a modulator that may influence neuronal growth in the uteri of these patients.

This study evaluated the eutopic endometrium in women with and without endometriosis for the presence of nerve growth factor (NGF), a well characterized neurotrophin known to regulate nerve growth in many tissues. The eutopic endometrium is the lining of the uterus that is normally shed during a menstrual cycle. This tissue is postulated to be the origin of the lesions in endometriosis patients. While this is the most popular theory, controversy remains, as discussed below.

My study looked for a potential role of NGF in the modulation of nerve growth in women with endometriosis by comparing the levels of NGF in endometriosis patients to controls without the disease. The null hypothesis of the study was that there is no difference in NGF levels in the eutopic endometrium of two groups of women, those with endometriosis versus controls without the disease.

To test the feasibility of the hypothesis, the presence of NGF in endometrial tissue was first established by reverse transcription-PCR (RT-PCR). Once NGF mRNA was identified, a quantitative ELISA was developed to measure NGF. Due to the skewness among the control group, a Mann Whitney test was used to detect differences in the NGF levels of the two groups. Receiver operator characteristic (ROC) curves also were created in order to determine the utility of

using NGF as a screening test for endometriosis. Logistic regression models of NGF concentrations and several clinical correlates were created to attempt to establish a predictive model of endometriosis.

BACKGROUND

Ten to fifteen percent of all reproductive age women have endometriosis (2) (3) and the most common and most specific symptom of this disease is pelvic pain (4). In fact, this disease is associated with several varieties of pelvic pain including: painful periods (dysmenorrhea), painful intercourse (dyspareunia), painful defecation (dyschezia), and chronic pelvic and lower back pain (5). Chronic pelvic pain is the indication for 40% of diagnostic laparoscopies (6) and of all the women who suffer from chronic pain, it has been reported that 97% have endometriosis (2). Direct costs to the United States are estimated at 880 million dollars (7) and when work absenteeism and other indirect costs are factored, annual expenditures are estimated at over two billion dollars (8). Despite the prevalence of the disease and the significant morbidity caused by the associated pain, a general lack of understanding remains regarding the etiology of endometriosis as well as the causes of pain in this disease (5).

Sampson's theory of retrograde menstruation remains the most widely accepted theory to explain the etiology of endometriosis (9). In this theory, refluxed endometrium implants on the peritoneal surface of the pelvic cavity (9). While the hypothesis supports the histogenesis of the majority of the lesions, the theory does not explain the existence of endometrial tissue outside of the pelvic cavity, nor does it explain the etiology of the pain associated with the disease. Prior studies emphasized the role of inflammation due to the cyclic sloughing of endometrial cells, recruitment of immune cells and prostaglandin release as a

cause of pain and other associated symptoms (3). New research has explored possibilities beyond inflammation and has revealed an association between nerve fibers and endometriosis.

These recent studies have documented that nerve fibers are present in greater amounts in endometriotic implants than in normal peritoneum (5) (4) (10). There is also evidence that nerve fibers are present in the superficial endometrium of patients with endometriosis but not in normal controls (11) (1) (12). This apparent difference in nerve growth is an area of research that can potentially delineate the etiology of pain associated with endometriosis, evaluate modulating factors of endometriosis, and may provide an opportunity to diagnose endometriosis by their presence.

Tokushige et al. evaluated nerve fibers in peritoneal endometriosis by immunostaining tissue sections with antibodies that probed a variety of neural proteins including, protein gene product 9.5 (PGP9.5), neurofilament (NF), nerve growth factor (NGF), NGF receptor p75 (NGFRp75), substance P (SP), calcitonin gene-related peptide (CGRP), acetylcholine (ACh), and tyrosine hydroxylase (TH) (5). They used different markers to characterize the populations of nerve fibers in the tissues and identified a high concentration of unmyelinated sensory C fibers (11) (1). A semi-quantitative and qualitative evaluation of nerve fibers stained with PGP9.5 and NF showed a significantly higher density of nerve fibers in endometriotic lesions than in normal peritoneum ($P<.001$). There was also a

qualitative increase in the presence and intensity of NGF immunoreactivity near endometriotic glands in peritoneal endometriotic lesions (5). From their findings, the authors postulated the increased expression of NGF and NGF receptors in endometriosis lesions may stimulate the in-growth of nerve fibers, ultimately leading to painful symptoms (5).

Previous unrelated work had already shifted focus from the peritoneum to the endometrium as a possible inflammatory nidus in the genesis of endometriosis. The abnormal expression of several genes in the so-called eutopic endometrium of women with endometriosis led researchers to focus on this tissue (13). The Tokushige group extended their peritoneal evaluation to the eutopic endometrium in women with endometriosis and compared it to controls. They reported a high density of small nerve fibers in the functional layer of the endometrium in women with endometriosis (1). This is a significant finding as previous research showed that nerve fibers are absent from the superficial two-thirds of the endometrium in the normal human uterus (14). Their study showed positive PGP9.5 antigen in the functional layer of the endometrium in endometriosis, whereas they found none in the control group (i.e., 100% specificity) (1). It is plausible that nerve fibers in this location may provide a mechanism for dysmenorrhea and central pelvic pain reported by these subjects (1). These authors urged further exploration of the small nerve fibers in the functional layer of the eutopic endometrium as a potential diagnostic marker of this condition.

Within the past four months, these endometrial findings have been reproduced by an independent group of researchers in Belgium. They confirmed the presence of nerve fibers within the endometrium of women with endometriosis, but not in the endometrium of controls. While the latter study showed a slightly lower specificity (98%) this remains a remarkable finding (12).

Presently almost nothing is known regarding the mechanism(s) of endometrial nerve fiber growth in endometriosis patients. Research involving nerve growth in other areas of mammalian biology has identified numerous neurotrophins, a family of proteins which promote survival, growth, and maintenance of neurons (15). NGF was the first neurotrophin identified and is the most widely studied (15). It was discovered by Levi-Montalcini (16) in *1983* who was awarded the Nobel Prize in 1986 for her work. Tokushige and colleagues hypothesized that nerve fibers in endometriotic plaques may originate from nerve fiber progenitors in the functional layer of the endometrium or from in growth of local nerve fibers due to secretion of nerve growth factors and increased expression of two NGF receptors (TrkA and p75) (1). It is possible the implanting endometrium may be programmed to secrete large amounts of nerve tropic factors resulting in in-growth of unmyelinated small nerve fibers (1).

METHODS

General Hypothesis: Increased nerve density has been observed in the superficial endometrium of women with endometriosis. We postulated that this phenomenon may be due to increased neurotrophin production in the endometrium of women with endometriosis relative to normal controls.

Null hypothesis: There is no difference in nerve growth factor protein levels in the endometrium of women with endometriosis compared to women without disease (controls).

Study Design: This was a cross sectional study of women undergoing gynecologic surgery with and without endometriosis.

Patient Selection:

Subjects were recruited from Emory University Hospital Midtown (EUHM) in Atlanta, Georgia from July 2008 until February 2009. EUHM serves a racially diverse group of women. Both academic and private practice physicians operate and provide care at this institution with over 2000 gynecologic procedures performed each year. Subjects were recruited from this diverse population. Women were identified for study participation by their physicians and were screened for eligibility.

Inclusion criteria included women of reproductive age (18-45) undergoing gynecologic surgery for infertility, pelvic pain, tubal ligation, or fibroids.

Exclusion criteria included women who had been on any estrogen, progesterone containing medications or other pituitary suppression therapy (e.g. Lupron, danazol) in the previous 3 months, were less than 18 or greater than 45 years of age, had not resumed normal menstruation after delivery, or were unable to understand or give written consent prior to participating.

This study was approved by the Institutional Review Board at Emory University, and all women gave written informed consent prior to participation

Outcome Variable: NGF protein levels in endometrial homogenates were measured by a quantitative ELISA.

Measurement of variables:

After written consent was given by the participants, they answered several questions to determine history of hormone use, as well as presence of infertility, pelvic pain, dyspareunia, and dyschezia. Next they were taken to the operating room and underwent general anesthesia induction. Endometrial biopsy specimens were collected under anesthesia via a 1.5 mm silastic Pipelle catheter, prior to placement of an intrauterine manipulator or full antiseptic prep. Tissue was placed in normal saline and transported to the laboratory on ice.

Tissue was then blotted, divided into three equal portions, and placed in 1x PBS for endometrial cell culture, protein lysis buffer for protein evaluation, and RNA later® for RNA preparation. The specimens in RNA later® and lysis buffer were immediately frozen at -80 degrees Celsius.

At surgery, a comprehensive visual evaluation of the pelvis, cul-de-sac and diaphragmatic domes was undertaken. Following the surgery, women were classified as having endometriosis (n=21) if their surgeon noted any evidence of endometriosis at the time of surgery. Women were classified as controls (n=16) if there was no visible evidence of endometriosis at the time of surgery.

Biochemical evaluations: Fresh frozen biopsy specimens were thawed on ice and subjected to the following analytical procedures.

Reverse transcription-PCR (RT-PCR): RT-PCR with sequence-specific primers was performed to detect NGF mRNA. Total RNA extraction from samples stored in RNA later® was performed. After preparation of complementary DNA, nested NGF-specific primers were used for amplification according to the previously described method (17). (5'-TGAAGCTGCAGACACTCAGG-3' (sense), 5'GACAAAGGTGTGAGTCGTGGT-3' (antisense) had been previously shown to successfully generate NGF amplicons. GAPDH was used as an internal control of mRNA quantity and integrity. NGF mRNA transcripts were evaluated in the human endometrial cells from primary culture, endometrial tissue, and an

established human endometrial stromal cell line (HESC). HESC cells are telomerase-immortalized human endometrial stromal cells provided generously by Drs. Krikum and Lockwood from Yale University.

RT-PCR findings established excellent quality mRNA, based on OD 260/280 ratios and expression of GAPDH transcripts. The presence of NGF mRNA transcripts in endometrial tissue indicated that its protein product also was likely to be detectable and supported the hypothesis that neurotrophin production in the endometrium might drive innervations of this tissue.

Protein Homogenates:

Freshly thawed biopsy fragments were placed in approximately 300 µl of lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40 (NP40), 0.02% NaN_3) and homogenized by vortexing until the tissue was dispersed. The samples were then placed on ice for 30 minutes. Samples were centrifuged and the homogenate supernatants were frozen in 50 µl aliquots at -80 degrees Celsius. Prior to freezing, 5 µl of homogenized material was used to determine protein concentration for future Western blot and ELISA assays.

Western blot:

Previously frozen protein lysates, prepared as described previously were thawed and resuspended. A total of 60 µg of protein from each specimen was separated

on 4-12% SDS-polyacrylamide gradient gels and transferred to PVDF paper. The membranes were then blocked at room temperature for 1 hour in TBS-T (10 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween-20) with 5% Carnation non-fat milk powder and probed with affinity-purified rabbit polyclonal anti-NGF IgG (Cell Signal) at a dilution of 1:1000, rotating overnight at 4 C. After washing with TBS-T, membranes were incubated with goat anti-rabbit IgG secondary antibody (Pierce Biotechnology) at a dilution of 1:20,000 for 1 h at room temperature. After washing with TBS-T, bands were visualized by chemiluminescence using Kodak Biomax film.

ELISA

A quantitative ELISA was developed and performed on acidified and non-acidified endometrial tissue lysates to measure total and mature NGF, respectively. NGF protein levels discussed in this paper are total NGF levels unless otherwise specified.

Previously frozen protein lysates, as described previously, were thawed and resuspended. NGF protein levels were measured by two-site, sandwich ELISA using affinity purified rabbit anti-human polyclonal NGF detection antibody purchased from Promega. The samples were treated with 1N HCl to acidify the samples to pH<3 and then neutralized with 1N NaOH to a pH of 7.6 to enhance the sensitivity of detection. The samples and known standard protein preparations were incubated in 96-well plates precoated with mouse anti-human

NGF monoclonal antibodies. All analyses were performed in duplicate according to the manufacturer's instructions. Purified NGF of known concentration was treated in parallel with samples and served as an internal standard. Mouse liver homogenates at the same protein concentrations were used as negative controls.

Endometrial Stromal cell cultures: Primary endometrial stromal cell cultures were prepared from endometrial biopsies of endometriosis and control subjects, as we have described previously (18). Glandular epithelial cells were separated from stromal cells and debris by filtration through narrow gauge sieves. Endometrial stromal cells (ESC) were subcultured at least three times to eliminate contamination by immune cells, but were used before the sixth passage for these experiments. Prior studies in our laboratory characterized ESC cultures prepared using this protocol and confirmed that they were more than 95% pure and retained functional markers of their endometrial origin in vitro (18).

Statistical analyses: A sample size calculation was performed for the main outcome variable, NGF protein levels obtained from ELISA. We calculated the sample size to detect an 80% difference in total NGF protein levels with $\alpha=0.05$ and $\beta=0.20$. Fifteen patients in each group (N=30) were needed to reach appropriate power to detect a difference, based on the assay variance.

The study population characteristics were evaluated using SAS version 9.1. Differences in age, history of infertility, and characteristics of pelvic pain were

evaluated between the two groups. Student's t-test was used to detect differences in continuous, normally-distributed characteristics. Cochran Mantel Haenszel was used to evaluate differences in binomial outcome characteristics.

For the main outcome variable, total NGF protein concentration, levels were determined from absorbance readings in the validated ELISA. NGF concentrations were performed in duplicate for each specimen. NGF levels are reported as pg of NGF per 100 mg of homogenate protein. During the establishment and optimization of the assay, sensitivity of the ELISA was determined to be 15 pg. The lowest endometrial lysate-NGF protein concentrations were consistently within the linear aspect of the standard curve and the coefficients of variance ranged from 2-11%.

Shapiro-Wilk and Kolmogorov-Smirnov tests were used to test for normal distribution. Given the relatively small sample sizes, statistical analysis using nonparametric tests were used to compare the difference in means between the two groups.

Receiver operator characteristic curves (ROC) were plotted using several cut off points for NGF protein concentrations to evaluate the protein as a screening test for the diagnosis of endometriosis. Sensitivity and specificity were calculated for each cut-point and were plotted accordingly.

A logistic regression was used to establish a predictive model for endometriosis using patient characteristics reported by the subject at the time of recruitment as well as the primary outcome variable (NGF levels). We studied the presence of infertility, pain, severe pain, dyspareunia or dyschezia, family history of endometriosis, and total endometrial NGF concentrations. Additional variables were created that combined pain with dyspareunia or dyschezia and pain with infertility to determine if a combination of clinical characteristics was more predictive.

RESULTS

Study population:

Thirty seven patients were recruited to participate in the study and gave informed consent (21 patients with endometriosis and 16 controls). Due to inability to obtain tissue (in 3 subjects) and loss of specimen during preparation (in 1 subject), 33 specimens were evaluated. During the course of the research, portions of the specimens were used for measurement of RNA, Western blot and ELISA. A total of 28 specimens were evaluated for NGF protein levels (17 endometriosis patients and 11 controls). The subjects ranged in age from 21-45. The mean age (±SD) of endometriosis patients was 34.1± 6.6 and the mean age of the controls was 33.7 ± 6.3. Of the patients consenting to the study, 34 of 37 answered the questionnaire (92%). In the endometriosis population, 55.6% of the population reported being infertile, 80% reported dysmenorrhea, and 60% reported dysmenorrhea was severe enough to affect activities of daily living (ADLs). In the control population, 42.9% reported infertility, 57% reported dysmenorrhea, and 28.7% reported severe enough pain to affect ADLs. (Table 1).

Overall, the population was similar in age. There were no statistically significant differences in the characteristics of the population. Although, endometriosis is normally associated with pain and infertility, our control population did not differ, perhaps because they also were recruited from gynecological practices treating women with other causes of infertility or pelvic pain. However, the combination

of multiple characteristics including pain, infertility and dyschezia or dyspaerunia was exclusively seen in the endometriosis patients.

RT-PCR

Total RNA was extracted from biopsied endometrial tissues stored in RNA later® and also from cultured endometrial stromal cells. Amplification of mRNA with RT-PCR identified NGF message in all samples. The results of the PCR confirmed the presence of NGF at the mRNA level in the endometrium, but were not quantitative enough to discern differences in NGF mRNA between the two groups.

Western Blot: After confirming the presence of NGF mRNA in the endometrium, we performed Western blot analysis to determine the quantity and molecular weight of NGF protein in the endometrial tissue lysates. We confirmed differential processing of NGF protein from an immature NGF precursor, Pro-NGF (37kDa) to a smaller, 13kDa mature NGF protein isoform. There was consistently very little fully processed, mature NGF in the biopsy specimens. (Figure 2). One Western blot appeared to show reduced processing of NGF to the mature, 13kDa band in an endometriosis patient, but this observation was not consistent in all specimens. Although these experiments confirmed the presence of NGF protein in the endometrium, they were unable to demonstrate any differences between endometriosis patients and controls.

ELISA: An NGF ELISA was developed to obtain the most quantitative assessment of NGF protein expression in endometrial biopsy lysates. An NGF sandwich assay was constructed using E_{max}® ImmunoAssay System reagents from Promega, according to the manufacturer's instructions. NGF ELISA was performed on 17 endometriosis samples and 11 normal samples. [Six of the other samples collected were used for RT-PCR, Western blot, or the initial development of the ELISA assay and were not used in the final analysis of NGF levels]. As the NGF precursor (37kDa) isoform appeared to dominate on Western blots, measurement of total NGF in the ELISA was optimized by pre-treating the lysates with HCl and neutralizing the solutions prior to running the assay. Acid treatment increased total detectable NGF and allowed for a more standard evaluation. Nonacidified specimens were detected inconsistently. NGF levels were expressed as pg/100 mg of protein to normalize the concentrations and allow for accurate comparisons. Levels were normally distributed in the endometriosis group, but not in the control group. The range of NGF in the endometriosis patients was 10.6-276.8 pg/100 mg of protein with a mean of 100.0 pg/100 mg and a standard deviation of 74.2. The NGF levels in the control patients ranged from 17.4-276.8 pg/100 mg of protein with a mean of 93.4 pg/100 mg and a standard deviation of 83.0.

A Mann Whitney test was performed to test for differences in the means of the two groups, but no difference was observed ($p=0.83$).

ROC curve:

A receiver operator characteristic curve was performed to evaluate the feasibility of using NGF as a screening test for endometriosis. Cut of values of 50, 100, and 150 pg/100 mg protein were used and the sensitivity and specificity were calculated for each level. (Figure 4). Based on this analysis, the highest sensitivity and specificity were 69 and 57 percent, respectively.

Logistic regression:

A logistic regression using presence of endometriosis as the outcome variable, was performed to evaluate a predictive model of diagnosis endometriosis. NGF protein concentration, age, dysmenorrhea, severe pain (defined as severe enough to affect ADLs), infertility, dyspareunia, dyschezia, and family history of endometriosis were placed in the model. Combinations of characterisitics such as pain and infertility as well as pain and dyspareunia or dyschezia also were evaluated. The variables were placed in the model simultaneously, and subsequently were assessed in a step-wise fashion. With all of the variables in the model, none of the individual variables predicted endometriosis in a statistically significant manner. Pelvic pain and severe pain were both statistically significantly predictive of endometriosis when placed in the model alone ($P<.05$). The combination of characteristics (pain and infertility) or (pain and dyspaerunia or dyschezia) highly predicted the presence of endometriosis. These combinations were unable to be placed in the model as these combinations were only seen in the endometriosis patients in our study. A history of infertility was

similar between the two populations, and likely reflects the indications for surgery in our study cohort. (Table 2).

DISCUSSION

This study evaluated the endometrial concentrations of NGF as a modulator of nerve growth in endometriosis patients and for its potential utility as a diagnostic marker. Due to the easy accessibility of endometrial tissue, it is an ideal source to provide insight into differences between women with and without endometriosis. Ultimately, the detection and measurement of differentially-expressed neurotrophins in endometrial biopsies could predict the presence of endometriosis without the need for invasive surgery, allowing for improved diagnostic capabilities, widespread assessment of disease prevalence, and earlier treatments. Previous data have shown the presence of nerves within the endometrium of women with endometriosis, is highly specific. In recent abstracts from the two leading groups in this field, the specificity of endometrial nerves was 83% (19) and >90% (12) and sensitivity was >90% (12, 19). However, a lack of understanding remains as to why the nerves are restricted to subjects with endometriosis. We postulated that NGF, a common neurotrophin, might be a modulator of nerve growth in these patients and could serve as a potential easy biomarker for the diagnosis of endometriosis.

Through RT-PCR we demonstrated that NGF mRNA is expressed in eutopic endometrial tissues derived from patients with endometriosis as well as normal controls. Western blot analyses revealed the expression of NGF protein in the endometrial tissue in both of these groups, with the 37 kDa precursor representing the most common NGF isoform in tissue lysates.

Quantitative analysis of total NGF protein was established using the acidification method to disrupt NGF from interfering binding proteins (20). Using this method we observed no statistical difference in protein concentrations between the two groups. ROC curves showed a sensitivity less than 70% and specificity less than 60%. These numbers do not support the use of NGF protein levels as a screening tool to predict the presence of endometriosis.

The logistic regression model confirmed what is generally known about the characteristics of endometriosis. Pelvic pain and infertility as well as several other combinations of pain symptoms almost completely separated the two groups. Single characteristics did not predict endometriosis with statistical certainty, nor did endometrial NGF concentrations add any significant predictive value. It is likely that individual symptoms did not show a difference due to the baseline patient characteristics of this population. The population in this study had a high prevalence of endometriosis, likely because these patients were recruited primarily from an infertility population. Although, infertility and pelvic pain are normally more highly associated with endometriosis than controls, our cohort was biased towards women with this complaints. Also while certain risk factors were relatively higher in cases the difference did not reach statistical significance perhaps because of limited sample size.

Although endometrial nerves appear to be a highly specific finding in endometriosis, our findings indicate that their presence cannot be attributed to differential NGF protein concentrations in tissue lysates. While disappointing, these negative findings take us a step closer to help identify the possible etiology of these fibers by eliminating one neurotrophin (NGF) as a definite cause. Future research should continue this work by evaluating other neurotrophin modulators and their receptors as possible mediators of the increased nerve density in endometrium of women with endometriosis. Not only will these studies illuminate the pathogenesis of endometriosis symptoms they may provide relevant biomarkers for novel, relatively noninvasive strategies to diagnosis endometriosis.

TABLES

	Endometriosis (n=21)	Normal (N=16)	P value
Average age	34.1±6.64	33.71±6.29	.88
Infertile (%)	55.6	42.9	.48
Pain (%)	80	57.1	.15
Severe pain (%)	60	28.6	.08
Dyspareunia (%)	41.2	18.2	.13
Dyschezia (%)	35	27	.40
Family history of endometriosis (%)	16.7	21.4	.73

Table 1: Study population characteristics. There were a total of 21 endometriosis subjects and 16 control subjects recruited for the study. There were no differences in age between the groups and no statistically significant differences in any clinical characteristics associated with endometriosis. A higher proportion of subjects with endometriosis reported pain, severe pain, and dyspareunia or dyschezia, but the trends did not reach statistical significance. The proportion of patients with infertility and family history of endometriosis was similar between the two groups.

Variable	Odds ratio	95% CI	P value
NGF level	2.71	(.5,15.1)	NS
Infertility	0.67	(.0, 13.7)	NS
Age	0.99	(0.7, 1.4)	NS
Dysmenorrhea	4.29	(0.1, 140.5)	NS
Dyschezia	0.11	(0.0, 2.8)	NS
Dyspaerunia	5.04	(0.1, 229.5)	NS
Severe Pain	8.25	(0.3, 222.8)	NS
Family history	0.33	(0.0, 43.0)	NS

Table 2: Logistic regression was performed using all collected patient information and NGF ELISA. No single variable was significant to predict the presence of endometriosis.

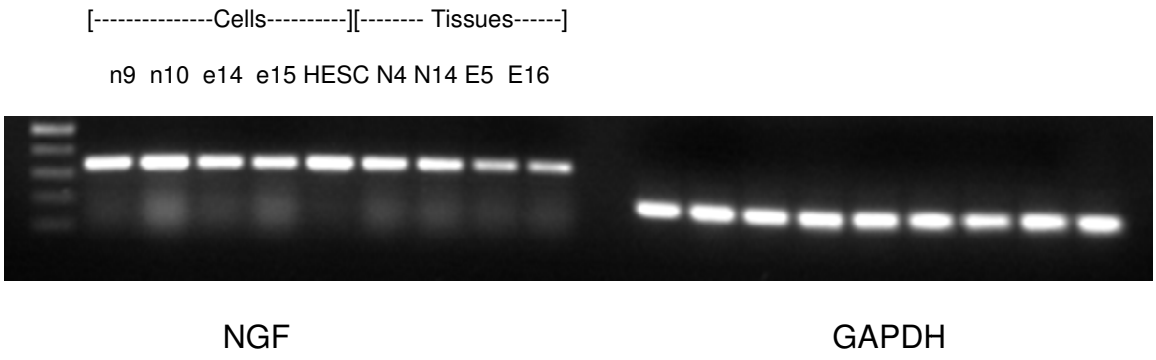

Figure 1. NGF RT-PCR. We demonstrated the presence of NGF mRNA in cultured endometrial stromal cells and biopsied endometrial tissue. There was no qualitative difference between normals and endometriosis patients. GAPDH was used amplified as an internal control for mRNA quality. HESC=Immortalized human endometrial stromal cells, n=control cells, e=endometriosis cells, N=control tissue, E=endometriosis tissue.

Normal

NGF Precursor (37kDa)

Mature NGF (13kDa)

Figure 2: A representative Western blot of NGF protein in endometrial tissue lysates. This normal patient showed both NGF precursor and the mature isoform. Most of the NGF was not processed (37kDa), and there was consistently a smaller amount of mature NGF (13kDA). When we compared endometriosis patients and controls, no difference existed.

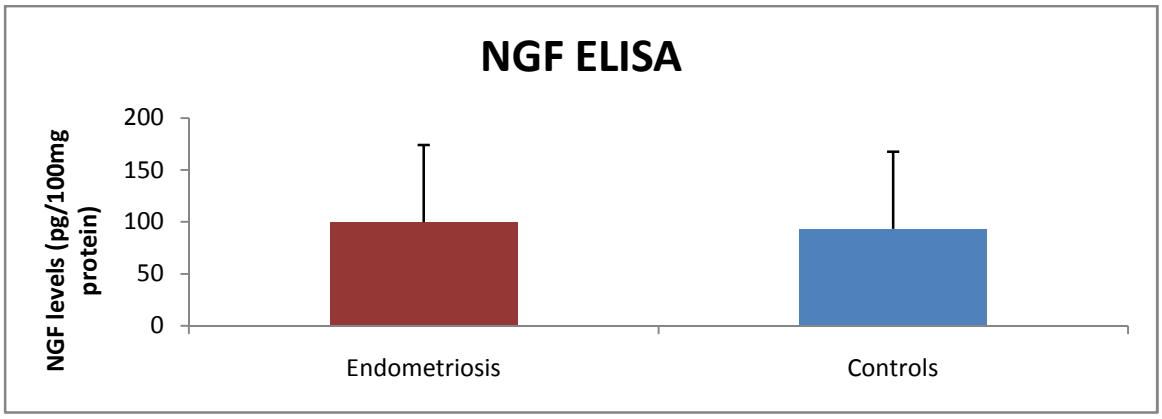

Figure 3: Results of NGF ELISA in endometrial tissue lysates showed no difference in NGF protein concentration between endometriosis patients and controls. p=0.83

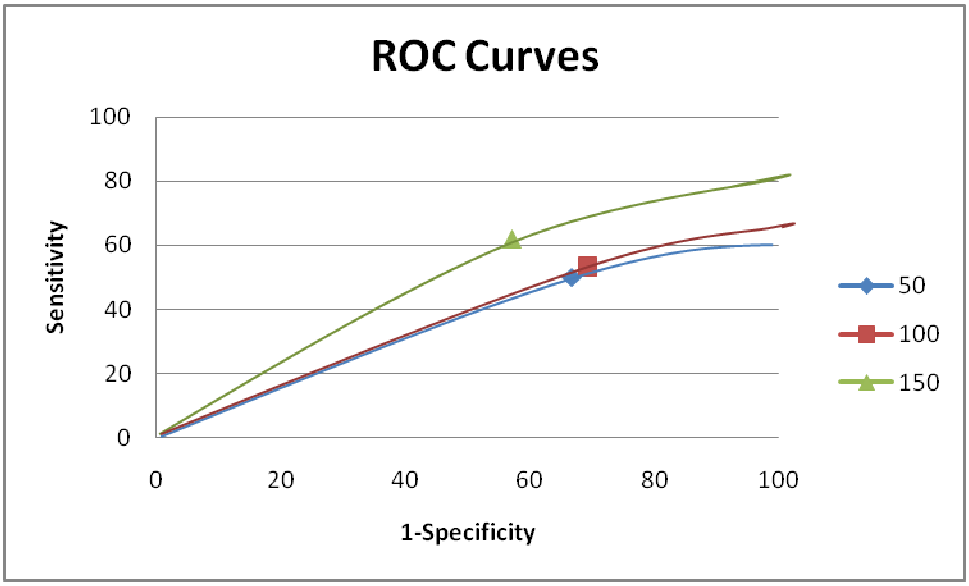

Figure 4: Receiver operator characteristic curves evaluating 3 separate cutpoints of NGF protein concentrations (50,100,150 pg/100 mg of protein). With low sensitivity and specificity, NGF protein concentration does not appear to be an adequate screening test for endometriosis.

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