Reviewer A

1. While in general the paper is laid out well there are numerous spelling and grammatical errors. Please revise accordingly.

Answer: Sorry for this, we apologize for these errors and an expert from Shine Write Services have polished our whole manuscript.

2. Important citations appear to be missing from the introduction. The relationship between neurotrophins and endometriosis was first demonstrated\(^1\) and subsequently confirmed by several other groups\(^2\)–\(^4\) including the relationship with pelvic pain\(^3\) well in advance of Ding an colleagues (2018) contribution who used an inappropriate matrix (serum) to quantify circulating BDNF concentrations. Perhaps more appropriate references should be used or at least included with Ding et al. (2018).

Answer: Thanks for your comments. We have added these important references about neurotrophins and endometriosis in revised manuscript.

3. A critical point in endometriosis research is the comparator group used as controls. In particular, using healthy women without evidence of pelvic pain or infertility, both hallmarks of endometriosis, is a weakness of the present paper. Moreover, incidental findings of endometriosis in asymptomatic women is a common occurrence (45.3 %)\(^5\). As such, pleased describe how women assigned to the control group were indeed free of endometriosis and include this limitation in the discussion section.

Answer: Good question. In this study, all participants underwent laparoscopic surgery to determine whether they had endometriosis and the staging of endometriosis. The specimen was confirmed by pathologists after operation. We have added this note in the Method Section.
4. Pain is an important issue in the present study; however, the method used to evaluate pain was not reported. It is unclear if the authors are referring to endometriosis associated pelvic pain in general or any pain at all. Similarly, it is unclear how severe the pain was for study participants. This is important as it could relate to how generalizable this is to all women with endometriosis. Please provide details on how pain was defined and measured.

Answer: Thanks for your good advice. We have added the description of endometriosis pain and the method for measuring the severity of pain in the Methods Section.

“Types of pain associated with endometriosis included dysmenorrhea, dyspareunia, dysuria, defecation pain and chronic pelvic pain. The severity of pain was documented using a standardized questionnaire with a visual analog scale (VAS, 0-10) as previously described (PMID: 28898282). Briefly, the pain scale was subdivided into ten grades, no pain was indicated at the left side of the scale and the maximum pain you could imagine was designated at the right side of the scale.”

5. Pain was reported as a dichotomous measure (e.g. present or absent). Please provide the actual pain scores and correlation with endometriosis related pain scores.

Answer: Thanks again for comments. We have described the severity of endometriosis-associated pain and analyzed its correlation with netrin-1 expression levels using Spearman analysis in Figure 1B, D in revised manuscript.

6. There is no mention of the procedures used to collect endometrial tissues. How was the eutopic endometrium collected and processed? How were endometriotic lesions collected and where they endometriomas, peritoneal lesions or deep infiltrating endometrial lesions? Did the study participants with endometriosis have only one type of endometriotic lesion or were they mixed and where were they lesions located in the pelvis as there is evidence that different lesions types are functionally distinct6-8. Were the lesions white, red, or bluish-black? Please be specific about the lesions as this will be important to be able to relate the results to women with endometriosis.
Answer: Thanks again. We have added the collection process and the location of endometrial tissues in the Methods Section.

“Of the 37 women with endometriosis, 16 (43.2%) women had ovarian endometriosis, 11 (29.7%) had peritoneal endometriosis and 10 (23.0%) women had deeply infiltrating endometriosis. Red fresh endometrial tissues (endometriotic lesions and eutopic endometrium) were collected during the surgical procedure.”

7. Details of the immunohistochemical and immunofluorescence staining are lacking. Specifically, please provide details of the host used to generate all the antibodies used, report whether they are polyclonal or monoclonal, and the dilutions used.

Answer: Sorry for this, and we have added the details of the immunohistochemical and immunofluorescence staining as well as details of relevant antibodies in revised manuscript.

IHC
Netrin-1 (1:50, ab122903, Goat polyclonal, Abcam)
DCC (1:300, bs-0592R, Rabbit polyclonal, Bioss)
UNC5B (1:300, bs-11492R, Rabbit polyclonal, Bioss)
A2BAR (1:1000, PA5-72850, Rabbit polyclonal, Invitrogen)

IF
CD68(1:200, ab201340, Mouse monoclonal, Abcam)
Netrin-1 (1:500, ab126729, Rabbit monoclonal, Abcam)

WB
Netrin-1(1:1000, ab126729, Rabbit monoclonal, Abcam)
Gapdh (1:1000, Mab5465-100; Multi Sciences)
Secondary antibody (1:5000, ab97051/ab97023; Abcam)

8. Was a secondary antibody used and what IHC technique was employed to detect
and image immunopositive cells?

Answer: Yes. We have added the details of IHC procedures in the Methods Section.

9. What was the chromogen used for the IHC procedures?

Answer: Yes. We have added the details of IHC procedures in the Methods Section.

10. The antibody details for flow cytometry are also not reported and should be included in enough detail so that the results of this study can be replicated by other labs.

Answer: Sorry for this, and we have added details of relevant antibodies used in cell flow cytometry in revised manuscript.

Fc Block (1:200, BD564219, BD Biosciences)
Fixation and Permeabilization Solution (BD54722, BD Biosciences)
anti-CD86 (1:200, BD561128, Mouse BALB/c IgG1, κ, BD Biosciences),
anti-CD163 (1:50, BD556018, Mouse BALB/c IgG1, κ, BD Biosciences)
anti-CD68 (1:200, BD562117, Mouse BALB/c IgG2b, κ, BD Biosciences)
PE-CyTM7-conjugated IgG1 (1:200, BD557872, BD Biosciences)
PE-conjugated IgG1 (1:50, BD555749, BD Biosciences)
FITC-conjugated IgG2b (1:1000, BD565379, BD Biosciences)

11. Please provide definitions for the first use of all abbreviations.

Reply: Sorry for this, and we have corrected it in revised manuscript.

12. Line 434 of the discussion is not quite accurate since the data present in this study is correlative only. Specifically, the authors have shown that Netrin-1 expression is greater in women with endometriosis and may be correlated with pain, although as mentioned above important details are lacking in the paper, mechanistic details are lacking on Netrin-1 and endometriosis associated pelvic pain.
Answer: Thanks for your comments. We have added the results of our further study on the role of Netrin-1 in endometriosis pain *in vitro and vivo* and rewritten this sentence in revised manuscript.

13. Please include a short paragraph in the discussion defining the strengths and limitations of the present study.

Answer: Thanks again for your advice. In revised manuscript, we have this part in the Discussion Section.

“Our study features some limitations, which should be taken into account when interpreting our findings. First, in this study a total number of 60 women were recruited. Different sample sizes often lead to differences in results. In order to make the results more accurate, the follow-up study should increase the sample size as much as possible to reduce the experimental statistical errors. Secondly, double immunofluorescence staining showed that there were about 62.2% percentage of Netrin-1 expressing macrophages (CD68+Netrin-1+) in total Netrin-1+ cells. This is another limitation in our study: what cells are responsible for the increased Netrin-1 in endometriotic lesions? Immunohistochemical staining results showed that Netrin-1 was not only expressed in epithelial and interstitial vascular endothelial cells, but also expressed in endometrial stromal cells in endometriotic lesions from women with endometriosis. In this study, we demonstrated that the expression levels of Netrin-1 in serum and endometriotic lesions were significantly higher in women with endometriosis, and Netrin-1 was co-expressed with CD 68 in endometriotic lesions, which was consistent with previously
reported studies that Netrin-1 is highly expressed in macrophage infiltrating in atherosclerotic plaques (PMID 22231519), adipose tissues (PMID 24584118) and inflamed aortic vessel wall (PMID 30479344). In vitro, we found that Netrin-1 was synthesized and secreted by THP-1 and NR8383 cells in process of M1 polarization. However, it has been reported that endothelial cells (PMID 25824964), as well as epithelial cells (PMID 19122655), can also synthesize and secrete Netrin-1. Thus, further research is needed on the origin of the up-regulation of Netrin-1 expression in endometriotic lesions. Last but not least, it this study we have not further studied the effects of Netrin-1 on different receptors, that is, how Netrin-1 cause endometriosis pain. In one of our further study, we demonstrated that Netrin-1 induced angiogenesis in ovarian endometriomas through interaction with CD146 in vascular endothelial cells and promoted neurite growth and sensitization through another receptor, neogenin (PMID 31953174). As this is a continuous study, we will further validate and deepen our research in the next animal experiments.”

Reviewer B
In fiure 1, the author need to quantify the percentage of Netrin-1 expressing macrophage in total Netrin-1 cells.

Reply: Thanks for your comments. Double immunofluorescence staining showed that there were about 62.2% percentage of Netrin-1 expressing macrophages (CD68+Netrin-1+) in total Netrin-1+ cells. This is one of limitations in our study: what cells are responsible for the increased Netrin-1 in endometriotic lesion? Immunohistochemical staining results showed that Netrin-1 was not only expressed in epithelial and interstitial vascular endothelial cells, but also expressed in endometrial
stromal cells in endometriotic lesions from women with endometriosis. In this study, we demonstrated that the expression levels of Netrin-1 in serum and endometriotic lesions were significantly higher in women with endometriosis, and Netrin-1 was co-expressed with CD 68 in endometriotic lesions, which was consistent with previously reported studies that Netrin-1 is highly expressed in macrophage infiltrating in atherosclerotic plaques (PMID 22231519), adipose tissues (PMID 24584118) and inflamed aortic vessel wall (PMID 30479344). In vitro, we found that Netrin-1 was synthesized and secreted by THP-1 and NR8383 cells in process of M1 polarization. In our further study, we demonstrated that Netrin-1 induced angiogenesis in ovarian endometriomas through interaction with CD146 in vascular endothelial cells and promoted neurite growth and sensitization by upregulating MAP4, TAU, and CGRP, Through another receptor, neogenin. The inhibition of netrin-1 using a neutralizing antibody reduced vascular and nerve infiltration in rat endometriotic lesions. However, it has been reported that endothelial cells (PMID 25824964), as well as epithelial cells (PMID 19122655), can also synthesize and secrete Netrin-1. Thus, further research is needed on the origin of the up-regulation of Netrin-1 expression in endometriotic lesions.

We have added this part in the Discussion Section.

In figure 2 and 4, The gene expression and markers of M1 should be consistently checked in vivo and in vitro.

Reply: Good question. Indeed, polarization is a dynamic process as the signals are temporally and dynamic, and the use of terms M1 and M2 is confusing due to the lack of specific phenotypic scoring criteria. Many physiological or pathological macrophages did not show a clear M1 or M2 phenotype, or macrophages with combinations of M1 and M2 markers can be found during M1/M2 polarization. New methods and technical advances are needed to reassess activation and classification of macrophage. Macrophages from different tissue sources, as well as different cell lines, often differ greatly in surface antigens. In endometriosis, the polarization of M1/M2 macrophages remains highly debated because of the use of different standards. In this study, we used CD68+CD86+CD163- to label M1 and CD68+CD86-CD163+ to label when analyzing the polarization phenotypes of the peritoneal macrophages, which is the most widely received. In vitro cell experiments, we used lipopolysaccharide (LPS)
and interferon gamma (IFN-γ) to induce M1 phenotype macrophages in THP-1/NR8383 cells, which is the most widely used (PMID24669294), and verify the macrophage polarization by detecting the expression of the inflammatory mediators. We have added this part in the Discussion Section.

The association between Netrin-1 (or Netrin+ macrophage) and pain should be analyzed by linear progressive analysis if there is a way to score the pain.

Reply: Thanks again for your good advice. We have described the severity of endometriosis-associated pain by using a visual analog scale (VAS) and analyzed its correlation with netrin-1 expression levels using Spearman analysis in Figure 1B, D in revised manuscript.

Reviewer C
The present study aimed to determine the role of Netrin-1 in endometriosis pain. This topic could be interesting, however, these authors failed to answer the question.

According to study design, 60 patients with or without endometriosis were recruited. Whether or not did the 23 endometriosis patients have related chronic pain were not declared. The study seems to be comparing between women with endometriosis and disease-free controls rather than women with pain symptoms and women who are not. Further essential information is needed to draw the conclusion of this study.

Reply: Thanks for your comments. In this study, a total of 60 women with (case group, n=37) and without endometriosis (other benign gynecologic diseases excluding chronic pelvic pain, control group, n=23) were recruited. All participants underwent laparoscopic surgery to determine whether they had endometriosis and the staging of endometriosis. The specimen was confirmed by pathologists after operation. We compared the differences of Netrin-1 expression between women with and without endometriosis. Besides, in women with endometriosis, we also compared the difference of Netrin-1 expression between women with and without endometriosis pain. Results showed that the expression levels of Netrin-1 in serum and endometriotic lesions were
significantly higher in women with endometriosis, and were positively correlated with the severity of endometriosis pain. Thus, we concluded that Netrin-1 in endometriotic lesions may play an important role in endometriosis pain.

We have added this part in revised manuscript.