Author's response to reviews

Title: Decreased Semaphorin3A expression correlates with disease activity and histological features of rheumatoid arthritis

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Author's response to reviews:

Dr. Francisco J. Blanco
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Re: Manuscript ID 2079539547803356: Decreased Semaphorin3A expression correlates with disease activity and histological features of rheumatoid arthritis

Dear Dr. Blanco,

We thank you and the reviewers for your comments and suggestions regarding the above-referenced manuscript (please note the revised title, as suggested). We have revised the manuscript and have included responses to the reviewers' comments on a point-by-point basis below. We hope that you find the revised manuscript acceptable for publication in BMC Musculoskeletal Disorders.

Sincerely,

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Responses to Reviewer Comments
Reviewer 1

Major comments:

1. The experiments done are sound, clear and well presented. The main problem of the data is that they lack novelty. To this reviewer, Sema3A immunohistochemical studies are one of the most novel revelations in this study, however they are weakly demonstrated. Probably these results would be clearly explained with a semiquantitative score for each immunohistochemical staining in order to test the statistical significance of these data. The representative photomicrograph of all determinations should be maintained.

Response: Thank you for the excellent suggestion. We quantified Sema3A immunostaining intensity in the lining layer and added this to the revised manuscript.

Text was added as follows:

Page 8 (Methods):
Quantification of Sema3A immunostaining
Tissue sections immunostained with anti-Sema3A antibody were analyzed for 12 patients with OA and 12 patients with RA. The total immunostaining intensity in the lining layer was measured using a BZ-9000 microscope (Keyence, Osaka, Japan) equipped with Dynamic Cell count software BZ-H1C (Keyence). Immunostaining intensity per unit was calculated as described previously [34].

Page 11 (Results):
We quantified Sema3A-immunostaining signal in the lining layer of RA and OA specimens. Sema3A-immunostaining intensity per unit area of lining layer was significantly less in RA patients compared with OA (Figure 1O).

In relation, it has been reported before that synovial tissue specimens derived from OA and RA patients exhibited very low levels of Sema3A mRNA expression without no significative differences between OA/RA synovial tissue versus healthy controls and even lower difference between OA/AR (Alfonso Catalano, J Immunol 2010). An explanation about this discrepancy should be included.

Response: These are interesting questions. As Catalano performed qPCR in a small number of patients, they could not exhibit significant differences between OA and RA synovial tissues. qPCR methods and sample populations may also have influenced the results. We have added new sentences to the discussion (pages 14 and 16), providing an explanation regarding this discrepancy and describing it as a limitation of the study.

Text was added as follows:

Pages 14 (Discussion):
Catalano reported synovial tissues derived from healthy controls, OA and RA patients exhibited no significant differences although the relative Sema3A
expression was lowest in RA samples[14]. This discrepancy might be explained by the difference in sampling numbers, which can influence statistical power, and/or by different qPCR methods used. We quantified Sema3A mRNA expression in a larger number of RA patients (n = 30) and OA patients (n = 23), whereas Catalano used relatively small numbers of RA (n = 10), OA (n = 10) patients and healthy controls (n = 5). For qPCR, we used the standard curve method for relative quantification, whereas Catalano used a comparative Ct method.

Page 16:
First, this study population may be influenced by environmental factors as well as genetic factors.

In the same way, the quantification of serum level of sema3A in RA and OA patients would be interesting to strengthen the results showing that reduction of Sema3A expression in RA synovial tissues may contribute to pathogenesis of the disease.

Response: Thank you for the excellent suggestion. Unfortunately, we do not have serum samples from the study patients’ serum before operation. We have added a new text to the discussion (page 14).

Text was added as follows:

Page 14 (Discussion):
Additional investigations to quantify the serum levels of Sema3A of patients with OA and RA patients and assess whether this level correlates with disease activity may contribute to further elucidation of pathogenesis of the disease.

2. How do you explain that VEGF expression levels did not exhibit a significant correlation with DAS28-CRP? And that the VEGF signal is so weak in RA synovial tissue? The authors should think of explaining in a satisfactory way the lack of correlation.

Response: Thank you for the interesting questions. We suggest VEGF expression levels at the cellular level and in the synovial fluid and/or serum might be different. Cellular VEGF expression levels between OA and RA were not significantly changed. RA synovial tissues had a higher cellularity, and therefore, total VEGF production was increased and VEGF expression levels in synovial fluid and/or serum were elevated. Hashimoto et al. reported that the staining intensity of VEGF expression did not differ between RA and OA synovial lining layer, similar to our results. This hypothesis may also be the reason for VEGF-A expression levels not exhibiting a significant correlation with DAS28-CRP scores in this study.

We have added a new sentence to the discussion (pages 15 and 16), providing an explanation regarding this discrepancy.

Page 15-16 (Discussion):
We found that mRNA expression of VEGF-A was not significantly altered in OA and RA synovial tissues. Hashimoto et al. reported staining intensity for VEGF expression did not differ between RA and OA synovial lining [46] and Lowin et al. reported that VEGF165 expression did not differ in the chronically inflamed tissue of RA patients and OA patients [47]. In contrast, several reports showed altered expression of VEGF in RA. Lee et al. observed significantly higher levels of VEGF protein in RA compared with OA synovial fluid and serum [48]. Kurosawa et al. observed significant correlations of serum VEGF levels with DAS28-CRP scores [24]. The elevation of VEGF protein in RA synovial fluid and serum may explain the higher total number of VEGF-producing cells in the region [46]. Indeed, our study showed no signification correlation between VEGF-A expression levels and DAS28-CRP scores (Fig. 2), but there was a marked increase of synovial tissue thickness of the lining layer in RA (Fig. 1). Thus, VEGF levels in RA serum may be increased as previously reported [24]. In an earlier study, Ikeda et al. reported VEGF165 was expressed in 41% of RA samples (17 patients) but not in OA samples (8 patients) using reverse transcription-PCR [49]. They also found NRP1 was up-regulated in RA synovial tissues. However, we did not observe significant alterations of VEGF-A (Figure. 2B) or NRP1 (Figure. 2C) between OA and RA specimens. Kim et al. reported that NRP1 expression was similar in OA and RA using immunohistochemical analyses [50]. These discrepancies might be explained by the difference in sampling numbers, disease duration of the populations studied, extent of inflammation, use of anti-rheumatoid drugs, and/or by different methods for analysis of NRP1 expression levels. Additional investigations in a large sample considering several different conditions are required to clarify these discrepancies.

Minor comments:

1. Check which is the most common abbreviation of neuropilin, NRP (your option) or NP.

Response: We checked the abbreviation for neuropilin in the NCBI genome database and it is NRP.

2. Include in the abstract that Sema3A was decreased in RA patient samples vs OA.

Response: We have included text in the abstract describing that Sema3A was decreased in RA tissues compared with OA samples.

Text was changed as follows:

Abstract results:
Immunohistochemistry revealed the density of Sema3A in synovial lining cells was decreased in RA tissues compared with OA samples. qPCR analysis demonstrated Sema3A mRNA levels in synovial tissue samples were significantly lower in RA than in OA and a significant correlation of the ratio of Sema3A/VEGF-A mRNA expression levels with DAS28-CRP.
3. The medication of each OA patient should be included. Since medication may affect the expression levels of several molecules this information should be inserted.

Response: As suggested, we have included the medication of each OA patient in Table1 (page 22). None of the OA patients received immunosuppressive treatment.

4. Since the first evidence as to the key role for Sema3A in autoimmune arthritis was published by Alfonso Catalano (Alfonso Catalano, J Immunol 2010) and Vadasz et al (Vadasz et al, Arthritis Res Ther, 2012), these references should also be included the first time the implication of Sema 3A is mentioned in the exacerbation of autoimmune diseases (line 24).

Response: Thank you for this suggestion. We have added these references to the text.

Text was changed as follows:

Page 5 (Background):
Several studies have indicated that a reduction of Sema3A expression is involved in the exacerbation of autoimmune diseases, such as RA and systemic lupus erythematosus (SLE)[13, 14].

5. Since in the first section of results (page 10) an evaluation of infiltration of immune cells has been performed, it would be better to also include in this section the results obtained with CD20.

Response: As suggested, we have included the results with those obtained with CD20 to the text (page 11).

Text was added as follows:

Page11 (Results):
B cells were also sporadically localized in the sublining layer of OA specimens and large numbers of B cells were observed in the lymphoid follicles of RA synovial tissues (Figure 1M, N). We quantified Sema3A-immunostaining signal in the lining layer of RA and OA specimens. Sema3A-immunostaining intensity per unit area of lining layer was significantly less in RA patients compared with OA (Figure 1O).

6. Since recently, it has been reported that sema3A promotes regulatory T cells by enhancing IL-10 production and as the authors discussed how the reduction of Sema3A may abrogate the functions of regulatory T cells, thus allowing for the infiltration and focal aggregation of autoreactive lymphocytes in the sublining layer, it could be interesting to know the levels of IL-10 expression by
imunohistochemistry in OA and RA synovial tissue as well as if a correlation between IL-10 levels and sema3A is present.

Response:
Thank you for the excellent suggestion. However, our attempts at IL-10 immunostaining in OA and RA synovial tissue during this revision period were unsuccessful. (We used rabbit polyclonal IL-10 antibody (ab34843, 1:200; Abcam). In so far, sections were not stained clearly including lymph node, positive control of immunohistochemical reaction with IL-10). We have added a new sentence in the discussion (page 14).

Page 14 (Discussion):
Further investigation of the correlation between Sema3A and IL-10 expression levels may increase our understanding of their roles in RA.

Reviewer 2

Discretionary Revisions:
1. My main concern in this paper is that the expression levels of VEGF165 was evaluated using VEGF-A primers in the qPCR experiments, meaning, no specific primer for the 165 isoform were used. I think the assumption that total VEGFA expression is a good measure of VEGF165 expression could be misguided, and I would suggest the possibility of repeating the analysis with other primers. Therefore, the correlation analysis between VEGF and Sema3A expression should be remade.

Response: Thank you for the excellent suggestions. We used VEGF-A primers in this study, since there are no VEGF165 specific primers available from Applied Biosystems LCC (ABI). We also performed qPCR analysis with an ABI 7500 Sequence detection system based on the Syber green method. Sequences of primers used in this study are as below.

VEGF165: product (254 base pairs) Forward: CCCTGATGAGATCGAGTACATCTT
Reverse: AGCAAGGCCCACAGGGATTT

#-actin: product (188 base pairs) Forward: AGAAAATCTGGCACCACACC
Reverse: AGAGGCGTACAGGGATAGCA

The mRNA expression of VEGF165 was not significantly different between RA and OA (Additional file 1: Figure S1A). VEGF165 expression levels did not significantly correlate with DAS28-CRP (R = 0.324, p = 0.114; Additional file 1: Figure S1B). We used pre-developed VEGF-A primers in this study, which we considered more accurate.

We have added a new sentence in the discussion (pages 11 – 12) and additional files.
We also obtained similar results with VEGF165 primer in preliminary study (Additional file 1: Figure S1A).

Additional file1: Expression of VEGF165 mRNA in synovial tissues: preliminary study.

Expression levels of VEGF165 mRNA were measured by real-time PCR. The mRNA levels were normalized to the expression of #-actin. The box plots demonstrate the 10th and 90th percentile (whiskers), the 25th and 75th percentile, and the median. VEGF165 mRNA levels were not significantly altered in RA (n = 25) synovial tissues compared with OA (n = 17) (A). VEGF165 expression levels did not significantly correlate with DAS28-CRP (B).

Methods
Quantitative real-time polymerase chain reaction (qPCR)
qPCR was used quantify VEGF165 expression in 17 OA samples and 25 RA samples. Total RNA was extracted from synovial tissues using an Illustra RNA spin Mini Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions. RNA was reversed transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara Bio). The cDNA synthesized from 1 µg of total RNA was used as the template in each reaction. qPCR analysis was performed in an Applied Biosystems PRISM 7500 Sequences Detection System (Applied Biosystems LLC) based on SYBER Green method using SYBR Premix Ex Taq II (Takara Bio). Sequences of primers used in this study are as follows:

- VEGF165 (254 base pair) forward primer (5'-CCCTGATGAGATCGAGTACATCTT-3'), VEGF165 reverse primer (5'-AGCAAGGCCCACAGGGATTT-3'), #-actin (188 base pair) forward primer (5'-AGAAATCTGGGCAACCACACC-3') and #-actin reverse primer (5'-AGAGGCGTACAGGGATAGCA-3').

The default Applied Biosystems PRISM 7500 amplification conditions were 30 sec at 95°C, followed by 5 sec at 95°C and 34 sec at 60°C for 40 cycles. A standard curve, derived from known serial dilutions of RA synovial tissue, was constructed to calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies. The obtained data were standardized using the reference gene, #-actin.

Minor Essential Revisions:
1. I would suggest the inclusion of a table with the characteristics of RA and OA patients, including demographic, clinical and data regarding current and past immunosuppressive treatment.

Response: As suggested, we have revised Table 1, showing the characteristics of RA and OA patients (page 22). None of the OA patients received immunosuppressive treatment.
2. In the discussion section (page 15, lines 14-15) the authors comment “the cohort number was not large enough for strong statistical analyses”. I would suggest a calculation of the statistical power of this study.

Response: Thank you for this suggestion. We performed a posterior analysis power using G power. We found that all statistical powers of this study were greater than 80%. We have removed the previous sentence and added new text to page 10, describing the explanation regarding statistical power of this study.

Text was added as follows:

Page 10 (Methods):
We calculated a posterior power of this study using G*Power (Faul, Erdfelder, Lang, & Buchner, 2007). All statistical powers in this study were greater than 80%.

3. Authors observed a similar VEGFA expression between RA and OA patients. How do the authors explain this discrepancy with the previous published data?

Response: Thank you for the interesting questions. We thought that VEGF expression levels on the cellular level and in synovial fluid and/or serum might be different. However, the cellular VEGF expression levels between OA and RA were not significantly different. RA synovial tissues had a higher cellularity, therefore, the total VEGF produced was increased and VEGF expression levels in the synovial fluid and/or serum was elevated. Hashimoto et al. reported staining intensity for VEGF expression did not differ between RA and OA synovial lining layer, similar with our results. This hypothesis may also explain why VEGF-A expression levels did not exhibit a significant correlation with DAS28- CRP scores in this study.

As suggested by Reviewer 1, we have added a new sentence in the discussion (pages 15 and 16), describing this discrepancy.

Pages 15-16 (Discussion):
We found that mRNA expression of VEGF-A was not significantly altered in OA and RA synovial tissues. Hashimoto et al. reported staining intensity for VEGF expression did not differ between RA and OA synovial lining [46] and Lowin et al. reported that VEGF165 expression did not differ in the chronically inflamed tissue of RA patients and OA patients [47]. In contrast, several reports showed altered expression of VEGF in RA. Lee et al. observed significantly higher levels of VEGF protein in RA compared with OA synovial fluid and serum [48]. Kurosawa et al. observed significant correlations of serum VEGF levels with DAS28-CRP scores[24]. The elevation of VEGF protein in RA synovial fluid and serum may explain the higher total number of VEGF-producing cells in the region [46]. Indeed, our study showed no signification correlation between VEGF-A expression levels and DAS28-CRP scores (Fig. 2), but there was a marked increase of synovial tissue thickness of the lining layer in RA (Fig. 1). Thus,
VEGF levels in RA serum may be increased as previously reported [24]. In an earlier study, Ikeda et al. reported VEGF165 was expressed in 41% of RA samples (17 patients) but not in OA samples (8 patients) using reverse transcription-PCR[49]. They also found NRP1 was up-regulated in RA synovial tissues. However, we did not observe significant alterations of VEGF-A (Figure. 2B) or NRP1 (Figure. 2C) between OA and RA specimens. Kim et al. reported that NRP1 expression was similar in OA and RA using immunohistochemical analyses [50]. These discrepancies might be explained by the difference in sampling numbers, disease duration of the populations studied, extent of inflammation, use of anti-rheumatoid drugs, and/or by different methods for analysis of NRP1 expression levels. Additional investigations in a large sample considering several different conditions are required to clarify these discrepancies.