Author’s response to reviews

Title: REGULATION OF SOMATOSTATIN RECEPTOR 2 BY PROINFLAMMATORY, MICROBIAL AND OBESITY-RELATED SIGNALS IN PERIODONTAL CELLS AND TISSUES

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Author’s response to reviews:

We are very grateful to the reviewers for their valuable time they spent on reading our manuscript and the critical and constructive comments. We have addressed all issues and made great efforts to improve our manuscript as recommended.

Sincerely yours

Dr. Svenja Memmert
Reply to Professor Dr. Elston (reviewer # 1)

1. Concern of the reviewer: In this study the authors describe increased SSTR2 mRNA and protein expression in diseased human gingiva compared to healthy patients, and increased SSTR2 mRNA and protein expression in cultured fibroblasts in response to IL-1beta, F. nucleatum, as well as the adipokines, leptin and visfatin. This was followed by a rat study also confirming SSTR2 increases in response to periodontitis (ligature induced), diet-induced obesity compared to control animals. The paper is well-written and the experimental protocol appears appropriate and the results consistent across the different experiments performed.

Minor comments

I note the age of the periodontally-diseased patients was quite different from the healthy donors but presume it was not possible to obtain tissue from a more closely matched group to the healthy donors.

Our response: First of all, we thank the reviewer for her positive comments and the valuable time she spent on reading our manuscript. The ages varied between the samples because, due to ethical reasons, only tissue was taken that would have otherwise been discarded. Healthy human gingival specimens were obtained during wisdom tooth removal or tooth extractions for orthodontic reasons, procedures mostly done in more youthful patients. Inflamed periodontal tissue was obtained during tooth extractions for periodontal reasons at the site of periodontal destruction, a treatment which is more frequent in older patients. Therefore, it was not possible to match the groups more closely as already mentioned by the reviewer.

2. Concern of the reviewer: From this work it doesn't appear that the increase in SSTR2 expression is just due to an increase in inflammatory cells in the diseased tissue. It would be interesting as the logical next step to see whether blocking and stimulating SSTR2 alters the disease process but this is outside the scope of this paper. Similarly assessment of the roles of the other 4 SSTRs.

Our response: We thank the reviewer for this constructive suggestion and it would indeed be interesting to perform blocking and stimulating experiments for SSTR2 in periodontal cells and tissues. We agree that more research regarding the SST/SSTR system including the roles of other SSTRs in periodontal cells and tissues is needed.

We have included the constructive comments of the reviewer and added the following statements to the Discussion section:
“Therefore, future studies should also focus on the actions of SST and on the roles of other SSTRs in periodontal cells and tissues.”

“Moreover, further studies should be dedicated to the intracellular signaling pathways, which are involved in the observed SSTR2 upregulation.”

Reply to Dr. rer. nat. Sielker (reviewer # 2)

1. Concern of the reviewer: In their study „Regulation of somatostatin receptor 2 by proinflammatory, microbial and obesity-related signals in periodontal cells and tissues“, the authors examined expression of somatostatin receptor 2 (SSTR2) on gene and protein level in 4 primary human periodontal ligament (PDL) cell lines after adding IL-1ß, by ultrasonication inactivated Fusobacterium nucleatum, and as obesity-related factors, leptin and visfatin. They observed a significant higher expression of SSTR2 compared to control and also dose dependent expression rate. In a second step, authors analysed gingival biopsies of periodontally healthy donors and periodontally-diseased patients. Here they also observed a higher expression rate of SSTR2 on gene and protein level. In an experimental periodontitis rat model, gene expression of SSTR2 was analysed. At four time points (6d - 8d - 12d - 20d) gene expression of SSTR2 was analysed in gingival tissue of rats with an artificial periodontitis compared to healthy rats. Authors found a significantly higher expression of SSTR2 after 12 days. Further gene expression of SSTR2 was analysed in rats feed with a high fat / high glucose diet compared to a control group. Authors found a significant higher expression of SSTR2 in the obese group. On the basis of this study, authors suggest that SSTR2 plays a critical role in the aetiopathogenesis of periodontitis. The subject of this work is interesting and relevant for scientific community. However, some issues must be addressed by the authors so that the study matches the scientific accuracy for publication.

Methods and Materials Culture and Treatment of cells / site 4 & 5:

IL-1ß, F. nucleatum, leptin, and visfatin were added to culturing media of PDL cells. How long was the incubation time with the added factors? In manuscript, only reference to previous studies was indicated. This straight information supports comparability with similar studies from other groups.

Our response: We are very grateful to the reviewer for reading our manuscript and assessing it in such a positive and detailed manner. We understand the reviewers concern and added the following information from the Figure legends to the text body in the Material und Methods section under “Culture and Treatment of Cells”: 
“PDL fibroblasts were exposed to the different stimulants for 1 d. Untreated cells served as control.”

2. Concern of the reviewer: F. nucleatum was inactivated by ultra-sonication. Authors observed none or slightly effects in gene expression of SSTR2 with different amounts of F. nucleatum. How was guaranteed that no cells survived this treatment?

Our response: Inactivation of bacteria was checked by subcultivation on Schaedler agar plates in anaerobic conditions. This statement was added in the Material and Methods section under “Culture and Treatment of Cells”.

3. Concern of the reviewer: Are there well-established information about concentration of pathogenic factors / cell debris of F. nucleatum and intense of immune response? Does the chosen concentration fit in it?

Our response: The concentration of F. nucleatum was about 10^7 in the experiments performed. Thus we had a MOI of 1:50, which is in the range of concentrations described by other research groups and showed a respective immune stimulation by PDL fibroblasts (Ahn et al., 2016; Lee et al., 2014; Yun et al., 2018). F. nucleatum ATCC 25586 used at this concentration has been demonstrated to significantly enhance the mRNA expression of cyclooxygenase 2 via toll-like receptors, as shown by our previous experiments (Nogueira et al., 2014).”


4. Concern of the reviewer: Regulation of SSTR2 Protein Levels by Interleukin-1ß, F. nucleatum and adipokines / site 9 & figure 2:

It is difficult to interpret figure 2. In chosen presentation differences between groups and control are weak and red staining of SSTR2 positive cells seemed to be washed-out. Is it possible to make the presentation more distinct? Authors observed strong differences between concentration of added factors and gene expression of SSTR2. Given information of protein expression of SSTR2 disallow a transfer between results of gene and protein expression. Was there also a provable correlated increase in protein expression?

Our response: We agree with the reviewer, that immunoreaction to SSTR2 is less strong than expected according to mRNA results. Nevertheless, it is pronounced and consistent. Especially in the pictures with higher magnification, the immunoreaction of the stimulated cells is undoubtedly stronger as compared to control cells. It is difficult to compare the changes in quantitative mRNA data to non-quantitative results obtained from immunocytochemistry. However, both, expression data and protein results, demonstrate an increase in SSTR2 expression after stimulation. We further agree with the reviewer, that the differences between the groups at protein level can hardly be assessed statistically. We did not want to enhance the visibility of our findings by software correction. Therefore, we would recommend keeping the figure file in the present form.

5. Concern of the reviewer: Regulation of SSTR2 in human and rat gingival biopsies/ site 9 & figure 3:

It seemed that SSTR2 is expressed in healthy tissue (figure 3b). Is this true or just interference of presentation? If yes, is it possible to calculate a basic expression level of SSTR2 in healthy tissue? If yes, does increased gene expression correlate with protein expression?

Our response: As correctly stated by the reviewer, SSTR2 is continuously expressed in healthy tissue. Nevertheless, as mentioned above it is hard to compare quantitative mRNA data to non-quantitative results. We added the following statement to the Results section under Regulation of SSTR2 in human and rat gingival biopsies:

“In parallel, a stronger immunoreaction to SSTR2 was found in inflamed biopsies as compared to the control tissues, but control tissues also showed weak but continuous immunoreaction to SSTR2 as expected (Fig. 3b).”

Furthermore we clarified the distribution of SSTR2 in the Background section:

“SST can bind to five ubiquitously distributed G protein-coupled receptors (SSTR1-5), which mediate the aforementioned effects of SST [26]”
6. Concern of the reviewer: Authors observed an increased gene expression of SSTR2 in rats with a high fat / high glucose diet compared to rats with a normal diet. Was there also periodontal alteration observable in these rats? If yes, what kind and strength of alteration?

Our response: We are grateful to the reviewer for giving us the opportunity to address this issue. In this context a recently published paper from our group was added to the manuscript. As in the present study diet-induced obesity was established in Wistar rats. Although metabolic and inflammatory changes where observed systemically, no changes were determined in the periodontium. Usually, to see periodontal changes, experimental periodontitis has to be induced, which would be aggravated in an obesity model


7. Concern of the reviewer: Authors studied expression of SSTR2 and its critical role in aetiopathogenesis of periodontitis. For this, human periodontitis biopsies and gingiva biopsies from rat models were analysed and compared. To identify stimulating factors, a cell culture model with PDL cells was chosen. Why not human gingiva fibroblast? Authors should comment this.

Our response: The reviewer raises a very good point. PDL fibroblasts play an important role in periodontal destruction and regeneration. In addition, our previous publications in the context of periodontal infection and obesity mainly involve PDL fibroblasts. Moreover, PDL cells are able to attain different cell types dependent on cell treatment. They can attain fibroblast like, cementoblast like and osteoblast like characteristics. Since we have not applied osteogenic media to the PDL cells they showed a fibroblastic phenotype, which corresponded very well with the gingival tissue biopsies. But gingival fibroblasts will be a very interesting cell type for future studies. Therefore, we added the following statement to the Discussion section.

“Additionally, further studies should analyze the role of the SST/SSTR system in different periodontal cells like gingival fibroblasts.”

8. Concern of the reviewer: Site 10: … Like in our experiments, the increased mRNA levels were paralleled at protein levels, as analyzed by immunocytochemistry…Given information of protein expression in this study are not quantifiable. A conclusion of this kind is with the given information not possible.
Our response: We agree with the reviewer and changed the manuscript accordingly.