Title: Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1

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Author's response to reviews: see over
"Increased endothelin-1 and diminished nitric oxide levels in blister fluids of patients with intermediate cold type complex regional pain syndrome type 1"

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Editorial Board comments:
The different sites of sampling in the affected versus the contra lateral limb, and lack of data from healthy matched subjects, raises concerns about the design and interpretation of the study.

As can be seen in the modifications to the Material and Methods section of our revised manuscript, artificial blisters induced by means of a suction method have been used in dermatological studies for more than 17 years, and this method has been adapted for research by our research group since 2000 [9, 17, 24, 26, 27], and it is described in recent proteomics research [25].

Up till now no reference data from healthy subjects are available from our group.

In the Methods we also explain that these different blister sites had been used in previous studies by our research group to reduce the inconvenience for the patient. [5, 9, 17, 22]

We used identical locations in order to enable a comparison with these previous data [28, 29].

Responses to referee 1’s comments:

In Abstract section:

1 - The introduction is long. It should be shortened.

The Introduction section has been rewritten and considerably shortened.

2 - Conclusion paragraph:

“Both IL-6 and TNF-α appear to be representative markers of disease activity during the initial stage of CRPS1.

During the course of the disease, however, the NOx / ET-1 ratio could be a worthwhile indicator of vascular involvement of the disease.”

These conclusions cannot be drawn from the results of this study. Because:

I - Authors did not perform any correlation analyses between disease activity and IL-6 or TNF-α in this study.

II - All the patients included in this study were in the intermediate cold phase between the acute inflammatory stage and the early atrophic stage. The measurements were done only once. If the measurements had been done in all stages of CPRS1, it might be suggested that during the course of the disease, the NOx / ET-1 ratio could be a worthwhile indicator of vascular involvement of the disease.

These conclusions have been removed from the revised paper.

In Introduction section:

The Introduction should be shortened.

The Introduction has been considerably shortened.

In Materials and Methods section:

2.1. Subjects

1 - The number of CRPS1 cases included in this study was small.
We agree with the reviewer that the number of CRPS cases was small. The incidence of CRPS 1 is only 5100 / year in the Netherlands, and we agreed to exclude all patients that do not 100% comply with the Bruehl diagnostic criteria for CRPS [3]. As can be read in the paper by Munnikes et al. [29] only 66 patients with CRPS were seen at the Pain Treatment Center of the Erasmus MC in the period between April 2001 and February 2004 . In their study on Inflammatory Cytokines in intermediate CRPS 1 only 25 patients could be included.

2. “…mean age 48 ± 11.3…” and “…mean duration of their disease of 2.8 ± 1.42…”
The expression of mean ± SD should be the same format. For example,
2.8 ± 1.42 is not valid, 2.8 ± 1.4 is valid.
This has been corrected in the revised paper.

2.2. Blister fluid collection
1- “A 3-hole (5 mm diameter per hole) skin suction chamber was positioned on the skin of the upper extremity, on the dorsal side of the involved hand and the flexor side of the contralateral forearm.”

Why was skin suction chamber not positioned on the same skin side of the both upper extremities?
As stated in the covering letter: “The measurement of cytokines in artificial suction blisters has been used in dermatology research for more than two decades. Huygen et al. were one of the first groups to investigate cytokines in blister fluid in patients with Complex Regional Pain Syndrome, thus providing evidence for a local inflammation [5]. Their results have been confirmed in later studies [9, 17, 22, 28, 29], and formed the basis for the successful treatment of the acute form of CRPS with anti-TNF-α [17]. Their method of sampling directly on the affected skin, and comparing it with a site on the contralateral extremity that is less inconvenient for the patients, has been used in several subsequent studies. In the present study we adapted this method in order to be able to compare our data with these previous studies”.

2- Is the measurement of cytokines in suction blister fluid samples reliable? Artificial blister is associated with iatrogenic tissue injury. This injury can result in cytokine production, so blister fluid samples might be contain cytokines. Because of vasomotor changes, edema, trophic changes etc, structural properties of skin in involved extremity can be abnormal. That’s why it is possible that tissue response to injury might be more susceptible and prominent in skin of the involved extremity than uninvolved. For that reason, cytokine level in suction blister fluid samples obtained from the involved extremity can be different from that obtained from the uninvolved extremity. Reliability of the measurement of cytokines in suction blister fluid samples should be discussed.
The reliability of cytokine measurement in artificial blisters has been discussed in numerous articles in the last decade. For a recent article on the subject we recommend the article on the Multiplex Assay from the Erasmus MC research group [28].

2.6. Statistical analysis
“The Wilcoxon signed ranks test was used for comparisons between measurements in blister fluid obtained from the CRPS1 and the contralateral extremity.”

If the extremities which measurements were done in blister fluid obtained from was the same, the Wilcoxon signed ranks test could be performed. Because the measurements were done in the different (CRPS1 and the uninvolved) extremities, the Mann-Whitney U test should be performed.
After consultation with our statistician, Dr. Dirk Stronks (Pain treatment Center, Erasmus MC) we would like to suggest another viewpoint. We are dealing here with dependent observations
measured in the same individuals, so the Wilcoxon matched pairs signed rank sum test must be considered as the adequate statistical test.

In Results section:
1- The total number of dots representing cases in scatter plots is inconsistent with the number of cases. For example, although 28 patients were included in this study, the total number of dots is 29 for IL-6 in Figure 1a, is 29 for TNF-α in Figure 1b. Although ET-1 concentrations were determined in paired blister fluid samples which were available for 24 patients, the total number of dots is 22 in Figure 2a. There was an error in the original paper. Initially this paper had been written as a paper combining the ET-1/NO results and a pilot with 5 patients receiving treatment with ISDN. This paper was rejected, and the reviewers suggested that we separate the two subjects, which we did. The statistics and the figures in the submitted paper were calculated using the correct numbers, but a mistake was made in describing the numbers in the Results section. This has been corrected in the revised paper.

2- Information that appears in the Results should not be repeated in detail in the legends of Figure 1 and 2. This extra information has been removed in the revised paper.

In Discussion section:
1- The Discussion should be shortened. The Discussion section has been considerably shortened.

2- The disease activity was measured in this study but not discussed.

In the Materials & methods – Subjects section “The mean disease activity of the patients was 35 on a scale of 0-100, calculated using the impairment sum score according to Oerlemans [32], representing a low-medium disease activity.”

In the Materials & Methods - Measurements of cytokines section “In order to determine the contribution of pro-inflammatory cytokines to the disease activity, both IL-6 and TNF-α were determined.”

In the Results, last paragraph “Significant correlations between IL-6, TNF-α and ET-1 were found (0.79, 0.44 and 0.67 for IL-6/TNF-α, IL-6/ET-1 and TNF-α/ET-1 and p-values of p<0.001, p=0.039 and p=0.001 respectively).” In the Discussion a new subsection has been added addressing the disease activity expressed as Impairment Sum Score in relation to proinflammatory cytokines.

3- In discussion, last paragraph, what is mean of “ISDN”? This line has been removed in the revised paper.

In Conclusion section:
The authors attempt to stretch their results well beyond the scope of the study. Following conclusions cannot be drawn from results of this study. Because:
1- “The stage of the disease, the acute or inflammatory phase, or the chronic and/or trophic phase, determines the involvement of inflammatory cytokines which could promote ET-1 production, leading to vasoconstriction and consequently to a diminished tissue blood distribution.”

All the patients included in this study were in the intermediate cold phase. And this was a cross-sectional study. The measurements were done only once. If the measurements had
been done in all stages of CPRS1, it might be suggested that “The stage of the disease, the acute or inflammatory phase, or the chronic and/or trophic phase, determines the involvement of inflammatory cytokines …” A longitudinal study could address this question further.  

*We entirely agree with the reviewer. This item has been removed from the Conclusion section and is addressed in the Discussion section. A longitudinal study as suggested is being prepared.*

II- “This process could be amplified by disuse. Therefore, both exercise and prolonged vasodilation induced by NO-donors could result in an improved blood distribution being the first step in remission of this severely invalidating disease. Therefore, both exercise and prolonged vasodilation induced by NO-donors could result in an improved blood distribution being the first step in remission of this severely invalidating disease.”

In this study, hand disability of the patients was not evaluated and it was not showed whether any effect of disuse on the production of both ET-1 and NO could be.  

*These lines have been removed in the revised paper.*

**Responses to referee 2’s comments:**

**Introduction**

On page 3, 1st paragraph, the authors state that “Central sensitization leading to exacerbations of pain is thought to be the result of neuroimmune activation of cells in the peripheral nervous system [8]”. However, reference [8] concludes that the data from the study is consistent with studies that suggest that the pathogenesis of CRPS is due in part to central (not peripheral) neuroimmune activation. Please change or use a different reference.  

*The reference has been changed to [5].*

On page 4, 3rd paragraph, the authors state that “It has been proven that the initially produced pro-inflammatory cytokines tumour necrosis factor alpha (TNF-a) and interleukin-6 (IL-6) play an important role in the initial and the intermediate phase of the disease [10, 23]. The word proven may be too strong a term since it does not allow for doubt, shown may be a better choice. In the following sentence the authors state that “These cytokines could counteract with the successively produced vasoactive mediators ET-1 and NO [24, 25].” As written, the sentence does not make sense. In addition, references 24 and 25 do not deal with interactions between IL-6, TNF-alpha and NO, ET-1. Once restated, the sentence must be properly referenced.  

*The word ‘proven’ has been corrected as suggested. The following sentence has also been changed, and the former reference nr. 25 has been deleted. The former reference nr 24, which is the paper by Alonso and Radomski [38] however, does contain information about the interaction between IL-6, TNF-alpha and NO, ET-1. We quote from Alonso and Radomski, The Nitric Oxide –Endothelin-1 Connection on page 109: “Furthermore, TNF-a has been shown to decrease eNOS mRNA levels by increasing the rate of mRNA degradation.” In the picture on page 111 the influence of ‘inflammatory mediators’ on the generation of ppET-1 is shown. The reference of Yoshizumi et al. [19] has been added to the revised paper.*

On page 5, the paragraph starting with “The therapeutic potential…”, needs a lead sentence in order to interpret the rest of the paragraph.  

*This paragraph has been deleted in order to shorten the paper.*

**Discussion**

Near the top of page 12, the sentence “In our observations an inverse relationship exists between increased ET-1 and diminished NOx in CRPS versus contralateral blister samples” is unclear. Please reword.
This has been changed to read “We found an inverse relationship between ET-1 and NOx in blister samples”. (page 10)

On the top of page 13, the sentence “From animal studies it could be learned that endogenous ET-1 participates in the redistribution of tissue blood flow [45]” should be reworded to “From animal studies it has been learned that endogenous ET-1 participates in the redistribution of tissue blood flow [45]” since reference [45] supports the allegation. This sentence has been corrected as suggested in the revised paper.

The last sentence on page 13 states that “Tumor necrosis factor-a (TNF-a) counteracts the activation of eNOS, whereas induction of iNOS in smooth muscle cells will be stimulated to generate NO [25]”. Reference [25] supports the last part of the sentence. However, reference [25] does not deal with TNF-alpha’s effect on eNOS. Please provide an additional reference to support the allegation. The reference of Yoshizumi et al. [19] has been added.

Conclusion

The first sentence should be reworded. First, “NO en ET” should be “NO and ET”. Second, “Without any doubt” is too strong a term. An alternative choice would be “There exists in the vascular system clear crosstalk between the NO and ET systems”. This has been changed as suggested.