Author's response to reviews

Title: Chlamydia trachomatis antigens in enteroendocrine cells of the small bowel in patients with irritable bowel syndrome

Authors:

Aldona Dlugosz (aldona.dlugosz@karolinska.se)
Hans Törnblom (hans.tornblom@karolinska.se)
Ghazaleh Mohammadian (ghazaleh.mohammadian-kermani.573@student.ki.se)
Gareth Morgan (gareth.morgan@ki.se)
Béla Veress (veress.bela@hotmail.com)
Benjamin Edvinsson (benjamin.edvinsson@smi.ki.se)
Gunnar Sandström (gunnar.sandstrom@smi.ki.se)
Greger Lindberg (greger.lindberg@ki.se)

Version: 2 Date: 3 November 2009

Author's response to reviews: see over
Dear Sir/Madam,

We are indeed grateful for the opportunity to re-submit our manuscript # 1706188665297073 which has been re-titled “Chlamydia trachomatis antigens in enteroendocrine cells and macrophages of the small bowel in patients with severe irritable bowel syndrome”.

These are our point-by-point reply to the comments and questions of the reviewers:

Reviewer #1.
There is some anxiety of the part of this reviewer about the methods. How did the authors get full thickness biopsies on so many IBS patients. Besides the ethical risks of such a procedure, who would agree to such a study. Agreeing to such invasive testing must have affecting the type of subject seen. Were these severe subjects. I would argue that they must have been severe since 2 had deficient actin staining which is sometimes attributed to visceral myopathy (not IBS). This may result in pseudoobstruction. Another suggestion that they were severe is the high rate of small bowel abnormalities consistent with enteric dysmotility. Can the authors be more specific? We need to know a lot more about these patients.

Reply: We agree that this is important, and it is correct that all our patients had severe symptoms of IBS. This information is already in the manuscript on page 5. It also contains a reference to ref #5, which details what we mean by severe IBS. This is a retrospective study of previously collected biopsy material (refs #5, #16, #17). At the time of biopsy, all patients had consented to biopsy taking and we did not take any new full thickness biopsies for this particular study. We have added a sentence about this under Ethical considerations on page 9. IBS was diagnosed according to symptom criteria and the presence of motor disturbances such as enteric dysmotility does not preclude a symptom-based diagnosis. We have not systematically investigated patients with IBS using small bowel manometry but there are data from other centres that indicate that a large proportion of IBS patients have enteric dysmotility, e.g Simrén et al. (Dig Dis Sci. 2000 Nov;45(11):2151-61) reported measurable dysmotility in 38%. In our material 40% of patients had enteric dysmotility.

Also, their sexual behaviors. Why would they have Chlamydia trachomatis in their GI tract?

Reply: Unfortunately, we do not have any information about sexual behaviours. At present we don’t know if genital infections are relevant or not in this context but since we found chlamydial antigens in macrophages we cannot exclude such a possibility. It is assumed that spread via macrophages may explain arthritis after genital infections.

Obviously these were not consecutive IBS patients given the proposed procedure of laparoscopy. How many patients were approached to get this number to consent? It might suggest how representative their population is. This is really important because this may not be applicable to IBS as a whole but only patients severe enough to do an invasive study like this.
Reply: We agree with these comments but since this is a retrospective study of archived biopsy material it is difficult to answer the question about the number of patients that were approached for biopsy. The material comes from different clinical and research series. All patients in our study had, as stated above, severe symptoms of IBS. This study cannot ascertain the applicability of results. We need to do a prospective study in a less selected population of IBS patients to answer that question. We have stressed the limitation of our study material to patients with severe IBS in the title and in the discussion.

I am very familiar with the previous work by this group but it is puzzling how they even came across the idea of looking for this. It might be helpful to know more specifically why they chose this since the rationale is very subtle in the introduction.

Reply: Since we had observed low-grade inflammation in mucosa and enteric ganglia we began exploring the possibility of an underlying persistent infection in the gut as a driving force for observed immune activation. We presumed that candidate bacteria should be compatible with an asymptomatic carrier-ship, have a preference for female gender, have the ability to become persistent and to live in bowel epithelium. Chlamydia trachomatis is an obligate intracellular pathogen with all these abilities. We have added a sentence about this in the Introduction page 3.

Back the results. The controls are odd also. Why so many obese subjects. Was the full thickness biopsy part of a bariatric procedure? How obese were they? I think since they brought it up, it might be good to see some demographic comparisons between IBS and controls such as BMI, age, etc.

Reply: We had 10 obese but otherwise healthy patients among our controls. It is correct that these subjects were biopsied during bariatric surgery. This information is in Methods page 6. This is so far the only control group in which functional bowel disorders could be excluded using the same methods as in healthy volunteers, and in whom we could take full thickness biopsies without additional risk. We have amended methods with data on BMI on these subjects. No patients or other controls had obesity.

I am confused about how the authors decided that the staining was positive. I know the readers were blinded (although I did not find out till the results that this was the case-this needs to be in the methods), but was one EEC with LPS considered positive or did they need more than one. I suspect any detectable staining was considered positive. This should be stated explicitly.

Reply: We are grateful for this question. We have amended Methods with our definition of positive staining (Page 7).

With regards to the 4 subjects that were previously positive and some time later re-biopsied, the authors state they were negative. In the discussion they suggest a possibility that over time the Chlamydia infection or affect may wane. This does not make sense. In the methods, the authors already state that the average duration of IBS
was 6.5 years. If C. trachomatis is important in IBS, why would it clear in 1 year and not clear in 6.5 years? This makes no sense at all.

Reply: We are sorry that we have confused the reader. New biopsies were taken from 4 patients who were positive for LPS and MOMP in previously taken biopsies. The new biopsies were also positive for LPS, and this was shown both with immunofluorescence and Western blot. However, the new biopsies were negative for MOMP, both in immunofluorescence and Western blot. (Page 11 and Table 1). In Discussion we discuss possible reasons why some patients can be or become MOMP negative (Page 14). The expression of MOMP can be downregulated in the persistent state and disappearance of MOMP expression is not the same as disappearance of infection.

Did the authors do PCR on all the original specimens or only on the 4 subjects stated in the last paragraph. This needs to be done in a larger group.

Reply: We agree that PCR needs to be done on a larger and prospective series. We did not attempt doing PCR on archived formalin fixed material. Chlamydia infection in persistent state is very difficult to prove with nucleic acid amplification techniques. There is no gold standard for PCR on Chlamydiae in tissue. There are many problems both regarding extraction and amplification methods. See for example Apfalter et al., J Clin Microbiol 2001;39(2):519-24. As stated on page 8 and page 11 we did PCR only on the new biopsies from 4 patients.

Maybe the LPS they are detecting is from other bacteria. Perhaps their assay is cross-reacting with the many intestinal bacteria that produce various forms of LPS. Other LPS antibodies should be tested before declaring C. trachomatis the main organism.

Reply: We have discussed the possibility of cross-reacting antibodies and also the reasons why we think this is an unlikely explanation to our findings (Page 12). We used two different genus specific antibodies (one monoclonal and one polyclonal) (Methods page 6) against Chlamydia LPS with similar findings. We used a species-specific monoclonal antibody for a membrane protein of C. trachomatis. A cross reacting antigen would therefore have to mimic not only Chlamydia LPS, but a membrane protein as well.

Were there differences between subgroups of IBS? It seems you might have enough numbers to look at D-IBS vs C-IBS, etc.

Reply: It is correct that we had patients from different subgroups of IBS. However, there was no difference in the prevalence of LPS positivity between the three groups and 19/21 with D-IBS, 22/22 with C-IBS, and 17/22 with A-IBS were LPS positive. This information has been added to Results on page 11.

Reviewer #2
Discretionary revisions
I would prefer a table or figure demonstrating the main finding of the article (number of C. trachomatis positive patients and controls, and statistics), which is well described in
the first section of “results”. This may very well be complemented by the current figure 1, showing examples of specific stainings.

Reply: We agree, but only repeating data from the text doesn’t in our view merit another figure. Also, univariate statistics would only add confusion, we think. The p-value for the univariate comparison of LPS positivity between patients and controls yielded a p <0.0001.

It is very difficult to see the arrows described in the figure legend (are there really any?) in figure 1.

Reply: We are sorry for having submitted a set of slides without the arrow layer. The correct set of slides for Figure 1 has been amended to the revised version of our manuscript.

The word macrophages might perhaps be included in the title of the paper?

Reply: We are grateful for this suggestion and we have added the word "macrophages" to the title.

The word “MOMP” needs an explanation in the abstract.

Reply: The meaning of the abbreviation MOMP has been added to the abstract.

Reviewer #3.
I read with great interest the paper by Dlugosz and coll. It reports the novel finding of chlamydial antigens in enteroendocrine cells and macrophages of the small bowel mucosa in patients with IBS. The limit of the study (it was unable to prove the presence of viable microorganisms) is clearly indicated. For this reason furthers studies to clarify the possible role of such antigens in the pathogenesis of IBS are strongly required. However the paper is well written, the results are novel and interesting.

Reply: Thank you!

For the authors
Greger Lindberg, MD, PhD