Author's response to reviews

**Title:** Anti-inflammatory effect of rosiglitazone is not reflected in expression of NFκB-related genes in peripheral blood mononuclear cells of patients with type 2 diabetes mellitus

**Authors:**

Marjolijn C.E. Bragt (m.bragt@hb.unimaas.nl)
Jogchum Plat (j.plat@hb.unimaas.nl)
Marco Mensink (marco.mensink@wur.nl)
Patrick Schrauwen (p.schrauwen@hb.unimaas.nl)
Ronald P. Mensink (r.mensink@hb.unimaas.nl)

**Version:** 3  **Date:** 14 January 2009

**Author’s response to reviews:** see over
Dear Mr. Todd,

Please find enclosed our revised manuscript entitled “Anti-inflammatory effect of rosiglitazone is not reflected in expression of NFκB-related genes in peripheral blood mononuclear cells of patients with type 2 diabetes mellitus”, which we would like to re-submit for publication in BMC Endocrine Disorders.

We would like to thank the reviewers for the critical and useful comments regarding our manuscript. The questions raised are discussed point-by-point below.

Yours sincerely,

Marjolijn C.E. Bragt
Reviewer's report

Title: Anti-inflammatory effect of rosiglitazone is not reflected in expression of NFκB-related genes in peripheral blood mononuclear cells of patients with type 2 diabetes mellitus
Version: 2 Date: 4 December 2008
Reviewer: Paresh Dandona

Reaction of authors on reviewer's comments:
1. MNC may not have shown the suppression of NFκB dependent genes but NFκB is suppressed by all TZDs. It is possible that the monocyte needs to be looked at specifically since it is central to the mechanisms of innate immunity and is also the cell that ‘takes’ inflammation to the atherosclerotic plaque and the adipose tissue and possibly to the skeletal muscle where it triggers and sustains inflammation.

   - We agree that monocytes may be highly relevant. However, because Mohanty et al. have shown earlier that roziglitazone suppressed NFκB in Peripheral Blood Mononuclear Cells (PBMCs) isolated from humans, we were specifically interested to evaluate if this was also reflected in changes in expression of NFκB-related genes in PBMCs. Since monocytes express PPARγ at higher levels than the other cell types in the PBMC mixture and, in addition, play an important role in inflammatory processes, it would have been very interesting to specifically look at monocytes in vivo in this respect. Therefore, we have raised the point in the discussion that it warrants further investigation if specified cell populations from the PBMC fraction are more suitable to study in vivo (page 15, line 315-318). We have now mentioned monocytes, preferably isolated from blood via cell sorter techniques to minimize the possibility to artificially change monocytes into macrophages, more specifically in this respect (page 15, lines 318-320).

2. From the point of view of the pathogenesis of insulin resistance, the deletion of IKKβ in myeloid precursors which include monocytes protects animals from diet induced obesity related insulin resistance (Karin). Thus, the monocyte is clearly central to inflammation related insulin resistance. Since MNC fraction includes not only monocytes (20%) but also lymphocytes (80%), it is important to look for changes in pro-inflammatory cytokines and mediators in pure monocytes. In the meantime, we can accept that the MNC do not give us a complete and precise picture.

   - Indeed, rosiglitazone did not exert effects on gene expression levels in PBMCs of type 2 diabetic subjects. The important role of monocytes in inflammation and insulin resistance makes these cells very interesting to look at more specifically. In the discussion, we now elaborate more on the study of monocytes regarding their role in inflammation and diet-induced obesity-related insulin resistance and refer to the study by Karin et al. (page 15, lines 320-322)

3. The authors should provide a complete list of the genes which were tested so that future investigators do not waste their time testing for those genes.

   - We have added the list of genes that were tested with the NFκB RT²Profiler PCR array as an additional file and refer to this table in the method section of the article (additional file 1) (page 8, line 152). In this table, we also indicate which genes could not be detected in PBMCs.

4. As far as their conclusions from the hyperinsulinemic-euglycemic clamps are concerned, like others, they assume that when they infused patients with insulin while co-infusing glucose to maintain euglycemia, that only insulin is exerting a biological effect. With the amounts of glucose usually infused during such a clamp glucose can exert both oxidative and inflammatory stress. 75g of orally administered glucose to normal subjects induces an
increase of ROS generation by MNC by 140% over the basal and an increase in NFκB binding by over 50% although the blood glucose concentration remains within the normal range. The anti-inflammatory effect of insulin was demonstrated with low dose infusions of insulin (2U/h) and 5–6g/h of glucose to maintain euglycemia (Dandona et al., JCEM, 2001; Chaudhuri et al., Circulation, 2004). It should also be noted that it takes longer than 10h of insulin infusion to demonstrate a fall in CRP concentrations (Chaudhuri et al, 2004; Wong et al, Diabetes Care,2004; Visser et al, Br J Anaesth, 2005; Kosenkari et al, Acta Anaesth Scand, 2006).

- We agree that the effects of glucose infusion while maintaining euglycemia during the hyperinsulinemic-euglycemic clamp should not be neglected. Even if levels are in a steady-state, the flux increases and may be different between subjects. Therefore, we now elaborate on the fact that glucose may still exert a biological effect, although levels were kept within the normal range (page 16, line 340-342). To emphasize the potential anti-inflammatory effects of insulin alone, we have now referred to a study of the reviewer (Dandona et al, JCEM, 2001) in the discussion of our manuscript (page 16, lines 342-349). In this study, the effects of insulin can be seen separately from glucose and insulin still exert potent anti-inflammatory effects at physiological concentrations. As an explanation for the fact that we did not observe reduced hsCRP concentrations upon insulin-stimulation, we have now mentioned in the discussion the relative short infusion period compared with studies that did find reductions in hsCRP (page 16, lines 337-340).

5. Please cite the work of Dhindsa et al (JCEM, 2003) in relation to the effect of rosiglitazone on both TG and FFA clearance.

- We have now cited the work of Dhindsa (JCEM 2005) in relation to TG and FFA clearance as a possible explanation for the reduced fasting plasma TG and FFA concentrations as observed in our study after rosiglitazone treatment (page 13, lines 262-264).

Reviewer's report

Title: Anti-inflammatory effect of rosiglitazone is not reflected in expression of NFκB-related genes in peripheral blood mononuclear cells of patients with type 2 diabetes mellitus

Version: 2 Date: 17 November 2008
Reviewer: Niels Juel J Christensen

Reaction of authors on reviewer’s comments:
1. The real time PCR assay is not a quantitative assay. Results are based on the fold-response. The data cannot be expressed in amol or zmol.

- Indeed, only relative quantitative data can be obtained with real time PCR. Therefore, we have removed the word “quantitative” preceding real time PCR (page 8, line 144) and have added the word “relative” preceding expression levels in the method section (page 8, line 154).

2. Gene expression levels were not measured after stimulation with e.g. LPS

- It would indeed have been interesting to measure the effects of rosiglitazone on LPS-induced NFκB related gene expression. In a very recent study, Necela et al. (Immunology, 2008) showed that rosiglitazone inhibited NFκB-mediated inflammation in unstimulated macrophages. LPS stimulation, however, activated TLR4 and strongly
reduced PPARγ expression, which could not be overcome by rosiglitazone treatment. So, the anti-inflammatory effect of rosiglitazone could be observed in the basal state, but not the LPS-stimulated state. Of course, it could have been of interest to choose a cytokine for stimulation, more related to metabolic disorders / obesity, such as TNFα. Nevertheless, the main reason for us not to stimulate peripheral blood mononuclear cells (PBMCs) ex vivo with LPS is that we focused on the true in vivo situation to examine the suitability of circulating PBMCs to be used as marker for the in vivo situation. Regarding this comment, we decided to give more emphasize in the abstract and conclusion of the article to the fact that we investigated the vivo situation (page 2, line 26 and 43 and page 17 line 361).

3. Gene expression was only studied in the fasting state and not after culture.

- Ex vivo handling and in vitro culturing of PBMCs quickly result in the differentiation of monocytes into macrophages. These latter cells express a larger amount of PPARγ. Therefore it is possible that after culture a change in gene expression can be found. This may indeed be an explanation for the discrepant findings between our in vivo study with in vitro studies, as discussed (page 15, lines 307-311). As already emphasized under the 2nd remark of the reviewer, a major research question was to investigate if these results could also be demonstrated in an in vivo situation.

4. Finally, I believe that PPAR is mainly but not exclusively expressed on macrophages. It would have been preferable if the mononuclear cells had been studied after culture and after development of macrophages from monocytes.

- We certainly agree that it would have been very interesting to study NFκB-related genes and PPARγ target genes in response to rosiglitazone in a more specified subpopulation of cells, namely monocytes. Because monocytes express PPARγ to a larger extent compared to the other cell types in the PBMC mixture and play an important role in inflammatory processes, it would be of great interest if in vivo changes can be demonstrated when isolating these specific cells. Therefore, we also mentioned in the discussion that it warrants further investigation if specified cell populations would be more suitable to study in vivo (page 15, lines 317-318). We have now mentioned monocytes, preferably isolated from blood via cell sorter techniques to minimize artificially changing monocytes into macrophages, more specifically in this respect (page 15, lines: 318-322).

5. The fact that blood mononuclear cells per se do not express PPARγ responsive genes is of course of general interest.

- Indeed, the main aim of the article was indeed to demonstrate the effect of rosiglitazone on PPARγ responsive genes in PBMCs in vivo.

General adaptations to the manuscript and in response to comments from the assistant editor:

- Email addresses of all authors are included in the title page
- Abstract has been divided in a “background”, “method”, “results” and “conclusions” section.
- “Introduction” is replaced by “Background”
- A “conclusions” section has been added, as well as “Competing interests” and an “authors’ contribution” section
- The “acknowledgement” section has been completed
- The references were given the heading “References”.
- The revised manuscript has been adapted to the journal style as given in the template for BMC medicine journals
- Order of tables changed (table 2 and 3 switched) and are now in order that they are referred to in the text
A heading “Additional files” have been added to the manuscript, because an extra table has been inserted as “additional file 1” and referred to in the method section.

We declare that we all contributed to the design, execution, analysis of the study and writing this manuscript. Furthermore, all authors have seen and approved the final version of the manuscript. The data in this manuscript has not been published elsewhere and is not under consideration for publication elsewhere. We also declare that we have no conflicts of interest in connection with this paper.