Author’s response to reviews

Title: Differential effects of dietary protein sources on postprandial low-grade inflammation after a single high fat meal in obese non-diabetic subjects: A randomized acute clinical intervention study

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Author’s response to reviews: see over
Dear Sir,

Thank you for the positive and constructive criticism of our article entitled “Differential effects of dietary protein quality on postprandial low-grade inflammation after a single meal in obese non-diabetic subjects: A randomized acute clinical intervention study” by Holmer-Jensen et al. We have answered the issues point by point below. The manuscript has been revised as proposed.

All references to pages and line numbers refer to final manuscript – not mark-up manuscript.

Kind regards

Jens Holmer-Jensen

Referee 1:

1) The researchers saw differences in CCL5/RANTES and MCP-1. But are these really the best measures of postprandial inflammation? Why wasn’t hsCRP, SAA, PAI-1, Fibrinogen, TNF-a, IL-6, IL-8, ICAM-1 or VCAM-1 measured or shown?

1: We agree that low-grade inflammation in obese subjects is traditionally evaluated by measuring up-stream cytokines e.g. hsCRP, TNF-a, IL-6 and IL-8. However, only slight changes in postprandial concentrations of hsCRP (Tholstrup, 2010; Blackburn, 2008; Poppitt, 2008), TNF-a (Blackburn, 2008; Poppitt, 2008), IL-6 (Tholstrup, 2010), PAI-1 (Mortensen, 2010), fibrinogen (Mortensen, 2010) and iCAM-1/vCAM-1 (Rubin, 2008; Lundman, 2007) have previously been reported. Consequently, we did not expect these cytokines to show marked postprandial dynamics in our study and therefore we decided to primarily focus on down-stream cytokines in order to present novel data on postprandial low-grade inflammation. We did, however, measure IL-6, IL-8 and TNF-α, but more than 9% of the concentrations were below detection limit and no statistical analyses were consequently performed as stated at p 10 l 170-172.
2) The conclusion may be a bit overstated as the changes in chemokines (MCP-1 and CCL5/RANTES) were not shown to actually modify any other specific inflammatory molecule/pathway that was measured. The author’s saw differential modifications to two pro-inflammatory chemotactic protein levels, but not necessarily in the inflammatory molecules/pathways (TNF-alpha, neutrophil recruitment, macrophage infiltration) associated with these chemotactic changes.

3) The results state that there were ‘overall anti-inflammatory effects of whey’, but whey was the only protein that showed an increase in MCP-1 at any time point, which could also potentially make it the most pro-inflammatory protein as well (especially if you looked at 2hr AUCs instead of 4hr AUCs.) It would be helpful to pose these results in comparison to what happens with a normal mixed meal or an iso-caloric control meal without added protein. If those meals cause an increase in MCP-1 above that of whey then you could say whey has the potential to be anti-inflammatory.

4) The study says no participants took lipid lowering drugs. Were anti-inflammatory, immune modulating and insulin sensitizing medications also controlled for?

5) Not enough attention is paid as to why there are opposing effects of whey on MCP-1 and CCL5/RANTES and what they mean and also not enough attention is paid to what happens with a normal mixed meal or an iso-caloric control meal without added protein. If those meals cause an increase in MCP-1 above that of whey then you could say whey has the potential to be anti-inflammatory.

<table>
<thead>
<tr>
<th>2: The comment is well taken. We have now modified the conclusion to more clearly distinguish between the terms “risk marker” and “risk factor”. P 15 l 274-277 and p 16 l 281-282.</th>
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<tr>
<td>3: We agree. Throughout the manuscript we do not try to emphasize the effects of whey protein on postprandial low-grade inflammation. The comment “overall anti-inflammatory effects of whey” has been deleted. P 15 l 259-261.</td>
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<tr>
<td>4: Medical treatment was noted for all subjects at screening. No subjects took anti-inflammatory, immune modulating or insulin sensitizing medications. This is now mentioned at P 7 l 97-99</td>
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<tr>
<td>5: We are not able to explain the opposing effects of whey on MCP-1 and CCL5/RANTES. Other unmeasured metabolic effects of whey</td>
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paid to the potential for cod and gluten to suppress MCP-1.

6) Three of the proteins were heated before serving (casein, gluten and cod), while the protein that appeared to show the most unique dynamics in MCP-1 and CCL5/RANTES (whey) was not heated. It is possible that the heating process denatured some of the proteins in the casein, gluten and cod and was therefore responsible for some of the specific chemokine changes in response to those meals. If the whey protein was similarly heated it may have led to different results as heating milk has been shown to change the allergenic/inflammatory properties of some of its proteins (Rytkonen 2006). Jani Rytkönen. Effect of heat denaturation of bovine milk beta-lactoglobulin on its epithelial transport and allergenicity. Acta Universitatis Ouluensis Medica. University of Oulu, Finland. 2006. D 883, ISBN 951-42-8119-5, ISSN 0355-3221.

7) What is the measurement of protein quality as stated in the title? Is it based on amino acid composition or just different protein sources? It does not appear that the quality of the proteins is actually assessed in this article. The author’s may be better off saying ‘protein sources’ instead of ‘protein quality’

Other unmeasured metabolic effects of whey may be responsible for these interesting diverging results. Correlations have been tested to explain causality. However, it would be rather speculative to suggest underlying mechanisms other than what have been tested.

6: The impact of heat denaturation of dietary protein is indeed relevant. The meals were heated briefly to a maximum temperature of 65°C. However, heating of beta-lactoglobulin at even higher temperature (67.5°C) for a prolonged period (15 minutes) only caused minor denaturation of the protein (Bauer, 1998). A much faster rate of denaturation of beta-lactoglobulin is obtained at 78.5°C (Schokker, 1999) which underscores the time/temperature relationship needed to structurally alter dietary protein. Due to the low heating temperature and the short heating duration of our test meals, we do not find it likely to have significant impact on the test results.

7: Point taken. The phrase “protein quality” has been changed to “protein sources”. Thank you.

Manuscript title and P 3 l 15 and P 16 l 280
8) The 17 cytokines measured via multiplex assay should be mentioned in the methods section, not just in the results section.

8: All cytokines measured are now also mentioned in the methods section. P 10 l 149

9) The title of the paper fails to mention that subjects were fed a ‘high-fat’ meal. This information is important since it is not a normal macronutrient spectrum meal and the high saturated fat content is largely responsible for the induction of the postprandial inflammatory response.

9: The term “high fat meal” is now included in the manuscript title, abstract (p 3 l 6) and discussion (p 12 l 208).

10) It would be nice to know the normal 4 hour postprandial response of MCP-1, CCL5/RANTES and other markers in a non-protein control so we can see how much influence the protein has on the overall spectrum of change.

10: We agree. However, to have a control meal without protein would either make the control meal non-isocaloric or iso-caloric but compensatory higher in either fat or carbohydrate content compared to the other meals. In either way, the inclusion of a non-protein control meal would not adequately describe the influence of protein as the control meal would be markedly different from the other meals in aspects other than just the protein quantity. As normal meals consist of both carbohydrates, fat and protein we aimed at detecting differential effects within the protein fraction rather than detecting differences between macronutrients.

11) Since these were obese subjects who normally have chronic low-grade inflammation, it would be good information to mention if the baseline levels of the biomarkers measured were higher than normal levels to begin with.

11: It is the impression of the authors, that no valid reference levels of these inflammatory markers have been established. Only a within-study comparison between the obese subjects and a lean control group would provide a decent estimation of any potential increase in
12) Besides the researchers past experiments with these 4 proteins, it should be explained why each one was chosen to represent a variation in protein quality.

13) The sentence from lines 67-71 regarding postprandial inflammation seems to be a run-on sentence and is not very clear as to the point being made.

14) Minor grammar/wording issues on lines 45, 51-53, 119, 167, 229-233

15) The term ‘per se’ is used twice and is not necessary in either case (lines 68 and 203)

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Referee 2:

1. The main problem of this study is that it is a part of another study. There are missing informations: what about plasma lipid, glucose and insulin levels. These results should be given, since it has been cited in the results section lines 174-175 that there was a negative correlation between fasting plasma insulin and iAUC 240 min for insulin and triglycerides have now been added to table 2. We were not able to detect any correlation between early (240 min) postprandial lipaemia and the inflammatory markers. This has been clarified in the results.
correlation between insulin iAUc and CCL5/RANTES iAUC. Moreover, plasma lipid levels might have an impact on these results.

2) One of the problems of this study is the absence of a reference or control test meal (without proteins for example). The high fat test meal should be tested alone to be able to determine if the observed effect was due mainly to the fat content or the association of this high fat test meal with the different proteins.

3) The test meal is a high fat meal, containing 66% as energy; this should be cited in the abstract and in the discussion.

4) What was the rational to select the used 4 proteins?

2: We acknowledge the relevance of a control meal. However, to have a control meal without protein would either make the control meal non-isocaloric or iso-caloric but compensatory higher in either fat or carbohydrate content compared to the other meals. In either way, the inclusion of a non-protein control meal would not adequately describe the influence of protein as the control meal would be markedly different from the other meals in aspects other than just the protein quantity. As normal meals consist of both carbohydrates, fat and protein we aimed at detecting differential effects within the protein fraction more than detecting differences between macronutrients.

3: The term “a high fat meal” has been added to the manuscript title, abstract (p 3 l 6) and discussion (p 12 l 208).

4: As stated in the introduction (p 7 l 84-90), these four proteins carry different properties regarding postprandial hormonal, lipid and glucose responses. This has been demonstrated not only by our group but of several research teams who has now been cited more clearly (p ??). The differential postprandial responses of particularly insulin and lipids may potentially influence the postprandial inflammatory response. We do not know the specific amino acid composition of the proteins.
5) The discussion must be focused only on the given results.

5: The discussion has been revised to focus on the results presented (p 12 L 207-216 and p 14 L 239-246 and p 15 L 259-272). Moreover insulin data and triglyceride data are now provided in table 2.

Referee 3:

1. The definition of low grade inflammation is usually based on more classical markers of inflammation, i.e. Hs-CRP, IL-6, TN#. None of these is given in the paper. If available, at least fasting levels should be added. This would help characterizing the subjects.

1: We agree that low-grade inflammation in obese subjects is traditionally evaluated by measuring up-stream cytokines e.g. hsCRP, TNF-a and IL-6. However, only slight changes in postprandial concentrations of hsCRP (Tholstrup, 2010; Blackburn, 2008; Poppitt, 2008), TNF-a (Blackburn, 2008; Poppitt, 2008), IL-6 (Tholstrup, 2010), PAI-1 (Mortensen, 2010), fibrinogen (Mortensen, 2010) and iCAM-1/vCAM-1 (Rubin, 2008; Lundman, 2007) have previously been reported. Consequently, we did not expect these cytokines to show marked postprandial dynamics in our study and therefore we decided to primarily focus on down-stream cytokines in order to present novel data on postprandial low-grade inflammation. We did, however, measure IL-6, IL-8 and TNF-α, but more than 9% of the concentrations were below detection limit and no statistical analyses were consequently carried out as stated at p 10 L 170-172.

2. Page 7, lines 89-92: Subjects are categorized as non-diabetics. However, OGTT was performed only in subjects with

2: All subjects having a fasting plasma glucose from 6.1 mmol/l and higher were subjected to an oral glucose tolerance test and were excluded if the 2-h plasma glucose level was ≥ 11.1 mmol/L. This is
impaired fasting glucose, leaving the possibility that someone with normal fasting glucose could have a diabetic OGTT.

3. The authors report the association between postprandial changes in MCP-1 and CCL5/RANTES and insulin. This is certainly interesting and suggestive. To this respect, however, insulin postprandial data should be added in the paper.

4. Page 15, lines 259-260: the conclusion “…………whey protein might enhance the anti-inflammatory benefits ……” is not completely supported by the results, because of the divergent effects of whey protein on MCP-1 and CCL5/RANTES. As suggested in the text (Page 12, lines 210-211; Page 15, lines 254-255), reducing MCP-1 levels may be beneficial.

5. Background: it could be made more clear whether findings refer to animal or human studies.

6. Page 6, line 77: “In accordance” is not appropriate to the two sentences.

7. Page 14, lines 241-244: the paragraph is not fully consistent.

8. Page 15, line 255: atherosclerosis is not an obesity related risk factor.

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<th>Addressed Changes</th>
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<td>4: The sentence has been rewritten to clarify that our results imply that different protein sources seem to have differential</td>
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<td>7. Page 14, lines 241-244: the paragraph is not fully consistent.</td>
<td>7: Thanks. We have now revised the paragraph. P 15 l 259-261</td>
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<td>8. Page 15, line 255: atherosclerosis is not an obesity related risk factor.</td>
<td>8: The risk factor “atherosclerosis” has been replaced by the risk marker “dyslipidaemia”. P</td>
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an obesity related risk factor.

9. The implications of these results on foods/alimentary patterns associated with the proteins investigated in this study could be further discussed.

15 | 275.

9: We find it too speculative and farfetched to include the long-term perspectives and implications of our acute findings. We think that results from long-term studies should be available to discuss this issue further.