Author's response to reviews

Title: Effects of acute ingestion of different fats on oxidative stress and inflammation in overweight and obese adults.

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Author's response to reviews: see over
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Dr. Nehme Gabriel  
Editor in Chief of *Nutrition Journal*

Dear Dr. Gabriel,

Please find a revision of our manuscript entitled “Effects of acute ingestion of different fats on oxidative stress and inflammation in overweight and obese adults” uploaded for continued consideration by the *Nutrition Journal*. We are pleased to have had the opportunity to revise our manuscript as we still believe that it is well suited for your journal. We understand that it may be reviewed by additional/new reviewers, however, we would also hope that we would have the opportunity to respond to our previous reviewers as we found their suggestions very helpful and feel as though we were able to adequately address concerns that were raised.

We do not have any potential competing interests. All authors contributed to the experimental design, interpretation of results, and manuscript preparation and all have seen and approved the final version of this manuscript.

Additional peer reviewers may be:
1) Kim-Tiu Teng, Department of Physiology, University of Malaya (kt.teng@gmail.com)  
2) Angelika Bierhaus, Department of Medicine, University of Heidelberg (angelika_bierhaus@med.uni-heidelberg.de)

I will look forward to hearing from you concerning the acceptability of our manuscript. Please do not hesitate to contact me if you have any questions. We have responded to the reviewers’ suggestions/comments following this letter.

Sincerely,

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Response to reviewers
We would like to thank reviewers for their time and careful analysis of our manuscript. We appreciate the feedback and have adjusted the manuscript accordingly, incorporating suggestions and clarifying points within the paper as requested. We have responded to specific concerns raised by each reviewer below with reference to changes in the text.

Reviewer #2:
1. The soluble ICAM-1 and NF-kB data are interesting but the differences are small and their clinical importance is difficult to interpret.
Response: Although we are reporting relatively small differences between treatments, they were statistically significant, and thus not likely to be random (with 95% accuracy with p<0.05). We agree that they are not high in magnitude, but still valuable to report that macronutrient composition of a meal can influence these factors as chronically elevated levels of inflammatory mediators play a key role in chronic disease development over time. Inflammatory events typically have a specific purpose and are supposed to respond to a stimulus and then resolve quickly. The body is not designed for constant over-stimulation of the immune system and endothelial cell activation. ICAM-1 is an adhesion molecule integral to monocyte transmigration into the endothelial wall and has been implicated in relation to coronary events (Ridker 1998). As NF-kB is a major transcription factor involved in the upregulation of many inflammatory cytokines and chemokines, even slight increases in NF-kB could result in significant downstream effects. Thus, the implications of different meals invoking different inflammatory responses could be significant over time.

2. What were the initial inclusion criteria for this investigation and the rationale behind them?
Response: Our inclusion criteria were overweight/obese (BMI>27kg/m2) adults without chronic disease diagnosis or taking medications that would interfere with metabolic or inflammatory parameters. It was expected that based on literature, these individuals would be more likely to begin with some elevated inflammation and have a hyper-response to a meal (Patel, JCEM 2007;92:4476; Manning, Obes 2008;15(9):2046). Since chronic inflammation has been connected to insulin resistance and other co-morbidities in the obese population (Obes Rev 2010;11(9):635 and Pradhan, Nutr Rev 2007;(65):S152), we were focused on this group. Based on the criteria, many people did not qualify due to medications or disease status, and the blood draws were also a deterrent (subjects also had to be willing to undergo 3 high fat meal challenges with 5 venipunctures per test), resulting in a population that was perhaps less homogenous than ideal in terms of age and metabolic phenotype.

3. There may be potential sex differences in these factors
Response: We provided the data by gender for exploratory purposes only, as we mentioned in the results on page 8. The data was provided due to potential gender differences in inflammatory variables (Diab Obes Metab 2008;10(11):1086). However, as our study was not designed (or powered) to test for differences by gender, statistical tests were not conducted in this regard. If providing this data is distracting or inappropriate, we can remove it from the table.

4. Providing ranges for each of the characteristics in Table 1 would be helpful to readers in assessing the variability. The mean ± SE data alone provide an unclear picture of the groups unless the data are normally distributed. For example, the fasting glucose data might suggest that at least some subjects were diabetic.
Response: The ranges for the variables do indicate some heterogeneity amongst subjects, one subject had high blood glucose and TG, and one had high insulin and CRP (different subjects). However, it should be considered that since each subject participated in each meal challenge, he/she served as his/her own control, thus minimizing differential responses between treatments based on subject differences. We
also accounted for baseline values by including initial levels as covariates in our models. We have added this to Table 1, if the reviewers agree that it is useful.

Mean (Range):
Glucose: 6.0 (4.8-8.5)
Insulin: 11 (4.8-26.4)
TG: 1.58 (0.63-5.5)
FFA: 0.33 (0.12-0.49)
CRP: 3.5 (0.3-10)
TNF: 1.3 (0.85-1.64)
ICAM: 210 (163-269)
VCAM: 677 (447-924)
Epi: 0.08 (0.05-0.14)

5. The authors should clarify whether all subjects were studied at a similar time of day, given the strong diurnal variation in several markers of inflammation. The authors should clarify whether all subjects were studied at a similar time of day, given the strong diurnal variation in several markers of inflammation.

Response: All subjects were studied first thing in the morning, in the fasted state. Blood samples were collected by repeated venipuncture. This information has been clarified in the methods section (page 5).

Reviewer #2:
1. Table 1 presents average fasting levels of inflammatory, oxidative stress, and metabolic variables – is this the average of all three baseline values? If so, please make this clear in the methods.

Response: Table 1 does present the average fasting levels of all 3 of the baseline values for each subject. This clarification has been made in the results section (page 8).

2. The data were analyzed with RM ANOVA using the baseline values as a covariate; were there baseline differences between trials detected with a 1-way ANOVA? Please indicate.

Response: The baseline levels of the variables were not different by 1-way ANOVA. As there was variability within the same subjects across different meal days in their starting values and between subjects as well, we included the initial value as a covariate for each measure. For example, one subject had a 4 fold difference in initial NEFA levels between two trials (0.08 vs 0.35), another had the same difference in insulin between two (6.5 vs 24).

3. The methods indicate that post hoc tests were only used to examine differences between treatments – was an adjustment made (Bonferroni) for multiple comparisons (alpha = 0.05/3 = 0.0167)? No mention of post hoc comparisons across time is mentioned. Figures 1 and 2 indicate that there were differences between specific time points for CRP, TNF-a, and 8-epi – was a correction made for multiple comparisons between time points? In line with this, only main effects of time and treatment are indicted in Table 2. It would be helpful to indicate any pairwise differences between treatments or between time points.

Response: Post hoc tests were used when significant effects of either treatment or time were detected by ANOVA. This has been clarified in the methods on page 8. No adjustments were made for multiple comparisons.

The information for which time points were different has been added to Table 2 variables. Specifically:
- NEFA: baseline value was greater than all time points except 6 h postprandial (PP)
- Glucose: 1h PP was greater than all other time points
- TG: all PP time points were greater than baseline, and MFA > SFA for a treatment effect.
- Insulin: 1hPP was greater than all the rest, 2hPP was greater than all except 1h PP.

4a. The authors speculate that the acute increase in NF-κB following omega-3 fatty acid consumption may be due to the lack of habitual dietary intake of omega-3 fatty acids. Was this assessed?
Response: The average intake of n-3FA (DHA + EPA) for the US population is approximately 100 mg/d (Kris Etherton, 2009 and Ervin, 2004), which is much lower than the dose that we were providing (4000 mg), and thus most people would be naïve to that level of n-3FA supplementation. We assumed that our subjects were average, although inclusion of a background assessment such as a food frequency questionnaire would be recommended for any future studies involving effects of n-3FA supplementation.

4b. Anti-oxidants were excluded for 2 weeks before the trial, did this include omega-3 fatty acids?
Response: Participants were asked to cease all dietary supplementation for at least 2 weeks prior to and throughout the trial (AOX, vitamins/minerals, omega-3s – although no subjects were not taking these), and we have clarified this point in the methods section.

5. The authors state in the discussion that their study supports the increase in ICAM-1 following acute saturate fat ingestion by lean individuals (3rd paragraph on page 11) and SFA relative to MFA negatively impacted ICAM-1 (2nd main conclusion). From the data (Figure 1d) this reviewer does not understand the basis for this conclusion as the change in ICAM-1 was similar for both the omega-3 fatty acid meal and the saturated fat meal. The variability trial to trial (baseline values) for this measure appears to as great, if not greater than, the effect of any individual treatment. Please either remove or clarify this point
Response: We are a little unclear as to the concern regarding SFA having a negative impact on ICAM-1 relative to MFA. We agree that the change in ICAM-1 was similar between the OFA trial and the SFA trial, but we did not make any statements regarding those two as different. There was also variability in starting values between those two treatments, however, for the SFA and MFA trials, the starting values were essentially the same, and yet they had different postprandial responses. We still feel that this is worthy of notation, please let us know how best to clarify this point.

6. The small sample size is an acknowledged limitation of the study. Although previous studies (as mentioned) have used similar sample sizes, it may be prudent for future studies to utilize larger sample sizes in order to detect significant effects. Was an a priori power analysis performed for this study? Please include. If not, what was the observed power for the current study? This would be pertinent for future studies undertaking similar investigations.
Response: In regards to whether we had enough statistical power to detect a difference in other inflammatory markers, we feel that it is unlikely that testing more similar subjects would provide a different result for those factors that showed almost parallel responses following meals (e.g. VCAM, CRP). We did, however, calculate the number of subjects that would be necessary to achieve statistical difference between meal trials with 80% power for TNF-α and 8 epi. A total of 359 and 74 subjects would have been needed to allow determination that the trials caused differential responses (area under the curve) in TNF-α and 8 epi, respectively. This was not feasible and suggests that any difference in effect of the fats on these variables is very modest, if it exists at all, and that the variability in the measures is substantial. It is recommended that future studies including these measures attempt to reduce variability by choosing subjects with similar baseline inflammatory markers to attempt to address this. We have included this information in the discussion on page 12.

Minor revisions requested
1. It is unclear why one data was missing for one subject, please include.
   **Response:** Missing data for one subject is due to the fact that over the time course of postprandial assessments, we are missing too many samples for this subject to feel confident in his area under the curve values. He was missing at least 2 samples for 3 measures. The reasons were variable and included phlebotomy issues, insufficient collection, dropped sample, and broken vacutainer. Removing this subject, however, is conservative, as keeping him in only strengthens the associations and significance level of results reported here.

2. Regarding the two subjects unable to finish the milkshake on the first day, how much was actually consumed? Did this affect their individual responses relative to the group?
   **Response:** For the two subjects who did not completely finish their meals the first day, their remaining meals were adjusted to match the reduced intake so that their trials would be comparable. These subjects still consumed 70-80% of their assigned meal, resulting in an intake of 9-10 kcal/kg, still a substantial meal challenge, which we have added to the methods (page 5). There were no notable differences in their postprandial responses compared to the group.

3. Was the omega-3 fatty acid supplement added to the milkshake or taken separately in pill form? Please clarify.
   **Response:** The n-3FA supplement was provided in pill form, which has been clarified in the methods section (page 5).

4. With 6 of the 10 subjects being female, were any of the subjects on birth control? Was the impact of menstrual cycle considered?
   **Response:** The impact of the menstrual cycle was not considered in this study.

5. Subjects were instructed to follow the same pattern of eating for 3 days prior to each test day. How was this assessed? In the results, physical activity level is reported as not different between trials; how was this assessed?
   **Response:** We instructed subjects to follow the same patterns of eating and exercise for 3 days prior to each test day. The subjects provided 3 day food records and physical activity was via self-report. Overall, the participants were considered sedentary with minimal regular physical activity.

6. Please acknowledge as a limitation that these results may only be applicable to overweight and obese individuals.
   **Response:** We have added this limitation to the discussion section as suggested (page 12).

Additional

1. Please be consistent when referring to omega 3 fatty acids. In some places it is ‘n3FA’ and other places ‘omega 3 fats’.
   **Response:** We have tried to make sure that we are consistent in using n-3FA for omega 3 fats in general and being sure to differentiate between them and OFA (the fish meal challenge).

2. Many of the references do not include the title of the Journal.
Response: We apologize for references lacking journal titles. This was an oversight during the transition from using one reference manager software program to another. We have corrected this issue.