Author's response to reviews

Title: An Increase in Dietary n-3 Fatty Acids Decreases a Marker of Bone Resorption in Humans

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Author's response to reviews: see over
Responses to Reviewer #1

**The authors need to make this statement stronger in the conclusions.**
An additional sentence has been added to the conclusion (last paragraph) to emphasize the beneficial effects of omega-3 fatty acids on both the cardiovascular and skeletal systems.

**It should be made clear that the LA was similar across the groups...suggesting that ALA is key...**
Sentences have been added (Page 5; first full paragraph and Page 13; first full paragraph) to emphasize the importance of ALA.

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**Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)**

There are some errors in citations and also some key papers missing such as Dodin et al (1) in which flaxseed was the source of ALA and bone mass studied. A study in Japan what contains epidemiological data plus a small intervention by Terano (2) should be included along with the pilot work of Kruger prior to the full study in Aging Milano. Lastly a study in Crohn’s disease should be mentioned (3). We appreciate the suggestions. These studies have now been included in the second paragraph of the introduction (see below).


**Title:** the title should be revised to accurately reflect the observations as related to bone resorption specifically.
The title was changed to “An Increase in Dietary n-3 Fatty Acids Decreases a Marker of Bone Resorption in Humans”

**Abstract:** the first line should include human studies; also have shown effects on n-3 fatty acids. Units for NTx are nM BCE.
The first sentence of the abstract was revised to include humans. The units for NTx have been changed from “nM” to “nM BCE” in the abstract and throughout the manuscript. We thank the reviewer for pointing this out.
Introduction: the reports above should be included in the second paragraph. It should also be pointed out that the study by Bassey used whole body and had little chance of observing a difference given the error of measurement and the 1 year study. The study by Kruger was better designed and used spine and hip scans and was able to show differences over time.

The reports of Dodin, et al., Terano, and Trebble, et al., as well as the Kruger pilot study, have been included in the second paragraph of the introduction. Two sentences were added to clarify the differences in the study designs of the Kruger and Bassey papers.

Methods: For the biological samples, were they stored in a manner to preserve the fatty acids?

Samples were stored at –80°C for less than one year. A sentence was added indicating that the lipid extraction was performed in the presence of BHT. A citation is provided to the more detailed methodological procedures of Zhao et al (J of Nutr 2004)

It is well known that blood samples oxidize and a statement as to storage would help the readership know how good the values are. How long were they in storage? Naturally with the cross-over design time is a consistent factor in the study but overall the values might be lower if not stored ideally. The duration of the trial relative to the bone remodeling unit should be rationalized. A sentence has been added to the methods (Page 6 - first paragraph of study design section) indicating that 6 weeks is sufficient for alterations in NTx and BSAP to be seen. Samples were stored for less than 1 year at –80C. This is an ancillary study taken from a sample of adults who were enrolled in a clinical trial with lipids and vascular function as the primary outcome. The 6 wk treatment durations were selected based on the time course of serum lipid responses to changes in dietary fatty acids. We acknowledge that this study is shorter than previous studies examining the effects of fatty acids on bone. In light of this, it is impressive that we saw statistically reliable reductions in NTx. In fact our results may underestimate the true effect obtained with longer-term treatment. We feel that the results are valuable even with this qualifying statement.

Table 2 is not just of macronutrient composition, change the title. Is en the standard abbreviation for energy? Was the assay used to chemically measure the nutrients provided in the methods and for vitamin D did the assay have a detection limit to 0.001 ug?

The title of Table 2 was changed to “Nutritional composition...”; The en abbreviation was changed to “energy” and is now included as a footnote at the bottom of the table (*); The values reported for Vitamin D are not assayed values, they are calculated values from the Nutritionist V nutrition analysis program. The “***” in the table refers to this in the footnote.
Results: The figure is a repeat of the values in the text. Otherwise clear. We believe it is important to keep these values within the text, as they provide the exact numbers represented in the graph. Since this is the major finding of the paper, we believe that readers would appreciate having this information.

Discussion: the over 4 pg discussion must be reduced in length. We have worked to make the discussion more concise and have reduced the length of it by about 2 pages.

Both the LA and ALA diet caused reductions in NTx. This is not well discussed. In Table 2, it is clear that the AAD was lower in LA and ALA with higher n-6 to n-3 ratio. Perhaps the best conclusion is that reducing the n-6:n-3 fatty acid ratio in the diet from 9 to 3.5 or lower has a positive outcome, reduced bone resorption. We have addressed these points more thoroughly in the discussion (page 12; last paragraph)

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Page 15, references 6 and 10 on line 4 should be 14,15. We apologize for this confusion. The citations have been fixed.
Responses to Reviewer #2

... the response of three women is not significant enough to justify the statement that there was no difference;
We did not mean to imply that there was no difference between the men and the 3 women, only that the statistical analyses yielded similar results whether the men were analyzed alone or if the women were included. Since the results were similar, the data from the men and women were pooled.

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Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)
Methodology:
How was the study population selected from the larger study? Was it random and what were the criteria? Why were only three women included?
A sentence was revised to indicate that all of the subjects from the larger study were included in the present study. Only 3 women were enrolled in the larger study because a primary outcome variable (vascular endothelial function) is known to be strongly influenced by the menstrual cycle. Therefore, all of the women enrolled in the study were postmenopausal. We excluded women taking hormone replacement therapy because of its well-known effects on CVD risk factors and because we have shown that use of HRT alters dietary responsiveness. At the time the study was conducted, it was difficult to find postmenopausal women who had elevated lipids who were not taking HRT. We enrolled as many women as we could find within the constraints of the vascular study.

A wash-out period of 3 weeks is too short for bringing membrane fatty acid composition back to baseline. It should have been 6 weeks.
We agree that membrane fatty acid composition does not return to baseline within 3 weeks. Stabilization would have been expected to occur at the end of the 6-week feeding period plus the 3-week “washout”. So, think about the total amount of time from the previous intervention as 9-weeks. This is how we have done all of our studies. Whereas, it is true that a short break between interventions may not have been long enough for certain endpoints to return to baseline values, the total 9-week period between the end of one intervention and another should be adequate.

What was the intra-assay CV for NTx?
The intra-assay CV for NTx is 5.5%. It was previously referenced as the inter-assay CV but it is in fact the intra-assay CV.
Results:
The percentage change in NTx is within reported intraperson variation. The observation may not be real. Any comments?
We appreciate the reviewer's concern. To minimize intra-individual variability serum NTx was used, which produces much less variability than does urinary NTx. Regardless, we recognize that markers of bone turnover can be highly variable for a variety of reasons. The cross-over design of this study was intended to help control for that. We also recognize that the reported decrease is less than the standard deviation of the mean of each treatment period, and must therefore be considered relatively small. The magnitude of our effects has been addressed in the discussion section (page 13; last paragraph). Although small, we believe that the results are "real" because of their internal consistency (reductions with both ALA and LA diets, appropriate alterations in serum fatty acid levels, correlation with TNFα, lawful nature of the results, i.e. somewhat 'dose-related' effect), and because of the controls imposed by the study design.

We sought the advice of a statistician here at Penn State (Dr. Michael J. Rovine, Methodology Consulting Center) who indicated that we could alter our statistical model to include the duplicate samples for each individual at each time point as a nested variable. This would allow us to determine the deviation of each individual around his/her own mean, as well as the deviation of each individual around the group mean, i.e. we could include an intra-individual variance component. However, even in the case of a nested model, the proper statistical test would still involve deviating individual average values around the group mean. Since this is the way the data currently are analyzed (assessing the deviation of the individual average of each duplicate around the group mean), we would expect the results to be the same. For this reason, we have elected not to include this analysis in the revised version. However, if the reviewer wants, we will re-do the analyses in this way.

Plasma levels of cytokines are also highly variable for individual people. The small number of samples (16) used to draw a correlation between TNFalpha and NTx makes it questionable. Could the graph showing the correlation be included in the results?
The crossover design of the study helps to account for the variation observed with individual people; because the correlations were run using each subject as the repeated measure this variability is accounted for, with each subject acting as their own control. Because the correlations were run across all three diets it is difficult to present this in a simple manner graphically. Because it is not the major finding of the study we do not believe that the figure would significantly add to the quality of the paper.

Discussion:
The discussion is too long, and can be shortened significantly. We have reduced the length of the discussion by about 2 pages.
Page 11; there is reference to studies done in humans with evening primrose oil, with no mention of fish oil. The statement is therefore not correct as there have not been any human studies done using only evening primrose oil. All studies used a combination of EPO and fish oil, or fish oil only. Please correct.
This is an important point and we apologize for this oversight. This sentence has been changed to read “supplementation in humans”.

Page 13: There is mention of the synthesis of PGE1 after supplementation with EPA. PGE1 is synthesized from GLA not EPA. The latter gives rise to PGE3. In the same paragraph it is stated that production of PGE1 is expected to reduce osteoclast activity, why?? There are no references given.
This section has been altered to reflect the correct conversion pathways (Page 14).