Author's response to reviews

Title: Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: A randomized, placebo controlled trial

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Version: 2 Date: 15 June 2009

Author's response to reviews: see over
Thank you for your careful review of our manuscript entitled “Effects of a novel whey-derived peptide on vascular responses in healthy individuals: a randomized controlled trial”. Below you will find our responses and changes made to the original manuscript submission based upon the reviewers' comments.

Thank you

Reviewer's report
Title: Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: A randomized, placebo controlled trial
Version: 1 Date: 29 April 2009
Reviewer: Margreet Olthof
Reviewer's report:
This study describes the effects of a novel whey derived peptide on endothelial responses in humans. The study was well designed and executed under controlled conditions. However there are comments and questions.

MAJOR
Supplement
1) Please justify the choice for NOP-47 and provide more details on the supplement NOP-47. What does ‘NOP-47’ stand for? Which bioactive peptides are in it that are supposed to act on endothelial responses? Are there previous studies in humans with this supplement? In addition, the amino acid composition in table 2 seems strange, in total there is 128 g of amino acids in 100 g powder?

NOP-47 is an internal name developed by Glanbia that stands for Nitric Oxide Peptide. We have limited knowledge of the peptide other than limited information shared by Glanbia Nutritionals. Their preliminary work showed a whey hydrolysate increased NO production in vitro. There are no published studies done with this particular peptide, although the company has performed a 7 day animal toxicity study. The bioactive peptides in this specific fraction are not exactly known. It is common to develop whey peptides/hydrolysate and screen for various bioactivities (see for example Clare DA, Swaisgood HE: Bioactive milk peptides: a prospectus. Journal of Dairy Science 2000, 83:1187-1195). There are a lot of bioactive peptides and this approach has identified many other bioactive peptides as summarized in this reference. At this point, we only know the amino acid composition which was tested by an independent laboratory (Siliker Labs) (Table 2). Note there was an error in the original amino acid table which is why the values added up to 128 g. This has been corrected.

Results
2) In general no comparisons should be made within intervention groups (e.g. compare effects within the placebo group compared to baseline). The advantage of the cross over design is that comparisons can be made between the placebo
and the intervention, within subjects. Therefore the statistical analyses should match this design and only these comparisons should be presented. All other significant comparisons ‘in time’ should be deleted from the results because they are not important if there is no difference between the intervention and placebo. For the hematological responses this means probably that there are no effects of the NOP-47? In that case the table might be deleted and results only mentioned in the text?

While we agree with the reviewer that the most important comparisons are between the placebo and intervention trials, according to our statistical consultant it is appropriate to include time as a factor in the model along with supplement trial (NOP-47 vs Placebo). If there is a different statistical approach you think is more suitable, we would be happy to consider it but this is the model we have used in previous cross-over studies based on our statistician. Although the temporal changes in the dependent variables was not the primary objective of the study, it does provide important information in order to contextualize the differences noted in vascular function between NOP-47 and Placebo. For example, the fact that this peptide improved vascular function in the context of little change in oxidative stress and inflammation is noteworthy, and we therefore think the blood variables listed in Table 3 should remain.

3) A lot of outcomes were tested. Please justify all outcomes (what were the hypotheses?) and explain that you adjusted for multiple testing (or not)?

The primary outcome variable was vascular function. As stated in the introduction, we hypothesized that a single dose of NOP-47 would enhance vascular function as measured by flow-mediated dilation (FMD) of the brachial artery using high-frequency ultrasound and reactive hyperemia forearm blood flow assessed by venous occlusion plethysmography. We added to the text that a secondary objective was to characterize the effects of NOP-47 on circulating markers of antioxidant capacity, oxidative stress, and inflammation since these factors have been demonstrated to influence vascular function through various biologic mechanisms. In respect to controlling for multiple comparisons, the Fishers LSD post hoc test accounts for multiple pair wise comparisons similar to the Bonferroni correction.

Discussion
4) The discussion is rather general and not focused on the results of this study. Specifically: potential effects of whey proteins on ACE inhibition are discussed, but in this study there is no effect of NOP-47 on blood pressure?

We understand the reviewer’s point. This is a new target of peptides. We could find no information in previous research where a peptide or protein was shown to impact NO or vascular function as measured by FMD. Considerable work has however linked peptides to ACE inhibition which is related to vascular health. We would argue that ACE inhibition needs to be tied into the complete biological pathway since ACE inhibition and NO production share some common pathways. If the reviewer thinks this information is
not germane or distracts from the findings, we could be persuaded to remove it but again we feel it is relevant and prefer to keep it in the discussion.

MINOR
Methods
5) The blinding is unclear. If the intervention was 5 g of whey protein and the placebo was sweeteners only then participants could see the difference? Did the NOP-47 and placebo look and taste the same after mixing with water? Please explain.

The goal of the study was to make it double blind, however based on feedback after the study most subjects were able to discern which supplement was the peptide. The two beverages were of identical color after mixing with water. However, after reviewing subject questionnaires administered at the end of the study, 19 out of 20 subjects correctly guessed which supplement was the peptide primarily based on taste. Subjects were instructed to not give any indication as to which beverage they were ingesting to main study personnel. Therefore, study personnel involved in data collection and analysis were not aware of the treatments until the end of the study. Thus we do not think this had a major effect on the study outcomes. This information has been added to the manuscript (pages 6 and 12).

6) Which artificial sweetener(s) was/were used and how much was added to the Supplements?

Individual packets of supplements were provided already packaged with Crystal Light which uses the artificial sweeteners aspartame and acesulfame potassium.

Results
7) pre and post occlusion diameters are presented and tested (fig 2). This seems unnecessary, FMD includes both and therefore you can present the FMD only.

Figure 2 has been removed from the manuscript.

8) Present the actual differences in FMD and blood flow in the text (mean difference and 95% CI).

Mean differences and 95% CI have been added to the test for FMD and blood flow responses.

9) In the last sentence on page 12: ‘individual responses…NOP-47 ingestion’ it is unclear relative to what the responses were greater? Please clarify

This sentence has been clarified as demonstrated below.
“Individual responses revealed that 15 out of 20 subjects had greater peak FMD at 60 min (Figure2, bottom) and 90 min post-NOP-47 ingestion compared to these same time points following ingestion of placebo”.

10) Please clarify in general in the manuscript and the title what ‘acute ingestion’ means? The word ‘acute’ suggests that supplements were tested after a single dose, but subjects ingested the supplements for 2 weeks already. So you don’t know whether the effects you found result from a single dose or the (build-up) effect of 2 weeks ingestion?

Going into the study we did not know if a preloading period on the peptide would be required to observe an effect on vascular function. Thus a two week supplementation period was chosen. If this supplementation period had an effect on vascular function it would have shown up as a significant difference in FMD at the vascular testing day at the pre-ingestion time point. As shown in Figure 2, the values were almost identical between NOP-47 and placebo indicating that the supplementation period had no effect on resting vascular function. The benefits occurred only after acute ingestion of the peptide. We made this point in the first paragraph of the discussion, “We demonstrated that 2 wk of supplementation had no effect on fasting measures of vascular function, but acute ingestion of NOP-47 significantly increased postprandial FMD at 30, 60 and 90 min post-ingestion and reactive hyperemia forearm blood flow measured at 120 min post-ingestion.”

Figures:
11) Too many figures are presented.

Figure 2 has been deleted.

12) The figure numbers/legends in the manuscript are not clear, and in addition the figures are displayed twice in the manuscript?

Duplicate figures have been removed from the manuscript. Thank you for noticing this oversight.

13) Figure 4: in the text on page 13 it is stated that there is a significant difference between NOP-47 and placebo. However, it is unclear from the figure. It would be more clear if the data are presented in 1 figure so placebo and NOP-47 data can also be visually compared.

We have merged the data into one figure and included only data representing the post-ingestion values.

14) Figure 4: in the legend it says 120 min post-ingestion in the figure 110 min?

This has been changed to 120 min.

Conclusion
15) Preservation of NO bioavailability is not an outcome of this study and should be deleted from the conclusions.
This statement has been deleted throughout the manuscript.

Citations
16) Please include literature references for all statements made. E.g. page 3, before last sentence: dietary proteins….may also impact vascular function’ and ‘in vitro experiments on NOP-47’ (page 4)

We deleted the sentence on Page 3 as it was simply a transition into the next paragraph but really unnecessary. The statement about the in vitro experiments on Page 4 was based on internal data generated by Glanbia nutritionals as so there is no reference. We included in parentheses (data provided by Glanbia Nutritionals).

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests

Reviewer's report
Title: Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: A randomized, placebo controlled trial
Version: 1 Date: 2 June 2009
Reviewer: Edward P Weiss

Reviewer's report:
The authors hypothesized that a whey derived peptide, NOP-47, improves endothelial function, in humans. Results indicate that vascular function was acutely improved after NOP-47 ingestion in both conduit and resistance blood vessels. However, the compound did not have a longer term effect on vascular function as measured in the fasted state. The study was very well conducted and the report is thorough and well written.

Major Compulsory Revisions
1. It is not clear what potential implications are for using NOP-47 to improve vascular function. Was the goal to study this as a pharmaceutical agent or a possible digestion/degradation product of whey protein ingestion? This needs to be clarified in the introduction and a paragraph addressing the implications of the findings should be added to the discussion.

The intention was not to develop a drug. The goal of Glanbia Nutritionals is to develop a nutraceutical with vasoactive properties. They are a major manufacturer of whey and have spent considerable time studying various hydrolysates of whey. Based on their in vitro work, they identified an isolate from whey protein hydrolysate (NOP-47) having an
effect on nitric oxide production. Thus these are not degradation products of whey proteins but a specific group of bioactive peptides isolated from whey. They approached us to perform a human study to see if the peptide retained any bioactivity in an in vivo model of vascular function.

We included additional sentences in the introduction and added a implications to the concluding paragraph of the discussion.

Minor Essential Revisions
1. Throughout manuscript: P values are often reported as "0.000" but this is impossible. These should be replaced with "<0.0001"

*P values have been changed to <0.0001 throughout the manuscript.*

2. Page 4, 1st sentence in 1st full paragraph: A reference is necessary for the study on HPAE-26 cells.

*This was data provided to us from Glanbia Nutritionals which has been added to the text.*

3. Page 7, lines 11-16: the fact that dietary records were kept and were used to replicate diets on 2nd trial is stated twice.

*This duplication has been removed from the test. Thank you for noticing this oversight.*

4. Page 17: The fact that the company that provided the supplements was also the funding agency is a major competing interest and should be moved to the "Competing Interests" section.

*The statement “Glanbia Nutritionals provided funding for the study and supplied the test supplements used in the study” has been added to the Competing Interests section. We would point out that the sponsor had no involvement in the data collection, data analysis and interpretation of the findings.*

5. Page 23 / Table 2: The primary structure of the NOP-47 peptide should be given. Or, if the primary structure is proprietary, this should be stated.

*The amino acid composition is shown in Table 2. The structure is proprietary.*

6. Is NOP-47 available to the general public as a dietary supplement or other form?

*The intention is to make it available as a dietary supplement but it is currently not commercially being sold.*

**Level of interest:** An article whose findings are important to those with closely
related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests