Author's response to reviews

Title: Low-dose menaquinone-4 improves gamma-carboxylation of osteocalcin in young males: a non-placebo-controlled dose-response study

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Author's response to reviews: see over
Dear Dr. Gabriel,

Herewith we are resubmitting our revised manuscript, titled “Low-dose menaquinone-4 improves γ-carboxylation of osteocalcin in young males: a non-placebo-controlled dose-examination study” for publication in *Nutrition Journal* as a short report.

We greatly appreciate the editors’ and reviewers’ valuable comments and critical suggestions, which have helped us to considerably improve our manuscript. As indicated in the responses in the following pages, we have taken the reviewers’ comments and suggestions into account in our revised manuscript. We have modified the title page, keywords, abbreviations and references, and inserted all Tables into the manuscript according to the “instructions” of *Nutrition Journal*. Also, we have corrected the grammatically mistakes. The revised sentences are highlighted in red font.

We have tried to make the manuscript as brief as possible, but the length is over 1,500 words owing to the need to address the reviewers’ comments thoroughly.

Additionally, we would like to change the corresponding author from Eriko Nakamura to Ayako Kamimura. In our first submission we put Eriko Nakamura as the corresponding author with regard to the submission process. However, the corresponding author as far as readers are concerned is Dr. Kamimura.

We now hope that our paper will be suitable for publication in *Nutrition Journal* and look forward to hearing from you concerning your editorial decision.

Yours sincerely,

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Responses to Reviewer 1 (Dr. Kiyoshi Tanaka)

(1) General comments

*In this paper, the authors have made the intervention studies with graded doses of menaquinone-4 (MK-4) with serum undercarboxylated osteocalcin (ucOC) level, a marker for skeletal vitamin K deficiency, as the endpoint. As the authors state in the Introduction and Discussion, vitamin K requirement is considered to be much higher in the bone than in the liver, yet the intervention study for skeletal dose requirement has not been reported.*

(2) Major revision

1. *Data on vitamin K intake (Table 2) have something strange. Median dietary vitamin K intake is approximately 50 microgram/week, which is incredibly low and inconsistent with their description that the vitamin K intake was similar to DRIs (line 109–110). Table 2 also shows the presence of the subject(s) with null vitamin K intake, which is quite difficult to imagine. Vitamin K intakes on day 29 and 36 are markedly decreased. Additionally, what was the vitamin K intake at baseline?*

   **Answer:** In response to your comments, we have modified the text to: “*In contrast, the dietary vitamin K intakes of some of our subjects were very much lower than the DRIs and the Japanese average dietary vitamin K intake.*” (Discussion, line 136 to 138).

   We think that one of the reasons for the very low or zero median dietary vitamin K intakes of our subjects was that the number of assessment items was limited. Accordingly, we have added some text and new references [9, 10]: “*Furthermore, because the assessment items for dietary vitamin K intake were limited to nine vitamin K–rich foods, the minimum dietary vitamin K intake was 0 and the dietary vitamin K intake tended to decrease during the study period. To make the dietary assessment more closely reflect the entire dietary vitamin K intake, in future we will have to select more sophisticated methods; for example, we may need to estimate each participant’s dietary intake of not only vitamin K but also other nutritional components [9, 10].*” (Discussion, lines 155 to 161).

   We reanalyzed our subjects’ dietary vitamin K intakes in response to your major comment #3 on statistical analysis, and the dietary vitamin K values have been changed from those in the first manuscript (Table 2). We cannot clearly explain why the dietary vitamin K intakes on Days 15, 29, and 36 were decreased: this may be related to the method of dietary vitamin K assessment.
Additionally, we are very sorry to say that we did not assess dietary vitamin K at baseline in this study. However, some of our subjects may have been vitamin K deficiency on the basis of serum undercarboxylated osteocalcin (ucOC) values. In relation to the vitamin K status at baseline, we added and modified the text to the Results (line 111 to 112) and the Discussion (lines 142 to 152).

2. The authors have made some discussion citing the Dietary Reference Intakes for Japanese 2010. Reference #7 is a book in Japanese. I think that citing the paper below, one translated to English, would be more suitable for the foreign readers.
Additionally, DRIs 2015 was released on March 31, 2014. Since their work was done before that, I think that making the discussion with regard to DRIs 2010 is not a major problem. The authors are advised, however, to mention the new DRIs.

Answer: Thank you for bringing the correct reference and the new edition of the DRIs to our attention. We have replaced the reference to the 2010 Japanese DRIs with a new reference [6] (Introduction, line 46 and Discussion, line 133).

Furthermore, we mention the 2015 Dietary Reference Intakes with new reference [16], as follows: “However, in 2015 the Japanese DRIs will be revised upward to 150 µg/day for both males and females aged 18 to 29 years [16]. The average dietary vitamin K intake in Japanese people in their 20s (201 µg/day) [17] satisfies the current and proposed future DRIs.” (Discussion, lines 133 to 136).

3. The authors have made the statistical analyses using Kruskal-Wallis test. This is a non-parametric test for unmatched sample. Since the data here are matched ones, I think that Friedman’s test is to be employed.

Answer: In response to your excellent suggestions, we have now applied Friedman’s test to the comparison of serum γ-carboxylated osteocalcin (Gla-OC), ucOC, the ratio of ucOC to Gla-OC (UGR), and dietary vitamin K intake. We have also re-analyzed the serum vitamin K fraction.

Because these statistical analyses required repeated sets of complete data, the data from one subject who missed his visit on Day 36 were excluded. Therefore, we
re-analyzed the data from 13 subjects by using Friedman’s test and the post hoc Bonferroni-adjusted Wilcoxon signed-rank test. Although we examined the use of Friedman’s test and a post hoc Scheffé test, we finally selected a Bonferroni-adjusted Wilcoxon signed-rank test in accordance with [Bogdan T, Magdalena S, Zbigniew T, Tadeusz L: Nonparametric Statistical Analysis for Multiple Comparison of Machine Learning Regression Algorithms, In Knowledge-Based and Intelligent Information and Engineering Systems Lecture Notes in Computer Science. Volume 6276, Edited by Rossitza S, Ivan J. Berlin: Springer; 111–120]. Therefore, we have modified the relevant text in the Abstract (lines 19 to 22), the Methods (line 93 to 95), the Results (lines 100 to 102, 104 to 107, 110 to 116, 117 to 119), the Discussion (lines 125 to 130), and Tables 1 to 3. The numbers of subjects and the results have thus slightly changed compared with those in the original manuscript, but there is no major impact on our conclusions.

4. The authors are advised to comment on the decreased PK level after the intervention.

Answer: In response to your suggestion we have added the sentence “Notably, also, serum phylloquinone and menaquinone-7 levels decreased during the study period, possibly reflecting decreases in dietary vitamin K intake on Days 15, 29, and 36, because dietary vitamin K consists mainly of phylloquinone (leafy green vegetables) and menaquinone-7 (fermented foods).” to the Discussion (line 138 to 141).

(3) Minor comments

1. Dietary Reference Intakes is usually used in the plural form, not Dietary Reference Intake (line 35, 106).

Answer: Thank you for your instructions about Dietary Reference Intakes (DRIs). We have corrected the term in the Introduction section (line 44). Additionally, we have used the abbreviation “DRIs” in the other sentences and have added DRIs to the list of abbreviations (line 172).

2. The description “each country determines a dietary reference intake or adequate intake” is rather confusing. Adequate intake is one of the parameters in the DRIs.

Answer: In response to your suggestion, we have integrated DRIs and adequate intakes in the words “In most countries, dietary reference intakes (DRIs) are based on
satisfaction of the coagulation system (75 to 120 µg for adult males)” (Introduction, line 45 to 46).

3. The authors state that larger amount of vitamin K may be needed (line 36). What do they mean by “larger”? Larger than what?

**Answer:** In response to your suggestion, we have modified the sentences and have added new references [8–10], namely “However, some epidemiological studies have suggested that vitamin K requirements for maintaining skeletal health might be higher than the current DRIs at various ages [7–10].” (Introduction, line 46 to 48).

4. The authors have mentioned a previous epidemiological study citing reference #7. Since this is a guideline, citing the original paper is recommended. If the authors prefer to cite this guideline, I think that it would be better written as below.

*Based on an epidemiological study on serum ucOC levels, recently published guideline recommends the daily vitamin K intake of 250 to 300 micrograms.*

**Answer:** Thank you for your suggestion. Unfortunately, the guideline (reference [7] in the first manuscript) does not clearly refer to the vitamin K requirement, and we could not find a reference that definitively recommended a daily vitamin K intake of 250 to 300 µg. Therefore, we have removed the old reference [7] and have added and modified text citing a report that refers to the required vitamin K intake, namely “Furthermore, approximately 500 µg/day of dietary vitamin K by phylloquinone supplementation is needed to significantly decrease ucOC levels [11].” (Introduction, line 48 to 50).

5. Also, the original paper should cited instead of the guideline at line 113.

**Answer:** In response to your useful suggestion, we have reconsolidated the sentences because the guideline did not appropriately explain the cut-off value of serum ucOC. We have removed the reference (reference [7] at line 113 in the first manuscript) and now cite the new reference [3] in Introduction (line 40), Discussion (line 144).

(Note that there is no minor comment 6 in the reviewer’s report.)

7. AI for vitamin K is 75 microgram/day for adult men, and 65 microgram/day for adult women in the DRIs for Japanese 2010. Specify the gender at line 108.
**Answer:** We have added an explanation regarding gender, namely “Currently, the Japanese DRI is 60 µg/day in females, and 75 µg/day in males, aged 18 to 29 years” (Discussion, line 132 to 133).

8. Reference in Japanese are advised to be specified.

**Answer:** We appreciate your useful suggestion. In the References section we have added “in Japanese” to those papers written in Japanese (lines 201, 236, 239, 245, 247).
**Responses to Reviewer 2 (Dr. Katharina Nimptsch).**

**Reviewer’s report:**

This is a nicely written manuscript describing the findings from a small dose-examination study, in which 15 healthy volunteers received daily Menaquinone-4 (MK-4), with weekly increasing doses. Authors observed that compared with baseline, γ-carboxylation status of the vitamin-K-dependent bone protein osteocalcin improved at MK-4 doses of 600 µg/day or higher. It is a limitation of the study that it is no randomized placebo-controlled trial, but as I understand from the discussion, such a study design will be the next step of the research group. Authors state that 600 µg/day is a dose that can be achieved with dietary intakes, at least in Japan (with consumption of the fermented soy product natto, with is a rich vitamin K source). My main concern with the manuscript is that the conclusions drawn from the study do not always reflect the study design (i.e. no placebo-controlled trial).

**Answer:** We are very grateful for your critical comments and summary. We have added sentences regarding the limitations of our study design and the representation of effective dose. See the Title (line 2 to 3), the Abstract (lines 17, 23 to 25), the Methods (line 71), the Results (lines 104 to 107, 110 to 119), and Discussion (lines 153 to 154, 165 to 168).

**Major Compulsory Revisions**

1. The exact role of osteocalcin in bone health is not completely understood. This should be recognized in the introduction and/or discussion.

**Answer:** In response to your suggestion we have added an explanation of osteocalcin, namely, “The precise role of osteocalcin is not known” (Introduction, line 33 to 34).

2. Introduction, page 5, line 37: A larger dietary reference intake may be beneficial not only for a better carboxylation status of osteocalcin but also for other vitamin K-dependent proteins. Please refer to these briefly in the introduction.

**Answer:** In response to your comment we have added information on vitamin K–dependent proteins and have modified the text in the Introduction as follows: “Vitamin K plays an important role in coagulation and bone homeostasis as a coenzyme that mediates γ-carboxylation of glutamate residues into γ-carboxylated proteins such
as coagulation factors, osteocalcin, and matrix Gla-protein.” (line 31 to 33).

(3) Introduction, page 5, line 44: Please state why finding the lowest dosage of supplemental MK-4 was the aim of this study. Is there a concern of toxicity?

Answer: We are sorry for not explaining the purpose of our study sufficiently clearly in our first manuscript. Our aim was to find the lower dosage of supplemental menaquinone-4 (MK-4) not because of toxicity concern in higher dosage.

The currently reported effective dosage of MK-4 for bone health is 1500 µg/day. However, in terms of intervention studies this amount is too large to realistically obtain from the diet or as part of dietary reference intakes.

In epidemiological studies, the vitamin K requirements for bone health have been higher than the DRIs (see reference [7] and those in new references [8–10]). Furthermore, there have been intervention studies of the efficacy of lower dosages of vitamin K analogs other than MK-4 (see reference [11]).

In this context, our study was aimed at assessing the potential effects of MK-4 in the lower dosage range (i.e. below 1500 µg/day) on bone health.

In the Introduction we have revised the purpose of our study (lines 45 to 59).

(4) Methods, page 7, line 63: Were the blood samples fasting? If not, was fasting status accounted for?

Answer: Blood samples were drawn from fasting subjects. Subjects were fasted (except for water) for more than 10 h before blood sampling. In response to your comments, we have added the words “all blood samples were taken under fasting conditions” to the Methods (line 74 to 75).

(5) Results: Dietary vitamin K intake and serum phylloquinone as well as MK-7 decreased substantially during the study period. Do authors have an explanation for that? This should be part of the discussion.

Answer: In response to another reviewer’s comment we re-analyzed the serum vitamin K fraction data along with our other parameters. Thanks to your helpful comments we have added text, namely “Notably, also, serum phylloquinone and menaquinone-7 levels decreased during the study period, possibly reflecting decreases in dietary vitamin K intake on Days 15, 29, and 36, because dietary vitamin K consists mainly of
phytolquinone (leafy green vegetables) and menaquinone-7 (fermented foods).” to the Discussion (line 138 to 141).

(6) Results, page 9, lines 88–89: Please rephrase the sentence: it cannot be known whether the higher serum MK-4 is due to the supplementation with 1500 µg/day or due to the MK-4 supplementation in the preceding weeks.

Answer: In response to your constructive criticism we have modified the wording on MK-4 to “serum MK-4 was significantly greater on Day 36 after graded MK-4 supplementation than at baseline.” (Results, line 105 to 106).

(7) Dietary vitamin K intake was exceptionally low and it is unclear why dietary vitamin K intake decreased (could this be due to a decrease in the participant’s motivation to answer the questions on vitamin K-rich foods week by week?). Also, in table 2, the lower range is 0, which is practically impossible. While weekly dietary records are a good dietary assessment instrument, the calculation of vitamin K intake only from vitamin K-rich foods has limitations. Using the dietary record data in combination with a food composition database with vitamin K values on various foods, including meat and meat products, would improve the dietary intake data. Please also give some information on the participant’s compliance in filling out the dietary records. In addition, please give more information on the face-to-face interviews on the amounts of vitamin-K rich foods participants had consumed each day (i.e. how were amounts specified; how many missing values?). Adding the questions on vitamin K-rich foods as a supplement may be helpful. Also, please specify which food composition database was used for the estimation of dietary vitamin K intake.

Answer: We appreciate your critical comments about dietary vitamin K intake and the method of dietary assessment.

In regard to the low or zero values in dietary vitamin K intake, we think that our dietary assessment was indeed limited. However, we did not assess how the motivations of our subjects could have affected the results of the dietary assessment. We have added the following sentences and new references [9, 10]: “Furthermore, because the assessment items for dietary vitamin K intake were limited to nine vitamin K-rich foods, the minimum dietary vitamin K intake was 0 and the dietary vitamin K intake tended to decrease during the study period. To make the dietary assessment more closely reflect the entire dietary vitamin K intake, in future we will have to select more...
sophisticated methods: for example, we may need to estimate each participant’s dietary intake of not only vitamin K but also other nutritional components [9, 10].” (Discussion, lines 155 to 161).

We did not measure the participants’ compliance in the dietary vitamin K assessment, but all of the subjects filled out the assessments of vitamin K intake and the other records of dietary patterns and medications every day.

We have revised our explanation of the daily records, the weekly face-to-face interviews, and the food-composition database with new reference [14], as follows:

“Subjects kept daily dietary vitamin K records of the amounts of vitamin K-rich food (natto, broccoli, bok-choy, garland chrysanthemum, spinach, Japanese mustard spinach, Egyptian spinach, Chinese cabbage, chicken) they consumed. At the blood-sampling visits every week, the daily records were collected and trained dieticians interviewed the subjects to estimate their consumption of vitamin K-rich food. Dietary vitamin K intake was calculated by using software (Excel Eiyo-kun ver. 6.0, Kenpakusha, Tokyo) and was based on the Standard Tables of Food Composition in Japan – 2010 [14].” (Methods, lines 81 to 88).

We apologize that we do not have any information on the use of vitamin K–rich foods as supplements. Although there are many vitamin K–rich foods in Japan, the Japanese normally get their vitamin K from everyday consumption of these foods.

(8) Discussion: first sentence does not reflect observations. From the study design it cannot be known whether a one-week daily dose of 600 µg/day is effective, since it was preceded by one week of 300 µg/day.

Answer: As you point out in your comments about the first sentence of the Discussion, we did not describe the precise results and limitations of the study design. Therefore, we have modified the text accordingly: “We showed here in our graded-dose examination that serum Gla-OC increased significantly with intakes of 900 µg/day or more and serum ucOC and UGR decreased significantly with intakes of 600 µg/day or more. Therefore, MK-4 supplementation at 600 µg/day after a week’s initial intake at 300 µg/day improved vitamin K status in terms of bone health, especially on the basis of serum ucOC as a marker of vitamin K deficiency and UGR as a sensitive marker of γ-carboxylation of osteocalcin.” (Discussion, lines 125 to 130).

(9) Please briefly discuss the advantages/disadvantages of using absolute ucOC or GlaOC versus UGR.
**Answer:** We consider that absolute undercarboxylated osteocalcin (ucOC) and γ-carboxylated osteocalcin (Gla-OC) have advantages as an index of vitamin K deficiency and a bone formation–related marker, respectively. In contrast, we consider that the ratio of ucOC to Gla-OC (UGR) indicates the overall balance of vitamin K status in the bone metabolism. In response to your suggestion, we have added explanations of the differences among these indices (Introduction, lines 33 to 42).

**Minor Essential Revisions**

(1) **Methods:** Please state which software was used for analysis.

**Answer:** We have added the relevant text: “Statistical analysis was conducted with JMP 11.0.0 (SAS Institute Inc., Cary, North Carolina)” (Methods, line 96 to 97).

(2) **Discussion, page 10, lines 102–104:** Please move to results section.

**Answer:** We have moved and modified the sentence on the relationship between dietary vitamin K intake and serum ucOC or Gla-OC to the Results section (line 120 to 122).
Response to Reviewer 3 (Dr. Masao Kaneki).

Reviewer’s report:
Minor Essential Revision

It would be helpful for readers to understand your data if the data in Tables 1 and 2 are shown in graphs.

Answer: We appreciate your helpful comment. We tried to show the data in Tables 1 and 2 in box plots. However, it would be difficult to see the changes in response to menaquinone-4 intake in box plots because of their large variation. In addition, we would like to publish the precise data values for use as reference data for determining the vitamin K requirements of the Japanese population. Therefore, we would like to represent the data as tables. We apologize for not following your suggestion.