Author’s response to reviews

Title: Changes in the vitamin D endocrine system and bone turnover after oral vitamin D3 supplementation in healthy adults: Results of a randomised trial

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

Thank you for your response to our submitted manuscript # 6490852005791606 entitled "Changes in the vitamin D endocrine system and bone turnover after oral vitamin D3 supplementation in healthy adults: Results of a randomised trial" by authors Kristin Holvik, Ahmed A. Madar, Haakon E. Meyer, Cathrine M. Lofthus and Lars C. Stene. We appreciate your thorough review and the relevant comments pointed out by the reviewers. Please find attached a revised version of our manuscript.

Below, we have addressed the specific comments from the two reviewers. In accordance with the suggestions from the reviewers we have made improvements to our manuscript wherever feasible. Additions made to the manuscript are highlighted in red font. Page and line numbers cited in the present document refer to page and line numbers in the revised version of the manuscript.

We look forward to your evaluation of our revised manuscript.

Kind regards,
on behalf of the Authors

Kristin Holvik, Ph.D.

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A) EDITORIAL COMMENTS

Please revise your abstract so that your trial registration number is included as the last line.

This has been done (page 2 line 22).

B) COMMENTS FROM REVIEWER JULIE WALLACE

Major revisions

1) In the background section, the authors refer to healthy vitamin D status (line 13) what is healthy?

Reply: The definition of a healthy vitamin D status is, and must be, vague, as there is no current consensus about the optimal individual level of 25(OH)D for health. Recent evidence seems to support considerably higher levels for prevention of multiple adverse health outcomes (ref 20) than the levels needed to prevent rickets and osteomalacia (ref 17). We have touched upon this in the discussion on page 10. Challenges in establishing any definite recommended 25(OH)D concentration include firstly, the large variations in assessment of 25(OH)D levels between laboratories, and secondly, that the requirements of elderly and young populations probably differ. Most studies have focused on risk of falls and fractures in elderly populations. When considering the healthy adult population, we recommend levels of above 50 nmol/l throughout the year (ref 19), in accordance with other Nordic recommendations (refs 6 and 18). In the current manuscript, our focus of interest is whether the provided supplemental dose would suppress PTH and maintain normal bone turnover in the absence of sun exposure.
We have now modified the following line in the manuscript (page 3, lines 12-14):

"However, there is uncertainty as to which supplemental dose is needed in order to maintain 25(OH)D levels associated with PTH suppression and normal bone metabolism."

2) Line 15 the authors state that 12.5 ug of vitamin D are required to maintain vitamin D status in wintertime…. To maintain it at what level?

Reply: We have looked into the cited paper by Heaney and colleagues, and thus modified the phrase into the following (page 3, lines 14-16):

"In an experimental study performed in Omaha (41°N) it was estimated that daily oral supplementation with 12.5 µg (500 IU) may be required in order to maintain autumn s-25(OH)D levels of 70 nmol/l through winter [7]."

3) Were the data normally distributed? If not it may be better to present median or geometric mean and analysis should be on transformed data. The statistical analysis should be reconsidered. Rather than using t-tests it would be much better to use an ANCOVA and thereby analyse all the data together to look for between and within group changes.

Reply: We have now scrutinized histograms, Q-Q plots, and P-P plots of the distribution of our dependent variables: ∆25(OH)D, ∆PTH, ∆1,25(OH)2D and ∆TRACP. There were no major deviation from normality, although there was a slight deviation for ∆1,25(OH)2D. When substituting the tests in Table 2 with their corresponding non-parametric options (Wilcoxon signed rank test and Mann-Whitney U test, respectively), we obtained similar results, and the conclusions were unaltered. Therefore, we have kept the means and the parametric tests.

The magnitude of the unadjusted individual pre- and post-intervention levels regardless of covariates, presented in Table 2, were compared using paired samples t test. The purpose of this was to take account of the individual changes during the intervention period, as there were large individual variation in baseline levels as well as increase. Beyond this, we feel that the linear regression analysis (presented in Table 3) takes care of what you are asking for. The t test used to compare the two supplement groups in Table 2 is equivalent to the ANCOVA or linear regression, only we are in the situation where there are simply two levels to compare (fish oil vs. multivitamin) as opposed to several groups or continuous variables.

We have now removed the following statement from page 8:
" The distribution of ∆25(OH)D was slightly skewed and median ∆25(OH)D was 36 nmol/l."

We have now included the following statement in the statistical analysis section, page 7 lines 6-8:
"According to normality plots, the dependent variables: ∆25(OH)D, ∆PTH, ∆1,25(OH)2D and ∆TRACP did not deviate substantially from a normal distribution, and non-parametric tests yielded similar results. Therefore, means and distributions are presented..."

We have also modified the following lines on page 7 lines 9-11:
"Pre- and post-intervention concentration of biochemical parameters were compared by paired samples t tests. Crude differences between the two intervention groups were tested using t test."

We have also modified the footnote to Table 2, page 17:
"Paired-samples t test for comparison of individual pre- and post-intervention concentration."
4) Was #25(OH)D associated with #1,25 or #PTH or indeed change in any other biochemical markers. I think that a lot of table 3 could be omitted and the significant findings reported in the text.

Reply: We feel that the data in Table 3 represent a substantial point in our manuscript, because this data follows up one of the two equally important objectives stated on page 4: to investigate whether the magnitude of change in s-25(OH)D and the other metabolites was predicted by ethnic background, body mass index, age, gender, or serum concentrations at baseline. Although it is based on small numbers and yields few significant findings, it may for example be of interest for future meta-analyses. If desired, we propose to include Table 3 as supplementary web material, i.e. not included within the main article manuscript.

5) Page 11 line 18 states that lower age was a significant predictor of a larger increase in 1,25, but this does not appear significant in the table.

Reply: Thank you for your observation; you are absolutely right. The statement is based on an earlier analysis of predictors of change in serum parameters that was adjusted for baseline level of the parameter. However, the decision to present unadjusted results in Table 3 for comparison of non-randomised variables was made, and in this analysis age was not a significant predictor of increase in 1,25(OH)2D. There is, however, indeed a tendency of a higher increase in 1,25(OH)2D in younger participants in our data, revealed by the scatter plot below (lowess curve included) with a Spearman's correlation coefficient of −0.3 (p=0.035). However, out of consideration for the next comment of this Reviewer (point 6), we will avoid discussing results that are not significant. We have therefore deleted the statement.
6) The authors should remove reference to trends in the data.

We have now deleted the following statements from the manuscript:

Page 8: "although it decreased slightly more in the multivitamin group"

Page 8: "The increase was slightly higher in the fish oil group, although this was not significant."

Page 9: "although the increase tended to be inversely related to baseline s-25(OH)D"

Page 9: "There was a tendency to an association between change in s-iPTH and baseline vitamin D status (Table 3). Although with large individual variation, stronger PTH suppression after supplementation was observed in those who had lower vitamin D status at baseline (Figure 1)."

Page 12 (Conclusion): "a tendency to an increase in s-1,25(OH)2D,"

The following line on page 8 lines 23-24 has now been modified:

"There was an overall borderline statistically significant increase in s-1,25(OH)2D."

The following line on page 9 lines 7-8 has now been modified:

"The changes in the biochemical parameters (except 25(OH)D) were inversely associated with their baseline concentrations."

7) The authors should comment on other possible nutrient effects, both supplements contained vitamin A, could this have had an impact on bone turnover?

Reply: We have now added the following paragraph to the discussion section, page 12, lines 1-6:

"It cannot be excluded that an independent effect of the supplementation on bone turnover regardless of the observed decrease in PTH could be brought about by factors other than vitamin D. Both intervention supplements contained vitamin A (retinol). Although the evidence of an effect of vitamin A on bone health is inconsistent, some in vitro studies have shown that retinoic acid directly stimulate osteoclastic bone resorption, and high intakes and serum levels of retinol have been associated with reduced bone mineral density or increased fracture risk in some population-based studies [28]."

We have included the following reference (page 15):

Minor revisions:

8) Vitamin D status appears quite good for a population who have been in the dark in terms of vitamin D synthesis for a number of months. Were individuals who went on holidays to sunny destinations included in the intervention?

Reply: No, they were not. As stated in the Methods section (page 5, lines 10-13), persons who already took a vitamin D supplement once a week or more, or had been travelling to sunny areas or used a tanning bed during the previous three months, were defined as ineligible to participate. This was assessed through self-report, by distributing a simple questionnaire prior to enrollment of subjects. The participants were also instructed to avoid such behavior during the course of the 4-wk intervention period. The intervention was completed prior to Easter which would take place in mid-April, when sunlight availability was expected to increase, especially in the snow-abundant mountain areas that are favored among Norwegians during Easter holiday. (It may be added that the majority of the fair-skinned ethnic Norwegian participants who would be expected to seek sun during spring completed the intervention in mid-March). It was a key goal to minimize any potential confounding of sun exposure in our study.

However, it has previously been observed that vitamin D status is relatively high in Norway and other Scandinavian countries, in spite of their high latitude. See for example review by P. Lips, J Steroid Biochem Mol Biol 2007;103:620-5 (PMID: 17287117). This is probably partly due to its coastal location with a longstanding tradition of fatty fish consumption. Common meals and bread spreads marketed in Norway are based on oily fish. In addition, all butter and margarine, as well as certain types of milk, cheese, and cooking oil marketed in Norway are currently fortified with vitamin D, based on previous findings of low vitamin D status in certain risk groups (elderly, children, and non-western immigrant groups) (ref 19). Due to the extensive media attention on vitamin D and its potential health effects, there is reason to believe that particularly health-conscious individuals ensure an adequate dietary vitamin D intake. It should be noted that a large proportion of our study sample were medical and nursing students, probably more health-conscious than the general population.

We have now included the following line in the Methods section (page 5 lines 9-10):

"The four-week intervention was thus completed in mid-April by the latest, i.e. prior to Easter holidays when sunlight exposure would be expected to increase."

In addition, we have included the following paragraph in the Discussion section (page 10 lines 5-9):

"In spite of the strict exclusion criteria, vitamin D status at baseline was not very low in ethnic Norwegian participants. A large proportion of the study sample were medical and nursing students, probably more health-conscious than the general population, and may have had a relatively high intake of vitamin D intake during winter."

9) Can the authors confirm from previous research that a four week intervention is sufficient to observe changes in bone turnover markers.

In trials with anti-osteoporosis drugs affecting bone turnover (teriparatide or bisphosphonates), rapid changes in biochemical bone turnover markers are observed, commonly after 1 month. See for example Blumsohn A et al., Osteoporos Int 2011; 22:1935-46, PMID 20938767; Burshell AL et al., Bone 2010; 46:935-9, PMID 20060081.

Such a strong effect is not commonly seen with vitamin D supplementation. In a study providing 500 µg of oral 25(OH)D3 monthly to women 24-72 years, a decrease in serum bone alkaline phosphatase was observed at day 30 (Russo et al., Calcif Tissue Int 2011;89:252–257. PMID: 21701937). The intervention is not directly comparable to our study.
In other vitamin D supplementation studies more similar to ours, bone turnover was not affected in spite of higher dosage and/or longer duration (refs 11 and 13). As stated on page 11, in the Finnish supplementation study published in 2009 (ref 8), TRACP5b, the same bone turnover marker as measured in the present study, decreased during wintertime in adult Finnish men regardless of whether they received placebo, 10, or 20 µg vitamin D. The bone formation marker bone alkaline phosphatase decreased in the groups receiving vitamin D supplementation but was unaltered in the placebo group.

C) COMMENTS FROM REVIEWER GUNNAR SIGURDSSON

1) Major Compulsory Revisions
The study subjects were recruited primarily among medical and nursing students in Oslo, ranging from the age 19-48, but 40% were younger than 25 years of age. The intervention started in mid-February 2005, but it is not mentioned how long the incubation period lasted, which could be of significance with regard to s-25(OH)D values.

Reply:

Information meetings and invitation of participants was a process that started in January 2005. The eligibility criteria for inclusion (cf. reply to point 8 of the previous reviewer) were already then communicated to potential participants, and inclusion into the study was based on a simple questionnaire prior to enrollment of subjects. At this stage, we excluded those who reported use of tanning beds, had been on sun holidays, or used vitamin D containing supplements during the previous three months. All ethnic Norwegian participants recruited through medical or nursing school attended baseline examination within a period of four days during 14-17 February 2005. All participants recruited through the Tamil Resource and Counselling Centre in Oslo attended baseline examination within a period of four days during 11-14 March 2005.

At 60 degrees north, cutaneous vitamin D synthesis will be absent for several months during winter, designating the "vitamin D winter" (Webb et al., JCEM 1988 (PMID 2839537); Engelsen et al., Photochem Photobiol 2005 (PMID 16354110)). We believe that the observed s-25(OH)D values measured at baseline examination reflect steady-state wintertime vitamin D status in individuals living in Oslo who have not been exposed to sun or taken vitamin D containing supplements during the previous three months. Baseline concentration of serum 25(OH)D differed between randomised groups. For that reason, the increase in serum parameters according to randomised groups was adjusted for baseline levels, as recommended when analyzing results in randomised studies (Vickers & Altman: Statistics notes. Analysing controlled trials with baseline and follow-up measurements. BMJ 2001;323:1123-4. PMID: 11701584)

We have now replaced the clauses containing the expressions "Mid-March" and Mid-February" with the following line in the Methods section (page 5 lines 6-9):

"The participants recruited through medical or nursing school attended pre-test baseline examination within the period 14-17 February, and the participants recruited through the Tamil Resource and Counselling Centre attended within the period 11-14 March."
2) Results
The participants completed a questionnaire at baseline, including vitamin D containing foods, and the number of participants taking different items is listed as percentage in table I. However, no estimate of total vitamin D intake is mentioned, which might be of interest, especially in comparison between the ethnic groups in that respect.

Reply: This is true. The questionnaire at baseline was not sufficiently detailed to calculate total intake of vitamin D. However, for the current purpose and given the randomised design, we feel that the levels of 25(OH)D measured in serum would be the most direct and valid measure of the steady-state vitamin D status of our participants during winter. This metabolite is relatively stable with a half-life of 3-4 weeks (P. Lips, JBMR 2007;22:1668-71. PMID: 17645404) which implies that during late winter in Norway it will reflect vitamin D supply from the usual diet. The participants were encouraged to maintain their usual diet during the study period.

We have now added the following line on page 5 line 28:

"Participants were encouraged to maintain their usual diet during the study period."

3) The main increment in s-25(OH)D in both study groups was similar, about 8 nmol/L per 100 units of vitamin D which is considerably higher than many other studies have found. This might suggest other effect than of the supplementation per os. Therefore it is of importance to clarify the time period of the study, when the last blood samples were taken.

Reply: As described, the four-week intervention was completed prior to Easter which would take place in mid-April, when sunlight availability was expected to increase, especially in the snow-abundant mountain areas that are favored among Norwegians during Easter holiday. (It may be added that the majority of the fair-skinned ethnic Norwegian participants who would be expected to seek sun during spring completed in mid-March). It was a key goal to minimize any confounding effect of sun exposure in our study.

The duration of the intervention period was four weeks (28 days; one capsule or tablet taken each day); the first blood sample was drawn at baseline (day 1) and the second blood sample was drawn on the day following the ingestion of the final tablet/capsule (day 29). The participants received an appointment for their second visit exactly 28 days after their first blood sample according to the calendar. Participants who for any reason were not able to meet on the expected date, were excluded from the analyses if they met more than two days early or late. This only applied to two participants who met three days prior to the schedule for the second blood sample, as stated on page 8 lines 5-6. This was carefully described and discussed in our previous publication where increase in s-25(OH)D according to type of supplement was the primary focus (Holvik et al., Br J Nutr 2007;98:620-5, PMID: 17456248).

As stated on page 3 lines 19-21 in the current manuscript and also discussed in our previous publication, we estimated from the review by Vieth (ref 9) that the average increase in s-25(OH)D in studies where a daily dose of 10 µg (400 IU) was provided, was 31 nmol/l (about 8 nmol/l per 100 units). However, there were large variation between different studies, suggesting that additional factors may influence the degree of increase. In our Background section, we provide rationale for investigating whether various factors influence the increase. We believe age may be an important factor. Most studies have been performed in elderly populations and not in healthy young individuals. Only one of the summarized studies in ref 9 had provided a small dose of oral vitamin D to healthy young adults. In that study, nine individuals with mean age 21 years received 10 µg (400 IU) daily for a period of 2.3 months, and their mean increase in serum 25(OH)D was 41.5 nmol/l, corresponding to
10 nmol/l per 100 units (Davie et al., Clin Sci 1982;63:461-72, PMID: 6288317). Also, as quoted on page 3, a more recent study from Northern Ireland providing 15 µg vitamin D3 to healthy students 18-27 years for 8 weeks during late winter achieved a mean increase in s-25(OH)D of 38.6 nmol/l, corresponding to 6 nmol/l per 100 units (ref 11). We do believe that the increase in our population is a realistic effect of the supplementation. However, we feel that this has been discussed in our previous publication.

We have now included the following line in the Methods section (page 5 lines 9-10):

"The four-week intervention was thus completed in mid-April by the latest, i.e. prior to Easter holidays when sunlight exposure would be expected to increase."

4) The authors found a significant decrease in s-iPTH and the decrease did not differ significantly by type of supplement. They observed a large variation in the increment in s-1,25(OH)2D. As an estimate of bone turnover they used s-TRACP but no other estimates such as alkaline phosphatase or osteocalcin. They found that s-TRACP increased significantly during supplementation. This assay measures the active isoform 5b derived from osteoclasts and as the authors mention in the discussion, this enzyme is specifically a marker of number of osteoclasts rather than of their resorptive activity. The Tamils had higher baseline s-TRACP and had higher increase during supplementation which the authors claim was not significant when adjusting for higher baseline values. As other studies have shown, they found a stronger PTH suppression after supplementation in those who had lower vitamin D status at baseline (fig.1). From those results the authors conclude that the effect of four weeks of daily supplementation with 10 µg (400 IU) of vitamin D3 did not differ by mode of administration. This conclusion with regard to decrease in s-iPTH seems to be sound. However, the conclusion that there is an increase in bone turnover as measured by s-TRACP is another matter as the study group was so heterogeneous in terms of age, ethnic background and baseline values in s-TRACP. Further comparison between the sub-groups (which might be difficult to perform because of small numbers) is needed to justify such conclusions. As mentioned above and the authors refer to, young people differ in the response and participants with extremely low s-25(OH)D (possibly with evidence of osteomalacia) respond differently, such as is known from the alkaline phosphatase flare in such individuals. Without other measurements of bone turnover it is hardly justified to make such conclusions but the results may show such tendency with regard to s-TRACP whatever that means.

Reply:

We acknowledge that it would be desirable to include several bone turnover markers as outcome in our study. The decision to measure serum TRACP 5b was based on basic knowledge of bone metabolism and recommended by endocrinologists. Due to the short follow-up time, we expected any effect of supplementation likely to be observed in indicators of resorption rather than formation. This is discussed on page 10.

We have now replaced the expression "bone turnover as measured by circulating TRACP" with simply "circulating TRACP" in our conclusion, page 12 line 26.

We have also replaced the expression "bone turnover" with "TRACP" in a subheading in the Results section, page 8 line 20.
D) OTHER CORRECTIONS MADE TO THE MANUSCRIPT

We have corrected an error detected on page 9, line 11:

"This was not significant when adjusting for the lower baseline s-TRACP in Tamils (p=0.07)."

For Table 3, page 18, we have moved the citation "(1)" from the heading into the first row.