Author's response to reviews

Title: Correlation between vitamin D and cardiac natriuretic peptide levels in vitamin D deficient women

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Author's response to reviews: see over
Dear Mr. Dunckely,

We have made formatting changes to the manuscript as recommended and provided the following details:

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

HS was responsible for overall design, administration and coordination of the project, analysis of results, and writing the manuscript; MGN organised measurements of NT-proBNP and PRA, and participated in analysis of results and writing the manuscript; CF performed the statistical analysis and participated in analysis of results and writing the manuscript; SB and JY were responsible for sample preparation and biochemical analyses.

All authors read and approved the final manuscript.

**Acknowledgements**

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We would also like to thank the reviewers for their insightful and helpful comments. We have modified the manuscript extensively as suggested by the reviewers and we now also report on the correlation between NT-proBNP and 25(OH)D at baseline in 63 nulliparous women (we did not have sufficient samples to measure NT-proBNP after vitamin D supplementation to nulliparous women). Although we found no significant correlations between 25(OH)D or PTH with NT-proBNP and PRA at baseline and following vitamin D supplementation we believe that the lack of correlation is definitely worth documenting. We also present original data on postpartum levels of NT-proBNP. Previous studies have assessed levels up to 28 hrs postpartum. Below is a point-by-point response to reviewers.
Reviewer 1: Dr. Vin Tangpricha

Reviewer's report:
Overall, I have no major comments to this manuscript. The major strengths of the manuscript are the investigation of vitamin D in an emerging area in the vitamin D field. The weakness is that the renin levels and BNP levels were not elevated at baseline, the levels of 25-hydroxyvitamin D did not reach levels >75 nmol/L, which is now considered an optimal level and the study design (i.e. a post-hoc evaluation of previously collected samples and lack of a control group). The authors have discussed these issues in their conclusions.

Suggested Revisions
Would suggest a table for baseline demographics (height, weight, vitals, ethnicity, vitals, co-existing medical conditions or medications, etc) of the study population. This would be important to know which subjects were under investigation.

Response: A table of baseline characteristics has been added.

Would suggest reporting blood pressure and pulse recordings before and after vitamin D.

Response: Mean systolic and diastolic blood pressure before and after vitamin D supplementation is now included in Table 3.

If PTH levels are available, it would be useful to have this information as an additional marker for vitamin D status.

Response: Mean serum concentrations of PTH before and after vitamin D supplementation is now included in Table 3.

Would be good to new if there were any differences in those subjects that had samples available for analysis and those who did not have samples for analysis. This would be useful to avoid the potential of selection bias.

Response: A statement regarding significant differences in baseline characteristics among subjects based on availability of sufficient stored samples is now included in the results section.
Reviewer 2: Dr. Armin Zittermann

Reviewer's report:

Major compulsory revisions

The major problem with this investigation is its study design. This was not a randomized placebo controlled trial. Note that there are profound hormonal changes postpartum, including sex hormones and the vitamin D hormone calcitriol. Therefore, the reason for the decline in NT-proBNP remains unclear and cannot be related to vitamin D supplementation.

Response: We fully agree that the reason for the decline in NT-proBNP cannot be definitively attributed to vitamin D supplementation. We have reanalyzed our data and found that in fact the decline is most probably related to postpartum blood volume changes and not vitamin D supplementation. We now also report on the correlation of NT-proBNP and 25(OH)D at baseline in 63 nulliparous women (we did not have sufficient samples to measure NT-proBNP after vitamin D supplementation to nulliparous women). Although we found no significant correlations between 25(OH)D or PTH with NT-proBNP and PRA at baseline and following vitamin D supplementation we believe that the lack of correlation is worth documenting. We also present original data on postpartum levels of NT-proBNP. Previous studies have assessed levels up to 28 hrs postpartum.

In the discussion section we now state the following: “Our results show no significant correlations between baseline 25(OH)D concentrations and NT-proBNP levels in vitamin D deficient nulliparous women or between baseline 25(OH)D concentrations and NT-proBNP and PRA in vitamin D deficient lactating women. Although vitamin D administration over a 2-month period in lactating women was associated with a statistically significant decline in NT-proBNP levels and a non-statistically significant decline in PRA, there were no significant correlations between the change from baseline in 25(OH)D concentrations or PTH with the change from baseline in NT-proBNP and PRA levels. This suggests that the decline we observed in NT-proBNP is unlikely related to vitamin D administration but is probably related to postpartum blood volume changes. Pregnancy represents a state of physiologic volume expansion as maternal blood volume increases ~40%–45% above non-pregnancy volumes [24]. By 1 week after delivery, the blood volume returns nearly to its non-pregnancy value [25]. NT-proBNP levels, on the other hand, increase by 2-fold within the first 28 hours after delivery suggesting a role in postpartum diuresis [26]. Our results suggest that NT-proBNP levels drop quickly thereafter until they reach a plateau at ~14 days postpartum.” In the conclusion section we also state “We found no significant correlations between 25(OH)D or PTH with NT-proBNP and PRA in vitamin D deficient women. There were also no significant correlations between the change from baseline in 25(OH)D concentrations or PTH with the change from baseline in NT-proBNP and PRA levels following vitamin D administration over a 2-month period. Further information is required to clarify the effects of vitamin D administration on cardiac structure and function and prevention of cardiovascular disease.”

The rationale for this investigation is unclear. Elevated levels of NT-proBNP are found in patients with cardiac diseases. However, these lactating women were apparently healthy. Others (Schleithoff et al., Am J Clin Nutr, 2006) did not find a decrease in NT-proBNP levels after
vitamin D supplementation in patients with high NT-proBNP levels. Why has this article not been cited in the present manuscript?

Response: We referred to the above mentioned work and added the following statement in the discussion section: “Other investigators also found that vitamin D administration for patients with congestive heart failure improved pro-inflammatory and anti-inflammatory cytokine levels but there was no significant decline in NT-proBNP levels [21].” Under limitations we also stated that “… neither PRA nor NT-proBNP was elevated at baseline, making physiological significance an unresolved issue.”

The mean increase in 25-hydroxyvitamin D, which is the hallmark for determining vitamin D status, was relatively small (approximately 12 nmol/l). The mean 25-hydroxyvitamin D concentration was still in the insufficiency range (< 50 nmol/l) after 2 months of oral vitamin D2 supplementation. This seems surprising, since others have demonstrated that an oral daily vitamin D supplement increases 25-hydroxyvitamin D concentrations by 60 nmol/l (Schleithoff et al., Am J Clin Nutr, 2006). The oral bolus of 1500 µg should also have been high enough to result in a higher mean increase in serum 25-hydroxyvitamin D than only 12 nmol/l. The most likely explanation for this surprising result is the fact that they used vitamin D2 instead of vitamin D3. There is evidence that the increase in serum 25-hydroxyvitamin D is lower after vitamin D2 supplementation compared to vitamin D3 supplementation (Trang et al., Am J Clin Nutr 1998). Vitamin D2 should not be regarded as a nutrient suitable for supplementation or fortification (Houghton & Vieth R, Am J Clin Nutr, 2006).

Response: We generally agree with this statement. We have added the following statement in the discussion section: “Vitamin D2 supplementation with 2000 IU daily or 60,000 IU monthly for 2 months in this study increased serum 25(OH)D concentrations significantly but these concentrations reached an acceptable level (≥50 nmol/L) in only a small proportion of studied women [14]. Although the mean increment observed in 25(OH)D concentration in our study was slightly higher than that reported by other investigators [28] (0.4 nmol/L per 100 IU of vitamin D2) it remained lower than that reported for equimolar doses of vitamin D3 [29-31]. This could be related to greater potency of vitamin D3 compared to vitamin D2 [32, 33] although this has been recently questioned [34].”

Minor essential revisions

I am missing some important publications concerning vitamin D and cardiovascular disease (Zittermann et al., Br J Nutr, 2005) and natriuretic peptides (Zittermann et al., J Am Coll Cardiol, 2003; Schleithoff et al., Am J Clin Nutr, 2006).

Response: The above references were added.

Patients and Methods section, page 4, 25(OH)D measurement: Note that 25(OH)D is not a hormone.

Response: Above was modified.

Response: We clarified that the assay used measures both 25(OH)D2 and 25(OH)D3 equally.

Results section: The effect of vitamin D administration on serum 25-hydroxyvitamin D should be given separately for the groups that were supplemented with daily vitamin D2 and with the oral vitamin D2 bolus, respectively.

Response: Table 3 now replaces the figure and stratifies subjects by the type of vitamin D regimen.
Reviewer 3: Dr. Erin D Michos

Reviewer's report:

MAJOR COMMENTS
This study suffers from 2 major limitations which I think severely limit the interpretation and utility of this study. The authors mention both of these problems in their discussion but warrants further discussion as these are critical limitations. Perhaps if you could pull from other references (if available), it might help boost the meaning of your findings, but right now I am not sure we can make any useful inferences based on these major study design limitations.

1. There was no control arm of lactating women who did not receive any vitamin D. We have no idea that the fall in NT-proBNP levels was directly related to the vitamin D supplementation (vs some other factor such as weight loss as the women were farther and farther postpartum with time). Are there any other studies that looked at cardiac natriuretic peptide levels in peripartum women that we can extrapolate from? Why did you choose to do this study in the lactating women rather than the nulliparous women in your reference 8? In the nulliparous women, it would have been more believable that a fall in NT-proBNP might be related to vitamin D supplementation since you could anticipate their weights to be relatively stable over a 2 month period. But 25(OH)D levels are strongly associated with weight, so in lactating women postpartum, I have no reason to believe that a change in NT-proBNP has anything to do with vitamin D supplementation vs simply time from delivery.

Response: We fully agree that the reason for the decline in NT-proBNP cannot be definitively attributed to vitamin D supplementation. We have reanalyzed our data and found that in fact the decline is most probably related to postpartum blood volume changes and not vitamin D supplementation. We now also report on the correlation of NT-proBNP and 25(OH)D at baseline in 63 nulliparous women (we did not have sufficient samples to measure NT-proBNP after vitamin D supplementation to nulliparous women). Although we found no significant correlations between 25(OH)D or PTH with NT-proBNP and PRA at baseline and following vitamin D supplementation we believe that the lack of correlation is worth documenting. We also present original data on postpartum levels of NT-proBNP. Previous studies have assessed levels up to 28 hrs postpartum.

In the discussion section we now state the following: “Our results show no significant correlations between baseline 25(OH)D concentrations and NT-proBNP levels in vitamin D deficient nulliparous women or between baseline 25(OH)D concentrations and NT-proBNP and PRA in vitamin D deficient lactating women. Although vitamin D administration over a 2-month period in lactating women was associated with a statistically significant decline in NT-proBNP levels and a non-statistically significant decline in PRA, there were no significant correlations between the change from baseline in 25(OH)D concentrations or PTH with the change from baseline in NT-proBNP and PRA levels. This suggests that the decline we observed in NT-proBNP is unlikely related to vitamin D administration but is probably related to postpartum blood volume changes. Pregnancy represents a state of physiologic volume expansion as maternal blood volume increases ~40%–45% above non-pregnancy volumes [24]. By 1 week after delivery, the blood volume returns nearly to its non-pregnancy value [25]. NT-proBNP levels, on the other hand, increase by 2-fold within the first 28 hours after delivery suggesting a role in postpartum diuresis [26]. Our results suggest that NT-proBNP levels drop quickly
thereafter until they reach a plateau at ~14 days postpartum.” In the conclusion section we also state “We found no significant correlations between 25(OH)D or PTH with NT-proBNP and PRA in vitamin D deficient women. There were also no significant correlations between the change from baseline in 25(OH)D concentrations or PTH with the change from baseline in NT-proBNP and PRA levels following vitamin D administration over a 2-month period. Further information is required to clarify the effects of vitamin D administration on cardiac structure and function and prevention of cardiovascular disease.”

2. You said that these lactating [and thus I presume they were young (although no mean age was given)] women had normal levels of NT-proBNP and PRA at baseline. Thus I suspect they did not have clinical heart failure at baseline. Can you reference any data that suggests that lowering cardiac natriuretic peptide levels in women without heart failure and with normal baseline levels has any clinical benefit?? In your dialysis population (reference 19) which already has elevated levels, I can see how this might be beneficial. But in normal women, what is the clinical utility of lowering levels even further?? You didn’t even find an association between 25(OH)D levels and cardiac natriuretic peptide levels in this cohort. As you state yourself, “physiological significant an unresolved issue.”

Response: We generally agree with this statement and we clearly state under limitations that “.. neither PRA nor NT-proBNP was elevated at baseline, making physiological significance an unresolved issue.” Our study however should be viewed on the basis of “proof of concept”. We also added the following statement in the discussion section: “Other investigators also found that vitamin D administration for patients with congestive heart failure improved pro-inflammatory and anti-inflammatory cytokine levels but there was no significant decline in NT-proBNP levels [21].”

Other MAJOR Comments
3. There is no Table I with the baseline characteristics such as important characteristics such as mean age, blood pressure, BMI – all of which are factors which are associated with 25(OH)D levels. This is a gross oversight – as how are we to extrapolate the results to other populations if this study population is not even described! While reference is made to earlier publication (reference 8), that study had 90 lactating and 88 nulliparous women while this only had around ~50 lactating women, so it is not exactly the same cohort sample. Thus we should have some summary of clinical characteristics of the women included in these analyses.

Response: A table of baseline characteristics has been added.

4. You said in your methods that this was a randomized clinical trial. Lactating women were randomized to receive oral vitamin D2 either 2,000 daily or 60,000 IU monthly. Was there any difference in the cardiac natriuretic peptides between the daily dosing vs monthly dosing? Did one vitamin D regimen raise 25(OH)D levels or lower NT-proBNP levels better than the other regimen?
**Response:** Table 3 now replaces the figure and stratifies subjects by the type of vitamin D regimen and shows that in both vitamin D regimens, the changes in 25(OH)D and NT-proBNP were significant.

5. **While there was no association of 25(OH)D with NT-proBNP or PRA at baseline, was there any correlation between 25(OH)D levels with NT-proBNP and PRA at followup? This should be reported. If there is no correlation, then if the reduction in NT-proBNP is causally related to vitamin D therapy (and not just time postpartum), then how do you explain this?**

**Response:** We reanalyzed our data and now report the correlations between the change from baseline in 25(OH)D and other parameters. We now clearly state that “There were also no significant correlations between the change from baseline in 25(OH)D concentrations or PTH with the change from baseline in NT-proBNP and PRA levels following vitamin D administration over a 2-month period. As we stated above although we found no significant correlations between 25(OH)D or PTH with NT-proBNP and PRA at baseline and following vitamin D supplementation we believe that the lack of correlation is worth documenting.”

**Minor comments**

6. **In the methods section, you describe where the assays for NT-proBNP and 25(OH)D were run and the interassay variability. However you do not mention how/where the samples for plasma renin were measured and the interassay variability for PRA.**

**Response:** Above clarified.

7. **In the United States and many European countries, vitamin D levels vary dramatically by season. Thus 25(OH)D analyses are often adjusted by season of lab draw. Did you see any seasonal variability of 25(OH)D levels in your cohort?**

**Response:** We added the following statement in the discussion section: “Our current study did not evaluate seasonal changes in serum 25(OH)D concentrations but our previous studies showed no significant seasonal variation in 25(OH)D concentrations between September and February in the UAE, where there is abundant sunshine year-round [15].”
Reviewer 4: Dr. Thomas J Wang

Reviewer's report:

Major compulsory revisions
1. In both the introduction and discussion, the authors imply that the reduction in Nt-proBNP with vitamin D administration reflects an improvement in cardiac function. However, as they acknowledge, this conclusion is largely speculative given the lack of supporting echocardiographic or other cardiac functional data. Indeed, based on the experimental studies, changes in Nt-proBNP (and PRA) could mainly reflect direct effects on gene transcription without specific changes in cardiac function or structure. The authors should consider a more nuanced discussion of these various possibilities, which might have different implications when it comes to the hypothesis that vitamin D deficiency is a cardiovascular risk factor.

Response: We agree with the above point that the decline in NT-proBNP (and PRA) could mainly reflect direct effects of vitamin D on gene transcription without specific changes in cardiac function or structure. However our re-analysis of the data cast doubt on any significant correlations between 25(OH)D and NT-proBNP and therefore the above point was not stressed in the revised manuscript. In the introduction section however we now discuss the potential mechanisms underlying the link between vitamin D deficiency and heightened risk of hypertension and cardiovascular disease and state the following “Potential mechanisms that may explain this link include involvement of vitamin D in the regulation of the renin-angiotensin system [9-11] and the negative vascular effects of secondary hyperparathyroidism [12]. In addition, activation of nuclear vitamin D receptors present in the myocardium can inhibit cardiac growth and hypertrophy whilst also suppressing the manufacture and secretion of the cardiac natriuretic peptides in both atrial and ventricular myocytes [13].”

2. Basic information on enrollment criteria and demographics would be useful to provide so that readers do not have to rely on the reference. Also, although echo data are not available, the authors should provide other clinical information relevant to the cardiovascular system, at baseline and follow-up, including blood pressure, presence of diabetes, etc. These data could be provided in a Table. Levels of glucose, insulin, and hsCRP would also be interesting to see, if available, given the connections between the vitamin D axis, insulin resistance, and inflammation.

Response: A table of baseline characteristics has been added. Levels of glucose, insulin, and hsCRP were unfortunately not available.

3. Do the investigators have any information on determinants of vitamin D status, including diet, sun exposure, and/or use of other supplements? There seems to be a substantial degree of vitamin D deficiency in this study sample.
Response: More information on the determinants of vitamin D status is now included in the results (in Table 1) and a whole paragraph is now included in the discussion on vitamin D deficiency.

4. The authors should note whether any differences (in Nt-proBNP or PRA response) existed between the 2 vitamin D arms.

Response: Table 3 now replaces the figure and stratifies subjects by the type of vitamin D regimen and shows that in both vitamin D regimens, the changes in 25(OH)D and NT-proBNP were significant.

5. More information should be provided regarding the statistical tests used. Nt-proBNP concentrations typically have a skewed distribution, and thus may be less suited for standard parametric tests; did the investigators consider log transformation or non-parametric tests?

Response: We agree with this point. Indeed, NT-proBNP concentrations had a skewed distribution. Therefore, non-parametric tests were used throughout and all the statistical tests used are now clearly stated in the Methods section.
Reviewer 5: Dr. Michael Holick

Reviewer's report:

Suggestions

1. The observation is interesting. However, as noted by the authors, the normal reference range for 25(OH)D is 50-150 nmol/L. All of the subjects appear to be severely vitamin D deficient and 2,000 IU of vitamin D/d or its equivalency on a monthly basis did not raise the blood level to at least 50 nmol/L which is considered to be the absolute cut off for vitamin D deficiency. Most experts now agree that a blood level of > 75 nmol/L is a more desirable level. The results suggest that the baseline levels were < 50 nmol/L, but also at the end of the study, they remained < 50 nmol/L. For the women that may have been above 50 nmol/L, or more importantly > 75 nmol/L, was there a significant decrease in the naturopathic peptide levels?

Response: We generally agree with this statement. We have added the following statement in the discussion section: “Vitamin D supplementation with 2000 IU daily or 60,000 IU monthly for 2 months in this study increased serum 25(OH)D concentrations significantly but these concentrations reached an acceptable level (≥50 nmol/L) in only a small proportion of studied women [14].” The following statement was also added under limitations “serum 25(OH)D concentrations at 2 months remained below the optimal level of 75 nmol/L in all subjects studied.”

We also stated the following in the results section: “In the 10 (18.9 %) women who achieved 25(OH)D concentrations of ≥50 nmol/L at 2 months, the changes from baseline in 25(OH)D and NT-proBNP also showed no significant correlation (r = 0.5, p = 0.2).”

2. Did the authors measure the serum calcium and PTH levels? This would be helpful, and it would be interesting to know whether the PTH levels decreased as a result of treating the patients with vitamin D.

Response: Mean serum calcium and PTH values are now shown in Table 3 and reported in the results section as follows: ”mean serum PTH concentration decreased but not statistically significantly (Table 3), and “Overall, there were no significant changes in mean serum calcium concentrations, .”

3. What these data suggests is that even 2,000 IU of vitamin D2/d for three months was totally inadequate in correcting vitamin D deficiency.

Response: We generally agree with this statement. We have added the following statement in the discussion section: “Vitamin D2 supplementation with 2000 IU daily or 60,000 IU monthly for 2 months in this study increased serum 25(OH)D concentrations significantly but these concentrations reached an acceptable level (≥50 nmol/L) in only a small proportion of studied women [14].” This was also discussed in details in one of our previous studies (Reference 14).
4. The authors should use the more standard terminology for the active form of vitamin D being 1,25-dihydroxyvitamin D rather than talking about the bioactive vitamin D which may be confusing to the reader. The authors also use circulating levels of vitamin D when they mean 25-hydroxyvitamin D and this should be changed throughout the Manuscript.

Response: The manuscript was modified as suggested.
Reviewer 6: Dr. D. Sudhaker Rao

Reviewer's report:
General: This is a small prospective interventional clinical study with 2 different doses of vitamin D replenishment. It is a well conducted albeit not necessarily well designed study. Nevertheless, the results provide a basis for “proof of concept”. The limitations of the study are appropriately acknowledged, a rarity nowadays. Finally, the study comes from a group of investigators who have published on the subject previously.

Major Compulsory: None:
Minor Essential:
1. It would be desirable if the sample recruitment is described a bit more detail. Consecutive, random, or whoever agreed to participate etc.
2. An explicit statement of ethnicity of the population would benefit the reader and also explains the high incidence of vitamin depletion.

Response: This is now described in more details in the methods section. It now reads as follows: “We recruited 88 generally healthy nulliparous Emirati women in the reproductive age group, many of whom were medical students and interns working at Tawam hospital in Al Ain city, and 90 lactating women (15 UAE, 61 other Arab, and 14 South Asian) at the time of their first postnatal visit to the Maternal and Child Health center in Al Ain city (latitude 24°N and longitude 55°E) [14]. Lactating women were eligible if they planned to continue breast-feeding for the next 3 months. Exclusion criteria included pregnancy, history of metabolic bone disease or calcium disorders and treatment with vitamin D (other than multivitamins) within the past 1 year. Enrolment began in September 2005 and finished in February 2006.”

3. Suggest moving the PRA bars, which show a non-significant change, after 25-OHD and NT-proBNP.

Response: Table 3 now replaces the figure and stratifies subjects by the type of vitamin D regimen.