Author’s response to reviews

Title: 25-Hydroxyvitamin D level is Inversely Associated with Serum MMP-9 in a cross-sectional study of African American ESRD Patients

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Author’s response to reviews: see over
Dear Editors,
The authors are gratefully indebted to the reviewers of BMC Nephrology for their helpful comments and suggestions.

You will find a submission of the revised manuscript and below the point-by-point response to the concerns.

Reviewer 1:
The only major concern is that there is an absence of information on whether or not any patients were taking vitamin D, calcitriol or its analogues [see 1. Below].

‘Major’ Points that need to be addressed.

1. The patients studied had end stage renal disease and were having renal dialysis. Many such patients would be on treatment with either calcitriol or a calcitriol analogue such as 1-alpha-calcidol. Since modest supplementation with vitamin D reduced plasma MMP-9 by a mean of nearly 70% in ‘healthy’ South Asians, [ref 18 in this MS], it is important that the reader is told whether any of these patients were taking such supplements. If they were we should be told whether the supplementation contributed to serum 25(OH)D. This is unlikely since the assay systems used for 25(OH)D would not have detected them. However, this point should be clarified and the use of any such medication reported and the effects of their use on the associations examined would need to be examined and reported. If patients on these supplements were not included in this study we should be told if this was an exclusion factor?

Of the patients in our cohort, 17 were not taking any form of vitamin D (activated or nutritional), while 3 patients were taking only nutritional vitamin D. The remaining subjects received activated vitamin D during hemodialysis. We have included this variable in our model, and rerun our analyses. In addition, we have included this information in the results section, under patient characteristics.

2. There is in fact considerable earlier work showing vitamin D to act upon MMP9 in bones, in joint tissues and in inflammatory joint problems, e.g. by Tetlow et al. some years ago, that should be mentioned in the first Para on page 5.

As requested, a reference by Tetlow had been added to the first paragraph. (Tetlow LC, Woolley DE. Expression of vitamin D receptors and matrix metalloproteinases in osteoarthritic cartilage and human articular chondrocytes in vitro. Osteoarthritis Cartilage, 2001 Jul 9:5.)

3. How long were the intervals between blood sampling for vitamin D status and for the routine biochemical testing that provided the other biochemical data including lipid profiles? If there is more than a few weeks between these samples vitamin D status is likely to have changed considerably [with season] and any associations that might exist might well be progressively weakened as this gap enlarges, thus these intervals should be given and they may need to be included as a likely confounder of the associations examined since adjustment for them could either strengthen or weaken the associations reported with vitamin D status, depending on the rates of clearance from the blood of the different factors.
The blood for all inflammatory biomarkers and vitamin D status were drawn at the same time, as part of the baseline blood work. Routine blood work (calcium, PTH, albumin, etc) are drawn monthly, and were obtained within 2 weeks of the baseline blood draw. We have clarified this in the methods section, page 7.

4. MMP9 measurement in plasma reflects tissue production, but, if serum is used the MMP9 concentrations increase greatly due to its release from white cells during clotting. It must be made crystal clear, therefore, whether plasma or serum was used for these analyses.

*Plasma was used, and this has been clarified in the methods section* (page 8).

‘Minor’ points that it would be helpful to deal with.

5. Circulating factors measured in blood, plasma or serum are measured as concentrations rather than levels. This paper uses the term concentrations correctly but in some places where ‘level’ is used, concentration would be more correct; this could usefully be tidied up with the aid of the search/find facility.

*All use of the word “level” has been changed to “concentration”.*

6. Page 8, last sentence of Para 1, Is the description of the hs-CRP kit as ‘BNII, correct?

Thank you for pointing out this oversight. The description now reads:

“The CardioPhase * hsCRP reagents from Siemens Diagnostics (Dade Behring product) were used to determine the levels of hsCRP in plasma samples on a BNII (nephelometry systems) System. The Internal Quality Controls (Controls SL/1 and SL/2 - lower-higher range of CRP) and Standard SL curve were run with the assay.”

7. Page 10, line 13, it would be useful to add ‘correlation’ before (Pearson) analysis.

We have made this change as suggested.

8. Were AVGs, associated with higher MMP9 values etc, recent? The more recent they are, the more likely that increases in plasma MMP-9 would be associated with their use rather than vitamin D?

*Unfortunately, we do not have the data to determine this.*

9. It is a pity, if no vitamin D treatment was being used in this cohort, that there is no follow-up data on the effects of vitamin D administration as there was in ref 18 where MMP-9, and hsCRP fell with supplementation, since falls in LDL-C and increases in IL-10 could
well be expected with supplementation from other published data. Such findings would add to the evidence for causality. If the authors have this data and are planning a second paper on it, I suggest it would be better to include it in this MS, or as a twin paper, in order to emphasize which of these associations may indeed be causal.

Unfortunately, we do not have follow-up data on this patient cohort. However, we are currently completing a placebo-controlled double-blind RCT of nutritional vitamin D in a dialysis patient cohort and will be able to answer this question.

Reviewer 2:

Minor Essential Revisions:
1. page 5- reference 12 is not in the US ESRD population but in the general population - you can just take out reference or add "within the US ESRD and general population"

We have made this change as suggested.

2. page 7 - under Definition of variables: it says that the values were obtained closest to the baseline blood sample – what is the range of time between the baseline bloods and the other labs. When possible, I believe it is better to have samples from before the baseline date than after (because things may change after the blood draw). Can the authors provide a time range for how close these are and how many are before and after?

The blood for all inflammatory biomarkers and vitamin D status were drawn at the same time, as part of the baseline blood work. Routine blood work (calcium, PTH, albumin, etc) are drawn monthly, and were obtained within 2 weeks of the baseline blood draw. We have clarified this in the methods section, page 7.

3. Page 9 - The sentence that starts "To adjust for these associations..." doesn't make sense to me - I think it is better to say "to adjust for possible confounding of these associations" we used... Next sentence - it should be variables significant at the "less than" 5% level.

We have made this change as suggested.

4. Under statistics - the authors should include a power calculation - what differences could they detect in their sample?

We now stress in the discussion that for two analyses (TNF-α and MMP-2), the available sample size provided a power <50% to detect a statistical difference, if any.

5. Page 10 - I'm not sure you need to point out the differences in 25(OH)D levels between <15 and >15 - isn't this fairly obvious that they should be very different?
We agree. We now provide the mean 25(OH)D values without testing the statistical difference between the two groups.

6. Overall, I think you need to add a table or columns to table 1 comparing AVG and AVFs - as they are very different usually - AVG generally sicker, older - and this may be the reason for the differences seen rather than just the difference in access.

We now provide a supplemental table with characteristics of participants by vascular access type. We report in the results section the main differences between cohorts by vascular access type.

7. Page 12 - last sentence - we did not observe a significant parallel increase in MMP-9 and CRP and concentrations? - not quite sure what this means, I think there may be an extra and - but also it should be "higher" levels not increase because it is cross-sectional - increase implies change over time

We have clarified the text (below) to more accurately reflect the cross-sectional nature of this study.

“..we did not observe significant comparable elevations in MMP-9 and CRP concentrations, as reported previously [19], likely because MMP-9 gene expression is upregulated by several factors that were not included in our analysis.”

8. Page 13 - Top paragraph - after reference 22 - would take out "Finally," because you only discuss one other reason.

The word “finally” has been removed.

9. Another reference you may want to add is Agarwal’s study in Hypertension where I think he showed lower CRPs after giving vitamin D.

This reference has been added to paragraph 2 of the discussion section. (Paricalcitol reduces albuminuria and inflammation in chronic kidney disease: a randomized double-blind pilot trial. Alborzi, P et al. Hypertension, 2008.)

10. The authors should also add a sentence that after log transforming these variables, they were normally distributed (if they were) because sometimes log transformation does not completely make variables normal.

The word “successfully” has been added, to indicate that log transformation caused a normal distribution of the variables.

11. The authors should also note whether the blood collection for this study was specifically for this protocol (was this the primary outcome looked at) or did they use available samples from another study. While the data is the same in both, if it was collected for a different study, readers may use stricter p-values because it is a secondary analysis.
The blood collection was specifically for this protocol, and this is now stated in the methods section.

Reviewer 3

Major compulsory revisions:

1. Statistical analyses need to be improved and/or explained in more detail! Some analyses are not entirely clear to me. For instance, the authors stratify the patients into those with 25(OH)D levels higher and lower than 15ng/mL and then they perform a statistical test to evaluate whether those two groups have significantly different 25(OH)D levels (?).

   We agree. We now provide the mean 25(OH)D values without testing the statistical difference between the two groups.

2. Data presentation in Table 1: The authors present e.g. a mean 25(OH)D level of 18.78 but I wonder whether their assay measures 25(OH)D that precise; I therefore guess that 18.8 would be correct, the same applies also for other parameters.

   We agree and have changed the level of precision of our results throughout the manuscript and the tables.

3. The authors categorised for some analyses patients into those with 25(OH)D levels higher and lower than 15 ng/ml and for other analyses they used three groups (<12, 12-20, >20). It is a little bit confusing for the reader to have these different classifications and it is unclear to me why the authors do not present their data consistently by the use of a single vitamin D status classification.

   We agree and now only use two groups (higher and lower than 15 ng/ml) Figure 2 has been changed accordingly.

4. MMP-2 measurements are presented in the methods section but the authors do not present further analyses on this interesting biomarker (should also be mentioned in the introduction and discussion).

   We now report on MMP-2 in the introduction, results (Table 2) and discussion sections (page 13).

5. Do the authors have data on active vitamin D treatment in these patients? If not please discuss and mention as a limitation.

   Yes, and these data are now included in the results section.

6. There was no significant difference in PTH levels between the vitamin D status groups. This also needs to be discussed because it could be expected from the literature that patients with lower 25(OH)D have higher PTH levels.
We have included the following sentence in the discussion section, page 13. “First, while PTH concentrations were greater among patients with 25(OH)D < 15 ng/dL, they were not significantly different among the vitamin D cohorts, as we would have expected to observe. possibly due to our relatively small sample size”.

7. The authors did not consider seasonal variation of 25(OH)D in their analyses. It might be interesting to see whether patients with sampling in summer had higher 25(OH)D levels compared to those with blood sampling in winter. The authors should at least discuss the possible impact of seasonal differences in 25(OH)D levels and may consider seasonal influences in their statistical analyses.

We have added season of vitamin D sampling into our model, as suggested.

Minor essential revisions:

8. Introduction: there are two rather redundant sentences: "..few studies addressed the association between 25(OH)D concentration and increased inflammation on biomarkers of vascular remodeling" and "little is known regarding the association between 25(OH)D and biomarkers reflecting vascular remodeling"

As suggested, the sentence, “little is known regarding the association between circulating 25(OH) D and biomarkers reflective of vascular remodeling in the ESRD population” has been removed.

9. The authors should carefully check for typos e.g. "25(OH) D" and for grammar and style.

This has been done.

10. What do the authors mean with "antimetabolite medications". Please specify.

Antimetabolite medications are those that inhibit a chemical that’s part of normal metabolism. Common medications include methotrexate, azathioprine, 5-fluorouracil, mercaptopurine, sulfadiazine. These are now detailed in the methods section.

11. What was the main study aim?
To test whether 25(OH)D concentration is associated with markers of vascular remodeling and inflammation in African American ESRD patients. This is stated in the abstract and introduction sections.

12. How was log transformation performed? log(10)?
Natural log. We have clarified this in the statistical analysis section.

Discretionary Revisions:

13. The authors stratify their analyses for vascular access type. Vascular access type may have an impact on inflammation per se but I wonder how vascular access type may influence the 25(OH)D inflammation link.
Tables 4 and Supplement Table S1 serve to inform the reader how vascular access type may play a role in the link between vitamin D and inflammation.

14. Was there any sample size calculation for that study?

We now stress in the discussion that for two analyses (TNF-α and MMP-2), the available sample size provided a power <50% to detect a statistical difference, if any.

15. I would like to see more discussion on the association of 25(OH)D and IL-10.

We have added another sentence to the discussion on Il-10 and vitamin D on page 12 of the discussion section.

16. The authors may want to comment on the KDIGO 2009 suggestion to correct reduced 25(OH)D levels in CKD.

We would prefer not to comment on KDIGO recommendations as they are specific to the CKD patient population, rather than to our prevalent ESRD patient population.