Author's response to reviews

Title: Higher pre-infection vitamin E levels are associated with higher mortality in HIV-1-infected Kenyan women: a prospective study

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

Re: MS: 2130750475129914
Title: Higher pre-infection vitamin E levels are associated with higher mortality in HIV-1-infected Kenyan women: a prospective study

Thank you and your reviewers for your thorough consideration of our manuscript. We have carefully reviewed the points raised by BMC editorial staff and the two reviewers, and have modified the manuscript in response to these points. Below, please find our responses to the questions raised, followed by a point-by-point listing of changes to the manuscript. In our responses to reviewer queries, the numbers in parentheses refer to changes detailed in the point-by-point listing.

REVIEWER 1
1. In this manuscript, S. M. Graham et al. established a correlation between pre-HIV infection vitamin E level in blood on one hand, and viral load 4 to 24 months post-infection and mortality on the other hand. By contrast, they found no correlation between vitamin E level and CD4 slope. This point is somewhat surprising. It is important to make sure that this absence of correlation is not a consequence of the fact that 2 different methods were used to numerate CD4+ T cells. For this purpose, the results obtained with these two methods should be compared.

As discussed in our methods section, absolute CD4 counts were measured by one of two methods (Cytosphere, Beckman Coulter Corporation, Miami, FL, USA, or Zymmune, Bartels, Inc., Issaquah, WA, USA). We switched from the Zymmune to Coulter methods when Zymmune stopped making its product in 2000. At that time, we ran duplicate samples and found a high correlation and good reproducibility between results obtained by either method (Pearson’s
correlation = 0.88, p<0.001, n=134, unpublished data). Other groups have published formal analyses of the comparability of these methods. For example, the National Heart, Lung, and Blood Institute Retrovirus Epidemiology Donor Study found correlation coefficients for absolute CD4+ counts as measured by Zymune, Coulter, and two other alternative methodologies (FACSCount, TRAx) that ranged from 0.84 to 0.92 for both fresh and 24- to 30-hour-old specimens.1 Average coefficients of variation for reproducibility ranged from 4.5 to 7.1%, and each method was considered to perform well relative to standard methods of flow cytometry. We have added a reference for this paper, and included text to clarify that these are comparable methods (3).

2. Moreover, the CD4 slopes reported are unexpectedly low. The way these slopes were calculated should be further exposed, and the reasons for their low levels discussed.

The method used to determine CD4 slopes was a simple linear regression of CD4 count on time since infection in years. All available CD4 cell counts for women having ≥2 measurements were used in this estimate (median 8 counts available). In this small study population, the median rate of decline was 25 cells/µl/year (IQR, +19 to -48 cells/µl/year). This result is similar to the one we observed when we used a mixed effects model to estimate the slope of CD4 decline in a larger subset of the cohort.2

To simplify the presentation of these data, we have reanalyzed the data and have opted to use progression to a CD4 count <200 cells/µl as an alternative marker of disease progression. As we found in the analyses presented in the original version of this manuscript, the power to detect a difference is limited by the number of women with available CD4 counts. There was no significant association between pre-seroconversion vitamin E levels and time to CD4 count <200 cells/µl. We have revised the paper to include time to CD4 count <200 cells/µl rather than the CD4 slope as the CD4 outcome measure of interest (1, 4-10, 12).

3. Finally, did the authors observe a correlation between these CD4 slopes and set point viral load? And if not why?

Each 1-unit change in log₁₀ set point viral load was associated with a decrease of an additional 15 CD4 cells per year, although this decrease did not reach statistical significance (95% CI, 43 cells decrease to 13 cells increase, p = 0.287). Because at least two CD4 cell counts (to get a slope) were available for only 52 of the 67 women in this study, our statistical power to detect associations with the CD4 slope was limited.

As noted above (see point 2), we have revised this paper to present time to CD4 count <200 cells/µl instead of the CD4 slope. Each 1-unit change in log₁₀ set point viral load was associated with a hazard ratio of 1.31 (95% CI, 0.83-2.07) for the risk for progression to a CD4 count <200 cells/µl. Again, these
results did not reach statistical significance \((p = 0.248)\), but the hazard ratio is in the expected direction.

4. The fact that none of the deaths may be linked to HIV infection is a major drawback. At least, the authors should compare the mortality in their cohort of HIV-infected sex workers with the mortality among their colleagues who did not get infected in order to link these deaths to the infection. Even though, in absence of proof that the deaths were due to the infection, it can not be ascertained that pre-infection vitamin E level is correlated with HIV-mediated mortality.

We agree that our inability to ascertain the cause of death in this study is a limitation, as we have mentioned in the discussion section. Indeed, this is a limitation that applies to most studies of HIV progression in sub-Saharan Africa, where diagnostic capacity is limited and autopsies are rarely performed. We cannot directly compare the survival of HIV-1-seronegative and -seropositive women in our cohort, as suggested by the reviewer, because we have not collected comparable survival data on non-seroconverters. Nonetheless, a recent study from South Africa provides some evidence supporting the hypothesis that HIV is causally related to death among those who are infected. The study compared mortality rates among HIV-negative mine workers versus those who seroconverted for HIV-1. The vast majority of deaths among the HIV-positive miners, from very soon after infection, were likely attributable to HIV-1 infection (as suggested by estimates of attributable risk percent). In that study, by far the largest measure of HIV-1 mortality in Africa, deaths were ascertained solely from employment rolls and national mortality indices. In our cohort, we have demonstrated that survival among HIV-positive women was similar to that among HIV-1 seroconverter cohorts elsewhere prior to antiretroviral therapy and was related to the set point viral load. We have added a citation for our survival paper to the discussion section (11).

5. The hypothesis that the link between vitamin E level and set point viral load is the consequence of the inhibition of RANTES production by the vitamin is interesting. It could be directly tested by comparing pre-infection vitamin E and RANTES levels in blood (and/or RANTES mRNA in PBMC).

We thank the reviewer for this suggestion. Unfortunately, there is not enough sample remaining in the specimens used for this study to test this hypothesis in our current study population. However, this suggestion could be tested in our laboratories at a later time on different blood samples.

**REVIEWER 2**

1. General Comments: This is a very well written manuscript that examines the relationship between blood vitamin E levels and progression of HIV among Kenyan female sex workers. There is some existing literature suggesting vitamin E levels are lower with more advanced HIV. However, vitamin E can decrease secondary to factors associated with progression of HIV, thereby
confounding the interpretation of the relationship between vitamin E and the natural history of HIV. The current study provides a unique perspective on this relationship as the investigators are able to determine vitamin E levels prior to seroconversion, given the nature of their cohort and their close follow-up. The hypothesis that vitamin E levels correlate with faster progression of HIV has a scientific basis, in light of data demonstrating that vitamin E supplementation can increase expression of CCR5. The methods are described clearly and in adequate detail; the data and data reporting seem sound. The discussion and conclusions are lucid and are supported by the results. The title and abstract provide an accurate synopsis of the study. The weaknesses of the study include the relatively small sample size and the fact that the majority of potentially eligible subjects had to be excluded for a number of reasons. These weaknesses are acknowledged by the authors in the discussion and do not represent “fatal flaws”. The major strength of the study is the recording of vitamin E status prior to HIV infection and the careful follow-up of the cohort.

We thank the reviewer for this general commentary. We hope that other readers also find this article well written and interesting.

2. Because of the potential effects of Vitamin E on CCR5, an important question the authors could address is whether vitamin E levels are associated with an increased risk of seroconversion. Although this is admittedly another question, one wonders if the investigators have examined vitamin E levels in their population of female sex workers who have remained HIV-seronegative.

We thank reviewer 2 for this suggestion, and should funding permit, may be able to test this hypothesis at a later date. As the reviewer acknowledges, this is a separate question and was not the focus of the present investigation.

The editors requested that we clarify whether written or verbal informed consent was obtained. From 1993 to 2000, we obtained verbal informed consent after participants either read or listened to a study nurse read a standardized consent document. This consent process was approved by the University of Washington, University of Nairobi, and Fred Hutchinson Cancer Research Center ethical review committees. As procedures for consent evolved internationally, we implemented written consent for all new and continuing participants beginning in 2000. We have included this longer explanation in the text, and leave to the editors’ discretion whether they prefer this longer version or the shorter statement that all participants provided informed consent (2).

We have reviewed the journal style information per the editors’ request, and carefully formatted our files according to the guidelines provided.

**Point-by-point listing of changes to the manuscript**

Changes to the manuscript are numbered in the order that they appear. Text from the manuscript is shown in italics, with revisions shown in bold italics. References are provided in square brackets, as they are numbered in the manuscript.
1. Abstract, pages 2. We have revised the sentence on statistical methods used to read “Regression analyses were used to estimate associations between pre-infection vitamin E and plasma viral load, time to CD4 count <200 cells/µL, and mortality.”

2. Methods, page 4. We have deleted the sentence stating that “all participants gave informed consent” and replaced it with this text: “From 1993 to 2000, we obtained verbal informed consent after participants either read or listened to a study nurse read a standardized consent document. Beginning in 2000, we implemented written consent for all new and continuing participants.”

3. Methods, page 5. We have revised the description of CD4 cell count determination as follows: “Absolute CD4 counts were measured by one of two methods (Cytosphere, Beckman Coulter Corporation, Miami, FL, USA, or Zymune, Bartels, Inc., Issaquah, WA, USA), which have been demonstrated to generate comparable results [12].” A reference to the methods comparison study is provided.

4. Methods, page 6. We have deleted the sentence: “The rate of CD4 lymphocyte decline was derived from the linear regression of CD4 count on time since infection in years.”

5. Methods, page 6. We have revised the sentence on linear regression performed to read “Linear regression was used to estimate associations between pre-infection vitamin E level and set point viral load,” deleting “outcomes including” and “and rate of CD4 lymphocyte decline.”

6. Methods, page 6. We have revised the sentence on Cox regression to read “Cox proportional hazards regression analysis was performed to evaluate the association between pre-infection vitamin E and time to CD4 <200 cells/µL or death.”

7. Results, page 7. We have deleted the sentence that read “The CD4 count declined at a median rate of 25 cells/µL/year (IQR, +19 to -48).”

8. Results, page 7. We have added information to the sentence on disease outcomes: “Nineteen women (28%) progressed to a CD4 count <200 cells/µL, and twelve women (18%) died during follow-up: ….”

9. Results, page 7. We changed the sentence on CD4 count outcomes to read: “No significant association was found between pre-infection vitamin E and time to CD4 count <200 cells/µL.”

10. Discussion, page 9. We revised the second sentence of the last paragraph to read “First, CD4 cell counts were not available before April 1998. Thus, for some women, the first CD4 measurement was <200 cells/µL, decreasing our ability to measure progression to this endpoint with precision.” We have changed the word “precise” to “reliable” in the following sentence to avoid redundancy.

11. Discussion, page 9. We added a sentence after the third point discussing cause of death, as follows: “However, efforts to trace women were rigorous and survival among HIV-1-seroconverters in our cohort is similar to that of other seroconverter cohorts prior to antiretroviral therapy [17].” A reference to our published survival analysis has been included.
12. Table 2, page 16. We replaced the data on CD4 lymphocyte decline with the estimates for \textit{CD4 count }\textless 200 \textit{cells/µL}. The unadjusted hazard ratio is 0.99 (95\% confidence interval, 0.86-1.13, \(p = 0.85\)) and the adjusted 1.02 (95\% confidence interval, 0.88-1.18, \(p = 0.79\)). We deleted the information on CD4 slope in this table.

Thank you for your consideration of this manuscript for publication in BMC Infectious Diseases. We feel that the changes described above have strengthened the manuscript, and hope that you will find it acceptable for publication. Please do not hesitate to contact us if you have any further questions.

Sincerely,

Susan M. Graham, MD MPH

References