PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women: a cross-sectional study
AUTHORS	MUNYAZESA, Elisaphane; Emile, Ivan; Mutimura, Eugene; Hoover, Donald; Shi, Qiuhu; McGinn, Aileen; Musiime, Stephenson; Muhairwe, Fred; Rutagengwa, Alfred; Dusingize, Jean; Anastos, Kathryn

VERSION 1 - REVIEW

REVIEWER	Jean Bosco Gahutu, MD, PhD, FHEA (UK), Associate Professor of Physiology, Faculty of Medicine, National University of Rwanda, Rwanda No conflict of interest.
REVIEW RETURNED	13-Jul-2012

THE STUDY	1. The exact altitude of the study sites (around 1,500 m) should be stated. 2. Specify the automated hematology analyzer for full blood count. 3. HIV- women cannot meet the criteria for Stage IV illness; the statement should read as follows: "It should be noted that such symptoms are not specific to HIV infection and are encountered in some of the HIV- women".
RESULTS & CONCLUSIONS	1. Table 1: Give full WHO reference for definition of anemia and marked anemia. 2. Odds Ratio for BMI: remove the units in the abstract and in the results section; an Odds Ratio does not have units because it is a ratio between two compared groups. 3. The effect of altitude on hemoglobin concentration is variable and population-dependent, particularly at moderate altitude. Nutrition also intervenes; this is to be considered because the study cohort is from the capital city and is likely to have a better nutritrional status then the population in the countryside. 4. The statements on the relationship between WBC and CD4 lymphocytes and the effect of altitude on hemoglobin concentration are self-evident and should be removed from limitations.
GENERAL COMMENTS	Throughout the text, correct the spelling as follows: sub-Saharan; naive; leukopenia

REVIEWER	Laura M Smeaton Senior Biostatistician Center for Biostatistics in AIDS Research Harvard School of Public Health Boston, MA USA
REVIEW RETURNED	16-Jul-2012

THE STUDY	Please check the cross references for the following statements from the methods section: "Anemia .. is one of the strongest predictors of HIV mortality and poor responses to antiretroviral therapy [10]. Neutropenia is frequently observed in advanced stages of HIV infection after development of AIDS, and has been associated with certain types of antiretroviral medications used to treat HIV infection [10]. " Reference #10: Firnhaber, et. al. does not report these results, and therefore cannot serve as a primary reference to these statements.
RESULTS & CONCLUSIONS	The methods section states the following: "This also includes symptoms and diagnoses that define World Health Organization (WHO) Stage-IV HIV illness. It should be noted that such symptoms are not specific to HIV infection, so some of the HIV- women also meet the criteria for Stage IV illness." However, the WHO guidelines state that these stages are defined only among those who are HIV positive. From INTERIM WHO CLINICAL STAGING OF HIV/AIDS AND HIV/AIDS CASE DEFINITIONS FOR SURVEILLANCE AFRICAN REGION (2005 - which is the date of this study), the definition is as follows: (Interim African Region version for persons aged 15 years or more with positive HIV antibody test or other laboratory evidence of HIV infection)b TABLE 1. REVISED WHO CLINICAL STAGING OF HIV/AIDS FOR ADULTS AND ADOLESCENTS (see http://www.who.int/hiv/pub/guidelines/clinicalstaging.pdf). The WHO guidelines from 2007 reiterate the same : http://www.who.int/hiv/pub/guidelines/HIVstaging150307.pdf. Therefore HIV- women should be undefined for WHO-HIV clinical stage. Groups of women being compared changes between the univariate and multivariate models. The univariate models compare HIV+ to HIV-, and then separately compare among CD4 count groups within the HIV+ subgroup. Then the multivariate models make 3 pairwise comparisons between each CD4 group within HIV+ to the reference group of HIV-. The methods did not state what motivated this change in parameterization for the groups being compared. This should be explained in the methods section. Were potential confounders (e.g. covariates such as age) assessed for effect modification on the estimated associations of HIV status?

VERSION 1 – AUTHOR RESPONSE

Reviewer 1:

1. Suggestion: The exact altitude of the study sites (around 1,500 m) should be stated.

Response: we have changed the manuscript as suggested, page 8, paragraph 3

2. Specify the automated hematology analyzer for full blood count.

Response: This has been clarified in the Methods section, page 5, paragraph 2.

3. HIV- women cannot meet the criteria for Stage IV illness; the statement should read as follows: "It

should be noted that such symptoms are not specific to HIV infection and are encountered in some of the HIV- women".

Response: In keeping with Reviewer 2's request that the AIDS-defining symptoms in HIV-uninfected women not be included, we have removed reference to these symptoms in the HIV-negative women, both in the text and in the tables.

4. Table 1: Give full WHO reference for definition of anemia and marked anemia.

Response: We have provided references for anemia (World Health Organisation: Nutritional Anemia: report of a WHO Scientific Group. Geneva, Switzerland: World Health Organisation; 1968) and marked anemia (Laboratory values of clinical importance. In Petersdorf RG, Adams RD, Braunwald E, et al., eds. Harrison's principles of internal medicine, 10th ed. New York, McGraw-Hill, 1983:A-3.)

2. Odds Ratio for BMI: remove the units in the abstract and in the results section; an Odds Ratio does not have units because it is a ratio between two compared groups.
Response: We apologize for creating confusion with this variable. BMI was analyzed as a continuous variable, with the OR describing the difference attributable to each change of 1 unit of BMI in kg/m2—e.g. comparison is between a person with 1 kg/m2 greater than the other person. We have corrected this in Table 2 such that it now says "BMI (per kg/ m2)". Thus the statement in the text is correct. "BMI (OR 0.87 per kg/m2, 95% CI 0.82-0.93; $p<0.001$).

3. Reviewer comment: The effect of altitude on hemoglobin concentration is variable and population-dependent, particularly at moderate altitude. Nutrition also intervenes; this is to be considered because the study cohort is from the capital city and is likely to have a better nutritional status then the population in the countryside.

Response: We thank the reviewer for this suggestion. We have added a statement to the discussion, 2nd paragraph (page 8) commenting on nutritional status as a possible contributor to anemia, and our finding of an inverse relationship of BMI with anemia.

4. Reviewer comment: The statements on the relationship between WBC and CD4 lymphocytes and the effect of altitude on hemoglobin concentration are self-evident and should be removed from limitations.

Response: We have removed these statements from the text.

5. Reviewer comment: Throughout the text, correct the spelling as follows: sub-Saharan; naive; leukopenia

Response: We have corrected these throughout the manuscript.

Reviewer 2: Laura M Smeaton
Senior Biostatistician
Center for Biostatistics in AIDS Research
Harvard School of Public Health
Boston, MA USA

1. Reviewer comment: Please check the cross references for the following statements from the methods section: "Anemia .. is one of the strongest predictors of HIV mortality and poor

responses to antiretroviral therapy [10]. Neutropenia is frequently observed in advanced stages of HIV infection after development of AIDS, and has been associated with certain types of antiretroviral medications used to treat HIV infection [10]. " Reference #10: Firnhaber, et. al. does not report these results, and therefore cannot serve as a primary reference to these statements.

Response: We have corrected the references and thank the reviewer for noting this error.

2. Reviewer comment: The methods section states the following: "This also includes symptoms and diagnoses that define World Health Organization (WHO) Stage-IV HIV illness. It should be noted that such symptoms are not specific to HIV infection, so some of the HIV- women also meet the criteria for Stage IV illness." However, the WHO guidelines state that these stages are defined only among those who are HIV positive. From INTERIM WHO CLINICAL STAGING OF HIV/AIDS AND HIV/AIDS CASE DEFINITIONS FOR SURVEILLANCE AFRICAN REGION (2005 - which is the date of this study), the definition is as follows: (Interim African Region version for persons aged 15 years or more with positive HIV antibody test or other laboratory evidence of HIV infection) b TABLE 1. REVISED WHO CLINICAL STAGING OF HIV/AIDS FOR ADULTS AND ADOLESCENTS (see http://www.who.int/hiv/pub/guidelines/clinicalstaging.pdf). The WHO guidelines from 2007 reiterate the same : http://www.who.int/hiv/pub/guidelines/HIVstaging150307.pdf. Therefore HIV- women should be undefined for WHO-HIV clinical stage.

Response: In response to this comment and to comment 3 from reviewer 1, we have removed from the text and tables all statements referring to Stage IV indicator illnesses in the HIV-negative women.

3. Reviewer comment: Groups of women being compared changes between the univariate and multivariate models. The univariate models compare HIV+ to HIV-, and then separately compare among CD4 count groups within the HIV+ subgroup. Then the multivariate models make 3 pairwise comparisons between each CD4 group within HIV+ to the reference group of HIV-. The methods did not state what motivated this change in parameterization for the groups being compared. This should be explained in the methods section.

Response: In Table 1 we want to 1) first identify if there are overall differences between HIV+ women and 2) then identify if among HIV+ women there are trends with CD4 count among the HIV+ women in order to try have some separation in evaluating potential confounders that are associated with HIV infection as opposed to those associated with progression to more advanced HIV disease once the person is infected. The multivariate models are specifically targeting severity of HIV infection as opposed to uninfected to assess a causal effect of severity of HIV infection and quantify thresholds of increased risk. If we were to fit two multivariate models … HIV+ Vs. HIV- and then severity of CD4 among HIV+ as was done in Table 1, this would be much more difficult to follow and we believe would blunt the interpretation of the results.

4. Reviewer Comment: Were potential confounders (e.g. covariates such as age) assessed for effect modification on the estimated associations of HIV status?

Response: With 3 levels of CD4, > 20 degrees of freedom for potential confounders and 3 outcomes, examination of such effect modifications would be extremely complicated and could lead to Type-1 error due to multiple comparisons. As we did not find a scientific basis in the literature as to why effect modifications should occur between HIV status and other factors. We thus feel that it is better to not explore this in the analyses presented in this paper.

VERSION 2 – REVIEW

REVIEWER	Laura M. Smeaton, MS Center for Biostatistics in AIDS Research Harvard School of Public Health Boston, MA USA
REVIEW RETURNED	22-Aug-2012

THE STUDY	Limitations of the study should include that because comparisons were not made between/among randomized groups, that observed differences attributed to either HIV status, CD4 cell count, (or the combination), could be partially attributable to other, confounding factors, measured or unknown. For example, the age distribution between HIV+ and HIV- women was significantly different. What was the association for anemia by HIV status before and after controlling for this potential confounder? It is not clear the the modeling process and results presented here address this question. Exploration for effect modifiers may be performed one at a time to prevent problems with model fitting when there are many potential co-variates. Assessment for effect modification need not use p-values (rather focusing on the relative parameter estimate difference between models). Moreover, any presentation of multi-variable modeling should include uni-variable modeling results as well for comparison.
GENERAL COMMENTS	The answer to the first review regarding potential confounders is not sufficient. There are ways to explore for effect modfication that do not introduce either technical modeling problems (e.g explore one at a time), or type 1 error (by focusing on the magnitude of effect modification on the parameter estimate, rather than a p-value). A scientific justification from literature is not needed, just evidence within the cohort (which was observed), that groups being compared were not similar on important potential confounding factors (like age). I focus on age as there is literature suggesting an association between female age and anemia (via menstruation). Therefore, this would be one factor to definitely investigate. Was the association between HIV groups and anemia similar before and after controlling for age?

VERSION 2 – AUTHOR RESPONSE

Reviewer comment:
Limitations of the study should include that because comparisons were not made between/among randomized groups, that observed differences attributed to either HIV status, CD4 cell count, (or the combination), could be partially attributable to other, confounding factors, measured or unknown.

Response: We agree this is a limitation as is true with all observational studies and now note this (lines 354-355). However, while this may not have been apparent, we did undertake multivariate modeling to adjust for potential confounding. We have split Table 2 into Tables 2A, 2B, 2C with each table presenting univariate and multivariate models for anemia, neutropenia and thrombocytopenia respectively to make this point clearer.

Reviewer comment:
For example, the age distribution between HIV+ and HIV- women was significantly different. What was the association for anemia by HIV status before and after controlling for this potential confounder? It is not clear the modeling process and results presented here address this question.

Response: We do agree that the general univariate results should have been presented along with the multivariate analyses in Table 2, and we have now broken Table 2 into three tables (Table 2A, 2B, 2C) to do this. We hope that the reviewer will find the presentation of the univariate analyses in Tables 2A, 2B and 2C to be informative and sufficient.

Reviewer comments:
Exploration for effect modifiers may be performed one at a time to prevent problems with model fitting when there are many potential co-variates. Assessment for effect modification need not use p-values (rather focusing on the relative parameter estimate difference between models). Moreover, any presentation of multi-variable modeling should include uni-variable modeling results as well for comparison.

There are ways to explore for effect modification that do not introduce either technical modeling problems (e.g. explore one at a time), or type 1 error (by focusing on the magnitude of effect modification on the parameter estimate, rather than a p-value).

Response: We prefer to distinguish between "effect modification" and confounding. Effect modification means that the relationship differs by level of age (for example if HIV/low CD4 were positively associated with neutropenia for women <30 years of age and at the same time protective against neutropenia for women over 30 years old). We do not see a biological basis nor are we aware of evidence in the literature for such a possibility (effect modification) for any combination of our variables. If the reviewer was referring to confounding rather than effect modification then we do hope that the inclusion of univariate and multivariate models in Tables 2A, 2B and 2C addresses this concern. We have also added some text to the Results section of the manuscript (page 7, lines 240-242) to describe the added analyses including the potential impact of age differences.

Age was not a strong confounder as it did not enter the final models for hemoglobin (Table 2A) or neutropenia (Table 2B) and in fact did not have a strong unadjusted association with hemoglobin (p=0.44). While age did enter the final multivariate model for platelets (Table 2C) this did not result in a substantial change in the point estimate for the association (Odds ratio) for the association of HIV / CD4 with platelets.

However, if the reviewer did mean “Effect Modification” the way we understood it, we believe that going one by one through 10 choose 2 = 45 possible combinations of variables x 3 outcomes searching for effect modification without such apriori evidence for this as noted above or arbitrarily selecting which combination to use would lead to inflation of Type-1 error as noted in our previous response. Thus numerous books warn against data driven examination of effect modification1-7. For example, looking at 45 combinations in each model at P = 0.05 would lead to an expected 2.5 positive results by chance alone. If we were to abandon p-values when making a determination it could be even worse. In addition, once we start looking at combinations of factors for effect modification, small cell size issues arise where there will be very few observations in some factor combination cells all of which have the same outcome. This will send the parameter estimate in the logistic model to ±∞ and make the model unstable5. It also creates other problems with collinearity that can destabilize estimates6. Other fallacies of examination of effect modification occur from logistic models if the underlying association is not multiplicative in the odds ratio7.

1. Schlesselman J. Case Control Studies Design, Conduct and Analysis Oxford University Press, New York NY 1982.
2. Kelssey JL, Thompson Wd Evans AS. Methods in Observational Epidemiology Oxford University Press, New York NY1986.
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1989.
4. Rothman KJ, Greenland S. Modern Epidemiology, Second Edition, Maple Press York PA 1998.
5. Hosmer DW, Lemeshow S. Applied Logistic Regression John Wiley & Sons New York, NY 2000
6. Kleinbaum DG, Kupper LL, Morgenstern. Epidemiologic Research Principles and Quantitative Methods, Van Norstrand Reinhard, New York 1982.
7. Szklo M Nieto JF. Epidemiology Beyond the Basics Jones and Bartlett Publishers Boston MA 2004.

Reviewer comment:
The answer to the first review regarding potential confounders is not sufficient.

Response:
We agree and hope that the inclusion of the univariate models in Tables 2A, 2B and 2C as described above addresses those concerns.

Reviewer comment:
A scientific justification from literature is not needed, just evidence within the cohort (which was observed), that groups being compared were not similar on important potential confounding factors (like age). I focus on age as there is literature suggesting an association between female age and anemia (via menstruation). Therefore, this would be one factor to definitely investigate. Was the association between HIV groups and anemia similar before and after controlling for age?

Response:
We thank the reviewer for pointing out the need for clarity on this point. We have added a sentence to the results section, lines 240-242, indicating that the associations of anemia with HIV serostatus and CD4 count were similar after adjusting for age: “It should be noted that despite the differences in age between HIV+ and HIV- women noted in Table 1, age was not associated with our hematological outcomes of interest or otherwise did not impact the association of HIV with these outcomes.”