BMJ open

Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001600
Article Type:	Research
Date Submitted by the Author:	04-Jun-2012
Complete List of Authors:	MUNYAZESA, Elisaphane;
Primary Subject Heading:	Infectious diseases
Secondary Subject Heading:	Haematology (incl blood transfusion), Infectious diseases
Keywords:	HIV & AIDS < INFECTIOUS DISEASES, Public health < INFECTIOUS DISEASES, Tropical medicine < INFECTIOUS DISEASES

RWANDA BIOMEDICAL CENTER

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National Reference Laboratory (NRL) Division

Kigali, June 1st, 2012

Ref. N°: NRL/2012

The Editor
BMJ Open Journal

Dear Editor,

We are pleased to submit this manuscript, entitled, "*Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan women"* for consideration for publication in *BMJ Open*.

On behalf of all other authors, I declare that the results of our manuscript nor any part of it have ever been submitted, or are under consideration elsewhere, and thus this is an original submission.

We declare that all ethical procedures and consent process were ensured before the study implementation. A written informed consent was obtained from each participant before participating in the study. The study was approved by the Rwanda National Ethics Committee, which approved research studies with international affiliations, and Montefiore Medical center, NY, US. All authors read this paper before submission, and approved it. No author declares a conflict of interest.

We thank you for your time and consideration. .

Yours sincerely,

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Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women

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Text: 2313 words
Abstract: 298 words
Tables: 3
Short Title: Hematological parameters in HIV+ Rwandan women

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Abstract

OBJECTIVES: Although hematologic abnormalities are common manifestations of HIV infection, few studies on hematologic parameters in HIV-infected persons have been undertaken in sub Saharan Africa. The authors assessed factors associated with hematological parameters in HIV-infected antiretroviral-naïve and HIV-uninfected Rwandan women.

STUDY DESIGN: cross-sectional analysis of a longitudinal cohort.

SETTING: Community-based women's associations.

PARTICIPANTS: 710 HIV-infected (HIV+) antiretroviral-naïve and 226 HIV-uninfected (HIV-) women from the Rwanda Women's Interassociation Study Assessment. Hematological parameters categorized as (abnormal vs. normal) were compared by HIV status and among HIV+ women by CD4+ count category using proportions. Multivariate logistic regression models using forward selection were fit.

RESULTS: Prevalence of anemia [hemoglobin (Hb) <12.0 g/dl] was higher in the HIV+ group (20.5% vs 6.3%; p<0.001), and increased with lower CD4 counts: ≥350 (7.6% %), 200-349 (16.0%%) and <200 cells/mm^3 (32.2%%). Marked anemia (Hb <10.0 g/dl) was found in 4.2% of HIV+ and none of the HIV- women (p<0.001), and was highest in HIV+ women with CD4+ <200 cells/mm^3 (8.4%). The HIV+ were more likely than HIV- women (4.2 vs. 0.5% respectively, p=0.002) to have moderate neutropenia with WBC <2.0 $X10^3$ cells/mm^3 and 8.4% of HIV+ women with CD4+ <200 cells/mm^3 had moderate neutropenia. In multivariate logistic regression analysis, BMI (OR 0.87 per kg/m^2, 95% CI 0.82-0.93; p<0.001), CD4 200-350 vs HIV- (OR 3.59, 95% CI 1.89-6.83; p<0.001) and CD4 <200 cells/mm^3 vs HIV- (OR 8.09, 95% CI 4.37-14.97; <0.001) had large independent associations with anemia. There were large independent associations of CD4 <200 cells/mm^3 vs HIV- (OR 7.18, 95% CI 0.78-65.82; p=0.081) and co-trimoxazole and/or Dapsone use (OR 5.69, 95% CI 0.63-51.45; p=0.122) with moderate neutropenia.

CONCLUSIONS: Anemia was more common than neutropenia or thrombocytopenia in the HIV-infected Rwandan women. Future comparisons of hematological parameters in HIV infected patients before and after antiretroviral therapy initiation are warranted.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a systemic disorder caused by the human immunodeficiency virus (HIV), and characterized by severe impairment and progressive damage of both cellular and humoral immune responses. Besides immunological complications of HIV disease [1], hematological abnormalities have been documented as strong independent predictors of morbidity and mortality in HIV-infected individuals [2]. HIV replicates not only in CD4+ lymphocyte cells, but also in macrophages and dendritic cells [1, 3, 4]. Such replication is followed by immune system depression, which can lead to life-threatening opportunistic infections. Hematological complications such as mild to severe anemia are associated with HIV disease progression and subsequent reduced survival [5].

Although numerous complications occur in HIV-infected patients [2, 6, 7], the most common hematologic abnormalities are anemia and neutropenia [6]. Anemia and neutropenia are generally caused by inadequate blood cell production because of bone marrow suppression by HIV infection mediated by abnormal cytokine expression and alteration of the bone marrow microenvironment [5, 8]. Anemia in HIV-infected persons is associated with CD4 cell depletion and progression to AIDS [9] and 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]. Thrombocytopenia is characterized by platelet counts below 125 x 10^3/mm^3, and also frequently occurs in HIV-infected patients [11-13]. Hematologic parameters mainly anemia and leucopenia in HIV-infected antiretroviral therapy (ART)-naïve patients result in poor ART treatment outcome and otherwise strongly predict mortality [2,14,15].

Although hematologic abnormalities are common manifestations of HIV infection and AIDS, and may have considerable impact on patients' wellbeing, treatment and care, few studies on hematologic parameters in HIV-infected persons have been undertaken in sub Saharan Africa. Such information for HIV-infected adults in Rwanda may help to inform treatment of HIV-infected individuals in this region. We therefore assessed hematological parameters in HIV-infected antiretroviral-naïve and HIV-uninfected Rwandan women.

MATERIALS AND METHODS

Study Design

Participants were from the Rwanda Women's Interassociation Study and Assessment (RWISA), a prospective observational cohort study on the effectiveness and toxicity of antiretroviral therapy (ART) that enrolled 710 HIV-infected and 226 uninfected women in 2005. Participants from RWISA were recruited from the Women's Equity in Access to Care and Treatment (WE-ACTx) clinical site in Kigali, community-based organizations, associations of people living with HIV/AIDS, and HIV Health Center clinical care sites in Kigali. Volunteers were included if they were ART naïve except for possible exposure to single-dose nevirapine to prevent mother to child HIV transmission; were ≥25 years of age, had resided in Rwanda in 1994, and if HIV negative would be willing to undergo voluntary counseling and testing for HIV at 6 month intervals. All participants provided information on medical history, demographic characteristics, psychosocial history, experience of trauma during the 1994 Rwandan genocide, and symptoms of depression and post-traumatic stress. 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. A physical assessment was performed and specimens were taken for CD4 cell count, full blood count and other laboratory studies. Written informed consent was obtained from each participant in the local language (Kinyarwanda) in accordance with this study's protocols and procedures approved by the Rwanda National Ethics Committee and the Institutional Review Board of Montefiore Medical Center, Bronx, NY, USA. Details of the RWISA study procedures and informed consent process including video and individual discussion have been previously described [16].

Laboratory data

CD4 T lymphocytes counts were determined using the Becton Dickinson (BD) FASCount system (Becton, Dickinson, Singapore) at the National Reference Laboratory, Kigali, Rwanda, and full blood count analyses were performed using standard automated methods at King Faisal Hospital in Kigali. Blood hemoglobin was determined by automated blood analyzer (CELL-DYN 1800). A modified Methemoglobin method was used for the colorimetric determination of hemoglobin. A portion of the lysed, diluted sample from the WBC Mixing Chamber was used for hemoglobin measurement. Blood samples were sent to the central laboratory within 2 hours

after collection where HIV-1 status and CD4 count were assessed. Blood samples were tested for HIV using the Abbott's Combo HIV Test. White blood cell count and platelet counts were performed using automated hematological analyzer (micro Cobas; Hoffman La Roche, Basel, Switzerland).

Statistical analysis

Leukocyte values, hemoglobin levels and platelet counts were analyzed as continuous variables and compared by HIV status and CD4 cell count category (<200, 200-349 and ≥ 350/mm^3) in HIV positive women. Anemia and marked anemia were defined as Hb<12.0 and <10.0 mg/dl respectively, and neutropenia was examined at two thresholds: white blood cell count<2000 and <1000 cells/ mm^3 while thrombocytopenia was defined as platelets<125.0 X 10^3 /mm^3 [17]. Unadjusted statistical comparisons between i) categorical predictors and categorical outcomes were made using chi-square tests, and ii) categorical predictors and continuous outcomes were made using t-tests and ANOVA. Multivariate logistic regression models with anemia, neutropenia and thrombocytopenia as outcomes were fit using forward selection and a P to enter of 0.2. Statistical analyses were performed using STATA version 11.0 and SAS 9.1.3.

RESULTS

Demographic Characteristics

All 936 (226 HIV- and 710 HIV+) women who participated in the RWISA study were included in this analysis. Table 1 presents characteristics of the HIV- women and the HIV+ women by CD4 count category: ≥350, 200-349 and < 200 cells/mm^3. HIV-uninfected, compared to HIV+ women were older (59% Vs 22% over 40 years, respectively, p<0.001), and more likely to be widowed (51% Vs 42%, p=0.001). Less than half (41%) of HIV-infected women reported a prior WHO stage IV condition. Use of dapsone or co-trimoxazole in the prior 12 months was reported by 87% of HIV+ and 19% of HIV-negative women (<0.001).

Univariate Analysis of Hematologic Parameters

Anemia: Anemia was more common in HIV+ than HIV- women (20.5% vs 6.3% respectively; p<0.001), and among HIV+ women the prevalence of anemia was higher in the lower CD4 count categories: ≥350 (7.6%) 200-349 (16.0%) and < 200 (32.2%) cells/mm^3 (Table 1). Marked

anemia, defined as Hb<10.0 g/dL, was found in 4.2% of HIV+ and none of the HIV- women (p<0.001), again with the highest prevalence in HIV+ women with CD4<200 cells/mm^3 (8.4%).

Neutropenia: Mean (± standard deviation) white blood cell count was lower in HIV+ than HIV-negative women (3.7±1.4 vs 4.5±1.4 X 10^3 cells/mm$^{3)}$; and among HIV+ women decreased from 4.3±1.6 in those with CD4 ≥350/mm^3 to 3.3±1.4 X 10^3 cells/mm^3 in women with CD4<200/ mm^3 p<0.001). Neutropenia, defined as WBC<2,000 X 10^3 cells/mm^3, was more common in HIV+ than HIV- women (4.2 vs. 0.5%, p=0.006) and most prevalent in HIV+ women with CD4<200 cells/mm^3 (8.4%, p<0.001). Only one HIV+ and one HIV- woman had profound neutropenia, defined as WBC<1,000 X 10^3 cells/mm^3.

Thrombocytopenia: Mean platelet count was lower in the HIV+ compared to HIV negative women: 223.2 ± 109.0 x10^3/mm^3 vs. 231.8± 84.5 x10^3/mm^3 respectively, p=0.051 with minimal differences in platelet count by category of CD4+ lymphocyte count in HIV+ women (p=0.55). Thrombocytopenia was more common in HIV+ compared to HIV- women: (13.5% vs. 8.6%, p=0.047), with no significant differences among the CD4 groups in the HIV+ women (=0.92).

Multivariate Analysis

Table 2 illustrates the results of multivariate logistic regression analyses with forward selection for all participants with anemia (Hb <12.0 g/dL, neutropenia (WBC <2,000cells/mm^3) and thrombocytopenia (platelets < 125/mm^3) as outcomes. Body mass index (OR 0.87 per kg/mm2, 95% confidence interval (CI) 0.82-0.93; p<0.001), CD4 200-350 vs. HIV- (OR 3.59, 95% CI 1.89-6.83; p<0.001) and CD4 < 200 vs HIV- (OR 8.09, 95% CI 4.37-14.97; <0.001) had large independent associations with anemia. Income had some independent association with anemia, but the trend and statistical significance across categories were not consistent. There were large independent associations of CD4 <200 cells/mm^3 vs. HIV- (OR 7.18, 95% CI 0.78-65.82; p=0.08) and co-trimoxazole/dapsone use (OR 5.69, 95% CI 0.63-51.45; p=0.12) with neutropenia. CD4>350 vs. HIV- (OR 2.62 cells/mm^3, 95% confidence interval (CI) 1.19-5.78; p=0.02), CD4 200-350 vs. HIV- (OR 3.14, 95% CI 1.41-7.01; p=0.005) and CD4 < 200 vs HIV- (OR 2.48, 95% CI 1.09-5.64; p=0.03) had large independent associations with thrombocytopenia.

DISCUSSION

We assessed hemoglobin levels, white blood cell counts and platelet counts in HIV-infected antiretroviral naïve and uninfected Rwandan women, and found that greater anemia neutropenia and thrombocytopenia were all associated with HIV-positive serostatus. While anemia and neutropenia in HIV-infected women were strongly associated with lower CD4+ cell counts, thrombocytopenia was not. To that end we have compared the prevalence of abnormal hematologic parameters we observed in HIV positive women with those seen in 5 studies in sub Saharan Africa and Western World (Table 3).

Most notably, although our findings indicated that the HIV-infected women had lower mean hemoglobin and were more likely to have anemia or marked anemia than HIV-negative women, the proportions of HIV infected (as well as uninfected) women with anemia here were lower than those from prior published studies of women in sub Saharan Africa and Western Countries [18-21]. For example, the mean hemoglobin observed here of 13.1 g/dL for HIV+ and 14.5 g/dL for HIV uninfected women were each about a full point higher in both the HIV-infected and the HIV-uninfected women compared to 2056 HIV-infected (Hb 12.3 g/dL) and 569 HIV-uninfected (Hb 13.0 g/dL) participants in the Women's Interagency HIV Study (WIHS) [18]. Lower hemoglobin levels in HIV-infected than uninfected individuals are nearly universally observed, and our finding is similar to prior studies from sub Saharan Africa, e.g. HIV-infected women were more likely to be anaemic than HIV negative women (23.6% vs. 12.8%; p=0.031) in a Ugandan study [19]. In a recent Rwandan study of 200 HIV-infected (of whom 50 were on ART) and 50 uninfected women, the prevalence of anemia was similar to our study (29.0% and 8.0% respectively) [17].

The higher hemoglobin levels in the Rwandan women, with and without HIV infection, than elsewhere may be attributable to the higher altitude of Rwanda, a mountainous country with an average of 1,700 meters above sea level and over 2,500 meters in some parts of the country [22]. The high hemoglobin observed in this cohort of Rwandan women may be due to acclimatization to higher altitude ensuring that women living in high altitude regions of Rwanda have similar physiologic adaptations as those living at lower altitude levels [23]. High altitude adaptations to fall in partial pressure of oxygen reduces the driving pressure needed for diffusion of oxygen across the alveolar-capillary barrier, and thus a fall in arterial partial pressure

of oxygen. This results in reduction of oxygen delivery to body tissues and thus potential cellular hypoxia and organ dysfunction [24]. Thus, living in higher altitude may have resulted in a fall of arterial oxygen content and reduced oxygen tissue delivery. This may have resulted in participants' adaptation to ensure restoration of arterial oxygen saturation, which increases hemoglobin concentration in individuals who habitually reside in high altitudes areas, a principle used by endurance athletes [25].

We have reported higher prevalence of neutropenia in the HIV-infected than uninfected Rwandan women, a common finding in sub Saharan Africa [19-21]. Neutropenia may be due to HIV suppression of bone marrow resulting in abnormal granulopoesis. Antigranulocyte antibodies have been described in HIV-infected persons [26], and neutropenia observed in HIV-infected adults may be attributed to decreased production of granulocyte colony-stimulating factor [27]. It should be noted that one study from Nigeria among HIV-infected persons found a mean WBC count that was higher than the mean values for HIV-infected women in our study [20]. This difference could be attributed to different stages of HIV illnesses in the study populations and the fact that participants in our study were ART-naïve, which was not true for the Nigerian study.

Neutropenia observed in HIV-infected women in our study was of higher prevalence in women with CD4+ lymphocyte count <200 cells/μL. Similarly, the Women's Interagency HIV Study found baseline neutropenia, defined as <2000 cells/mm^3 in 44% of women participants, and a longitudinal analysis found that worsening HIV disease was associated with subsequent neutropenia [28]. Neutropenia in an Ivory Coast study was observed in 21% of HIV-infected patients starting co-trimoxazole prophylaxis, but low grade neutropenia was not associated with adverse clinical consequences [29] as is also the case in other Sub-Saharan African countries. Neutropenia in our study is independently associated with low CD4+ lymphocyte count, and this suggests that the stage of HIV-infection is an important determinant to pre-treatment neutropenia.

We observed a higher prevalence of thrombocytopenia (platelets ≤ 125.0 x 10^3 /μL) in HIV-infected compared to HIV uninfected women, but no association between thrombocytopenia and CD4 count within HIV infected women. In developed countries, thrombocytopenia is generally

infrequent in healthy asymptomatic HIV-infected patients, and is associated with very advanced HIV disease and co-morbidities [30]. However, thrombocytopenia has been shown to be one of the common hematological abnormalities in patients before HAART initiation in sub Saharan countries [10]. Although HIV-infected women in our study were HAART-naïve, the majority were asymptomatic and few reported WHO stage IV illness, and as noted may not have had advanced HIV disease.

Our study has some limitations. Its cross-sectional design makes it structurally impossible to determine temporal direction or causality. In addition, CD4 cells are a component of WBC, and thus neutropenia could be influencing low CD4 count rather than vice-versa. Secondly, all participants in this study were women, and as hemoglobin levels differ between men and women, our findings cannot be extrapolated to men. Thirdly, the higher altitude of Rwanda as a mountainous country may have influenced hemoglobin levels in our participants. Finally, the small number of women with WBC<2.0 resulted in inadequate power to assess predictors of neutropenia. It is possible, or even likely, that the large odds ratios for the associations of co-trimoxazole use (OR=5.69 CI=0.63, 51.45) and CD4 count<200 cells/μl (OR=7.18 CI=0.78, 65.82) with neutropenia would be significant with a larger sample size.

In conclusion, we observed high prevalence of anemia in HIV-infected and uninfected Rwandan women. Anemia was more common in the HIV-infected than uninfected women, especially those with greater disease progression as indicated by lower CD4 cell counts. Neutropenia and thrombocytopenia were more common in the HIV-infected than uninfected Rwandan women. As anemia and neutropenia are the most common hematologic abnormalities in HAART-naïve HIV-infected women, it is important to routinely assess these parameters for timely and adequate clinical management.

Acknowledgements:
We acknowledge RWISA participants for their valuable time and commitment, and particularly acknowledge all research staff for their contribution to this study.

Funding sources:
All authors acknowledge support from the AIDS International Training and Research Program *(Fogarty International Center, NIH D43-TW001403.) This study was supported by supplements from the National Institute of Allergy and Infectious Diseases to the Bronx/Manhattan Women's*

Interagency HIV Study (WIHS), which is funded by the National Institute of Allergy and Infectious (UO1-AI-35004). The study was also supported in part by the Center for AIDS Research of the Albert Einstein College of Medicine and Montefiore Medical Center funded by the National Institutes of Health (NIH AI-51519) and by the National Institute of Diabetes and Digestive and Kidney Disease (DK54615), and the Chicago WIHS (U01-AI-34993).

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Table 1: Socio-demographic and hematologic characteristics by HIV status and CD4 cell count

	AMONG ALL WOMEN N (%)			*AMONG HIV+ WOMEN ONLY N (%)*			
Characteristics	**HIV-negative (N=226)**	**HIV-positive (N=710)**	***p*-value**	**HIV+ CD4>350 cells/µl (N=197)**	**HIV+ CD4 200-349 cells/µl (N=268)**	**HIV+ CD4 <200 cells/µl (N=245)**	***p*-value**
Age/years N (%)							
<30	34 (15%)	158 (22%)	<0.001	51 (26%)	50 (19%)	57 (23%)	0.35
30-40	59 (26%)	393 (55%)		107 (54%)	151 (56%)	135 (55%)	
40+	133 (59%)	159 (22%)		39 (20%)	67 (25%)	53 (22%)	
Income (Rwf)							
<10,000	92 (45%)	251 (36%)	0.02	66 (34%)	98 (37%)	87 (37%)	0.41
10,000- 35,000	79 (39%)	347 (50%)		105 (54%)	131 (50%)	111 (47%)	
>35,000	33 (16%)	97 (14%)		22 (11%)	35 (13%)	40 (17%)	
Level of education N (%)							
No schooling	67 (32%)	156 (22%)	0.02	46 (24%)	58 (22%)	52 (22%)	0.89
Some primary school	69 (33%)	269 (38%)		78 (40%)	99 (37%)	92 (38%)	
Secondary or University	76 (36%)	277 (39%)		71 (36%)	110 (41%)	96 (40%)	
Marital status N (%)							
Legally married/partner	80 (38%)	256 (36%)	0.004	86 (44%)	97 (36%)	73 (30%)	0.034
Widowed	108 (51%)	296 (42%)		71 (36%)	118 (44%)	107 (44%)	
Other	25 (12%)	153 (22%)		38 (19%)	53 (20%)	62 (26%)	
Body Mass Index (kg/m^2)							
Mean ±SD	(21.3±3.8%)	(21.6±3.9%)	0.22	(21.9±3.9%)	(21.9±3.9%)	(21.1±3.7%)	0.15
Alcohol use N (%)	56 (28%)	144 (21%)	0.04	39 (21%)	53 (21%)	52 (22%)	0.98
Smoking N (%)							
Yes	7 (3%)	18 (3%)	0.58	4 (2%)	7 (3%)	7 (3%)	0.86
WHO stage 4 N (%)							
Yes	32 (14%)	288 (41%)	<0.001	59 (30%)	105 (39%)	124 (51%)	<0.001
Co-trimoxazole/Dapsone use in prior year N (%)							
Yes	41 (19%)	612 (87%)	<0.001	145 (75%)	247 (92%)	220 (91%)	<0.001
Employed N (%)							
Yes	51 (25%)	171 (25%)	0.99	50 (26%)	63 (24%)	58 (25%)	084
Hemoglobin (g/dl,)							
N	223	669	<0.001	180	251	238	<0.001
Mean ±SD	14.3±1.4	13.1±1.6		13.5±1.3	13.1±1.7	12.7±1.7	
Anemia N (%)	14 (6.3%)	137 (20.5%)		15 (7.6%)	43 (16.0%)	79 (32.2%)	<0.001
Marked anemia	0	28 (4.2%)	<0.001	4 (1.1%)	6 (2.4%)	20 (8.4%)	<0.001
White blood cell count, X 10^3 cells/mm^3							
N	223	670		181	251	238	
Mean ±SD	4.5±1.4	3.7±1.4	<0.001	4.3±1.6	3.8±1.3	3.3±1.4	<0.001
Neutropenia N (%)							
<2000 cells/mm^3	1 (0.45%)	28 (4.2%)	0.006	2 (1.1%)	6 (2.4%)	20 (8.4%)	<0.001
<1000 cells/mm^3	1 (0.45%)	1 (0.15%)					
Platelet count (X10^3/mm^3)							
N	208	654	0.05	179	246	229	0.55
Mean ±SD	231.8±84.5	223.2±109.0		225.9±106.7	222.9±109.9	221.4±110.3	
Thrombocytopenia	18 (8.6%)	88 (13.5%)	0.0547	24 (13.4%)	36 (14.6%)	31 (13.5%)	0.92

SD=Standard Deviation; WHO=World Health Organization; Anemia is defined as hemoglobin<12.0 g/dL; Marked anemia is defined as hemoglobin<10.0 g/dL; Thrombocytopenia is defined as platelet counts <125.0 X 10^3 /mm^3 . The HIV+ and HIV- women were compared using chi-square (X^2) test, and similarly CD4+ cell count categories within HIV+ women were compared the X^2 test. Rwf=Rwandan Francs

Table 2: Multivariate Logistic Models with Forward Selection[1] for all women

Outcomes	Characteristics	OR (95% C.I.)	p-value
Hemoglobin (g/dl) < 12 g/dl (Anemia)			
	CD4 > 350 vs HIV-	1.47 (0.68, 3.21)	0.33
	CD4 200-350 vs HIV-	3.59 (1.89, 6.83)	< 0.001
	CD4 <200 vs HIV-	8.09 (4.37, 14.97)	<0.001
	Income 10-35 K vs <10 K	0.68 (0.45, 1.03)	0.07
	Income >35 K vs <10 K	0.94 (0.52, 1.70)	0.85
	BMI (kg/m^2)	0.87 (0.82, 0.93)	<0.001
WBC < 2000 (Neutropenia)			
	CD4 > 350 vs HIV-	1.03 (0.08, 13.15)	0.98
	CD4 200-350 vs HIV-	1.86 (0.18, 18.85)	0.60
	CD4 <200 vs HIV-	7.18 (0.78, 65.82)	0.08
	Co-trimoxazole or dapsone use	5.69 (0.63, 51.45)	0.12
Platelet count (X 10^3 / mm^3) < 125 (Thromobocytopenia)			
	CD4 > 350 vs HIV-	2.62 (1.19, 5.78)	0.02
	CD4 200-350 vs HIV-	3.14 (1.41, 7.01)	0.005
	CD4 <200 vs HIV-	2.48 (1.09, 5.64)	0.03
	Age Per 5-year	1.20 (1.03, 1.39)	0.01
	Widowed vs married/partner	0.49 (0.29, 0.80)	0.005
	Other vs married/partner	0.59 (0.32, 1.07)	0.08
	BMI (kg/m^2)	0.95 (0.89, 1.00)	0.06
	Co-trimoxazole or dapsone use	0.73 (0.40, 1.32)	0.30

Among all non-hemotological Variables in Table 1 and CD4 count category, p to enter = 0.20; Income is in Rwandan Francs;

Table 3: Prevalence of anemia, neutropenia and thrombocytopenia in HIV-infected women in 5 studies

Study (N)	Anemia			Neutropenia		Thrombocytopenia
	Hb<12.0	Hb<11.0	Hb<10.0	WBC<2000	WBC<1000	Platelets<125,000
RWISA (710) [Rwanda]	20.3%		4.9%	4.2%	0.1%	13.5%
WIHS* (2059) [North America]	37.0%		7.2%	44%	7%	14.6%
Uganda** (123)		23.6%				
APS*** (2197) [North America]	32.3%		6.8%			
PEARLS**** [Africa, Asia and Americas]					~15%	

*WIHS=Women's Interagency HIV Study[18,28]; **Uganda[19]; ***APS=Anemia Prevalence Study[21]; ****PEARLS=Prospective Evaluation of ART in Resource Limited Settings[10]

Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women: a cross-sectional study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001600.R1
Article Type:	Research
Date Submitted by the Author:	01-Aug-2012
Complete List of Authors:	MUNYAZESA, Elisaphane; Rwanda Biomedical Center (RBC), National Reference Division, Department of Laboratory Networks Emile, Ivan; Rwanda Biomedical Center (RBC), National Reference Division, Department of Laboratory Networks Mutimura, Eugene; Women's Equity in Access to Care and Treatment, Research and Scientific Capacity Building Hoover, Donald; The State University of New Jersey, Department of Statistics Shi, Qiuhu; School of Health Sciences and Practice, New York Medical College McGinn, Aileen; Albert Einstein College of Medicine and Montefiore Medical College Center, Musiime, Stephenson; Rwanda Biomedical Center (RBC), King Faisal Hospital, Kigali Muhairwe, Fred; Byumba District Hospital, North Province, Rutagengwa, Alfred; Nyamata District Hospital, Bugesera, Eastern Province Dusingize, Jean; Women's Equity in Access to Care and Treatment, Research and Scientific Capacity Building Anastos, Kathryn; Albert Einstein College of Medicine and Montefiore Medical Center, Bronx,
Primary Subject Heading:	Infectious diseases
Secondary Subject Heading:	Haematology (incl blood transfusion), Infectious diseases
Keywords:	HIV & AIDS < INFECTIOUS DISEASES, Public health < INFECTIOUS DISEASES, Tropical medicine < INFECTIOUS DISEASES

RWANDA BIOMEDICAL CENTER

A Healthy People. A Wealthy Nation

National Reference Laboratory (NRL) Division

Kigali, July 30th, 2012

Ref. N°: NRL/2012

The Managing Editor
BMJ Open Journal

Dear Editor:

On behalf of my co-authors I thank the reviewers and editors for their thoughtful review and suggestions. I am attaching a revised manuscript that incorporates the reviewers' and editor's suggestions and requests. These are delineated below:

Managing Editor:
Suggestion: Change Title to **Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women: *A Cross Sectional Study.*** Please include the study design in the title

Response: We have changed the title as suggested above and also indicated in the manuscript

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/m^2—e.g. comparison is between a person with 1 kg/m^2 greater than the other person. We have corrected this in Table 2 such that it now says "BMI (**per** kg/ m^2)". Thus the statement in the text is correct. "BMI (OR 0.87 per kg/m^2, 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
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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.

Again, we thank the editor and reviewers for their time and commitment.

Sincerely,

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Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women: a cross-sectional study

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Contribution: Study design, obtained funding, data analysis, manuscript preparation and writing
Conflict of interest: None

Text:	2346~~2313~~ words
Abstract:	300~~298~~ words
Tables:	3
Short Title:	Hematological parameters in HIV+ Rwandan women

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Abstract

OBJECTIVES: Although hematologic abnormalities are common manifestations of HIV infection, few studies on hematologic parameters in HIV-infected persons have been undertaken in sub Saharan Africa. The authors assessed factors associated with hematological parameters in HIV-infected antiretroviral-naïve and HIV-uninfected Rwandan women.

STUDY DESIGN: cross-sectional analysis of a longitudinal cohort.

SETTING: Community-based women's associations.

PARTICIPANTS: 710 HIV-infected (HIV+) antiretroviral-naïve and 226 HIV-uninfected (HIV-) women from the Rwanda Women's Interassociation Study Assessment. Hematological parameters categorized as (abnormal vs. normal) were compared by HIV status and among HIV+ women by CD4+ count category using proportions. Multivariate logistic regression models using forward selection were fit.

RESULTS: Prevalence of anemia [hemoglobin (Hb) <12.0 g/dl] was higher in the HIV+ group (20.5% vs 6.3%; p<0.001), and increased with lower CD4 counts: ≥350 (7.6% %), 200-349 (16.0%%) and <200 cells/mm^3 (32.2%%). Marked anemia (Hb <10.0 g/dl) was found in 4.2% of HIV+ and none of the HIV- women (p<0.001), and was highest in HIV+ women with CD4+ <200 cells/mm^3 (8.4%). The HIV+ were more likely than HIV- women (4.2 vs. 0.5% respectively, p=0.002) to have moderate neutropenia with WBC <2.0 X10^3 cells/mm^3 and 8.4% of HIV+ women with CD4+ <200 cells/mm^3 had moderate neutropenia. In multivariate logistic regression analysis, BMI (OR 0.87 per kg/m^2, 95% CI 0.82-0.93; p<0.001), CD4 200-350 vs HIV- (OR 3.59, 95% CI 1.89-6.83; p<0.001) and CD4 <200 cells/mm^3 vs. HIV- (OR 8.09, 95% CI 4.37-14.97; <0.001) had large independent associations with anemia. There were large independent associations of CD4 <200 cells/mm^3 vs. HIV- (OR 7.18, 95% CI 0.78-65.82; p=0.081) and co-trimoxazole and/or Dapsone use (OR 5.69, 95% CI 0.63-51.45; p=0.122) with moderate neutropenia.

CONCLUSIONS: Anemia was more common than neutropenia or thrombocytopenia in the HIV-infected Rwandan women. Future comparisons of hematological parameters in HIV infected patients before and after antiretroviral therapy initiation are warranted.

Comment [KA1]: This should be kept, it is correct

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a systemic disorder caused by the human immunodeficiency virus (HIV), and characterized by severe impairment and progressive damage of both cellular and humoral immune responses. Besides immunological complications of HIV disease [1], hematological abnormalities have been documented as strong independent predictors of morbidity and mortality in HIV-infected individuals [2]. HIV replicates not only in CD4+ lymphocyte cells, but also in macrophages and dendritic cells [1, 3, 4]. Such replication is followed by immune system depression, which can lead to life-threatening opportunistic infections. Hematological complications such as mild to severe anemia are associated with HIV disease progression and subsequent reduced survival [5].

Although numerous complications occur in HIV-infected patients [2, 6, 7], the most common hematologic abnormalities are anemia and neutropenia [6]. Anemia and neutropenia are generally caused by inadequate blood cell production because of bone marrow suppression by HIV infection mediated by abnormal cytokine expression and alteration of the bone marrow microenvironment [5, 8]. Anemia in HIV-infected persons is associated with CD4 cell depletion and progression to AIDS [9] and is one of the strongest predictors of HIV mortality and poor responses to antiretroviral therapy [~~10~~2]. 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]. Thrombocytopenia is characterized by platelet counts below 125 x 10^3/mm^3, and also frequently occurs in HIV-infected patients [11-13]. Hematologic parameters mainly anemia and leuk~~e~~openia in HIV-infected antiretroviral therapy (ART)-naïve patients result in poor ART treatment outcome and otherwise strongly predict mortality [2,14,15].

Comment [KA2]: The Levine reference (WIHS) currently #29 has this information. It should be moved up to replace #10.

Although hematologic abnormalities are common manifestations of HIV infection and AIDS, and may have considerable impact on patients' wellbeing, treatment and care, few studies on hematologic parameters in HIV-infected persons have been undertaken in sub-Saharan Africa. Such information for HIV-infected adults in Rwanda may help to inform treatment of HIV-infected individuals in this region. We therefore assessed hematological parameters in HIV-infected antiretroviral-naïve and HIV-uninfected Rwandan women.

MATERIALS AND METHODS

Study Design

Participants were from the Rwanda Women's Interassociation Study and Assessment (RWISA), a prospective observational cohort study on the effectiveness and toxicity of antiretroviral therapy (ART) that enrolled 710 HIV-infected and 226 uninfected women in 2005. Participants from RWISA were recruited from the Women's Equity in Access to Care and Treatment (WE-ACTx) clinical site in Kigali, community-based organizations, associations of people living with HIV/AIDS, and HIV Health Center clinical care sites in Kigali. Volunteers were included if they were ART naïve except for possible exposure to single-dose nevirapine to prevent mother to child HIV transmission; were ≥25 years of age, had resided in Rwanda in 1994, and if HIV negative would be willing to undergo voluntary counseling and testing for HIV at 6-month intervals. All participants provided information on medical history, demographic characteristics, psychosocial history, experience of trauma during the 1994 Rwandan genocide, and symptoms of depression and post-traumatic stress. 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 and are encountered in some of the HIV infected women.~~ A physical assessment was performed and specimens were taken for CD4 cell count, full blood count and other laboratory studies. Written informed consent was obtained from each participant in the local language (Kinyarwanda) in accordance with this study's protocols and procedures approved by the Rwanda National Ethics Committee and the Institutional Review Board of Montefiore Medical Center, Bronx, NY, USA. Details of the RWISA study procedures and informed consent process including video and individual discussion have been previously described [16].

Laboratory data

CD4 T lymphocytes counts were determined using the Becton Dickinson (BD) FASCount system (Becton~~,~~ Dickinson, Singapore) at the National Reference Laboratory, Kigali, Rwanda, and full blood count analyses were performed ~~using standard automated methods~~ at King Faisal Hospital in Kigali using~~.~~ ~~Blood hemoglobin was determined by automated blood analyzer (~~CELL-DYN 1800 automated blood analyzer (Abbott)~~)~~. A modified Methemoglobin method was used for the colorimetric determination of hemoglobin. A portion of the lysed, diluted sample from the WBC Mixing Chamber was used for hemoglobin measurement. Blood samples were sent to the

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central laboratory within 2 hours after collection where HIV-1 status and CD4 count were assessed. Blood samples were tested for HIV using the Abbott's Combo HIV Test. White blood cell count and platelet counts were performed using automated hematological analyzer (micro Cobas; Hoffman La Roche, Basel, Switzerland).

Statistical analysis

Leukocyte values, hemoglobin levels and platelet counts were analyzed as continuous variables and compared by HIV status and CD4 cell count category (<200, 200-349 and ≥ 350/mm^3) in HIV positive women. Anemia and marked anemia were defined as Hb<12.0[17] and <10.0 mg/dl respectively[18,19], and neutropenia was examined at two thresholds: white blood cell count<2000 and <1000 cells/ mm^3 while thrombocytopenia was defined as platelets<125.0 X 10^3 /mm^3 [17]. Unadjusted statistical comparisons between i) categorical predictors and categorical outcomes were made using chi-square tests, and ii) categorical predictors and continuous outcomes were made using t-tests and ANOVA. Multivariate logistic regression models with anemia, neutropenia and thrombocytopenia as outcomes were fit using forward selection and a P to enter of 0.2. Statistical analyses were performed using STATA version 11.0 and SAS 9.1.3.

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RESULTS

Demographic Characteristics

All 936 (226 HIV- and 710 HIV+) women who participated in the RWISA study were included in this analysis. Table 1 presents characteristics of the HIV- women and the HIV+ women by CD4 count category: ≥350, 200-349 and < 200 cells/mm^3. HIV-uninfected, compared to HIV+ women were older (59% vs. 22% over 40 years, respectively, p<0.001), and more likely to be widowed (51% vs. 42%, p=0.001). Less than half (41%) of HIV-infected women reported a prior WHO stage IV condition. Use of dapsone or co-trimoxazole in the prior 12 months was reported by 87% of HIV+ and 19% of HIV-negative women (<0.001).

Univariate Analysis of Hematologic Parameters

Anemia: Anemia was more common in HIV+ than HIV- women (20.5% vs 6.3% respectively; p<0.001), and among HIV+ women the prevalence of anemia was higher in the lower CD4 count categories: ≥350 (7.6%), 200-349 (16.0%) and <200 (32.2%) cells/mm^3 (Table 1). Marked

anemia, defined as Hb<10.0 g/dL, was found in 4.2% of HIV+ and none of the HIV- women (p<0.001), again with the highest prevalence in HIV+ women with CD4<200 cells/mm^3 (8.4%).

Neutropenia: Mean (± standard deviation) white blood cell count was lower in HIV+ than HIV-negative women (3.7±1.4 vs 4.5±1.4 X 10^3 cells/mm$^{3)}$; and among HIV+ women decreased from 4.3±1.6 in those with CD4 ≥350/mm^3 to 3.3±1.4 X 10^3 cells/mm^3 in women with CD4<200/ mm^3 p<0.001). Neutropenia, defined as WBC<2,000 X 10^3 cells/mm^3, was more common in HIV+ than HIV- women (4.2 vs. 0.5%, p=0.006) and most prevalent in HIV+ women with CD4<200 cells/mm^3 (8.4%, p<0.001). Only one HIV+ and one HIV- woman had profound neutropenia, defined as WBC<1,000 X 10^3 cells/mm^3.

Thrombocytopenia: Mean platelet count was lower in the HIV+ compared to HIV negative women: 223.2 ± 109.0 x10^3/mm^3 vs. 231.8± 84.5 x10^3/mm^3 respectively, p=0.051 with minimal differences in platelet count by category of CD4+ lymphocyte count in HIV+ women (p=0.55). Thrombocytopenia was more common in HIV+ compared to HIV- women: (13.5% vs. 8.6%, p=0.047), with no significant differences among the CD4 groups in the HIV+ women (=0.92).

Multivariate Analysis

Table 2 illustrates the results of multivariate logistic regression analyses with forward selection for all participants with anemia (Hb <12.0 g/dL, neutropenia (WBC <2,000cells/mm^3) and thrombocytopenia (platelets < 125/mm^3) as outcomes. Body mass index [~~(~~OR 0.87 per kg/mm2, 95% confidence interval (CI) 0.82-0.93; p<0.001~~)~~], CD4 200-350 vs. HIV- (OR 3.59, 95% CI 1.89-6.83; p<0.001) and CD4 < 200 vs HIV- (OR 8.09, 95% CI 4.37-14.97; <0.001) had large independent associations with anemia. Income had some independent association with anemia, but the trend and statistical significance across categories were not consistent. There were large independent associations of CD4 <200 cells/mm^3 vs. HIV- (OR 7.18, 95% CI 0.78-65.82; p=0.08) and co-trimoxazole/dapsone use (OR 5.69, 95% CI 0.63-51.45; p=0.12) with neutropenia. CD4>350 vs. HIV- (OR 2.62~~ cells/mm^3~~, 95% ~~confidence interval (~~CI~~)~~ 1.19-5.78; p=0.02), CD4 200-350 vs. HIV- (OR 3.14, 95% CI 1.41-7.01; p=0.005) and CD4 < 200 vs HIV- (OR 2.48, 95% CI 1.09-5.64; p=0.03) had large independent associations with thrombocytopenia.

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DISCUSSION

We assessed hemoglobin levels, white blood cell counts and platelet counts in HIV-infected antiretroviral naïve and uninfected Rwandan women, and found that greater anemia, neutropenia and thrombocytopenia were all associated with HIV-positive serostatus. While anemia and neutropenia in HIV-infected women were strongly associated with lower CD4+ cell counts, thrombocytopenia was not. To that end we have compared the prevalence of abnormal hematologic parameters we observed in HIV positive women with those seen in 5 studies in sub-Saharan Africa and Western World (Table 3).

Most notably, although our findings indicated that the HIV-infected women had lower mean hemoglobin and were more likely to have anemia or marked anemia than HIV-negative women, the proportions of HIV infected (as well as uninfected) women with anemia here were lower than those from prior published studies of women in sub-Saharan Africa and Western Countries [18,20-22~~4~~]. For example, the mean hemoglobin observed here of 13.1 g/dL for HIV+ and 14.5 g/dL for HIV- uninfected women were each about a full point higher in both the HIV-infected and the HIV-uninfected women compared to 2056 HIV-infected (Hb 12.3 g/dL) and 569 HIV-uninfected (Hb 13.0 g/dL) participants in the Women's Interagency HIV Study (WIHS) [18]. Lower hemoglobin levels in HIV-infected than uninfected individuals are nearly universally observed, and our finding is similar to prior studies from sub-Saharan Africa, e.g. HIV-infected women were more likely to be anemic than HIV negative women (23.6% vs. 12.8%; p=0.031) in a Ugandan study [20~~19~~]. In a recent Rwandan study of 200 HIV-infected (of whom 50 were on ART) and 50 uninfected women, the prevalence of anemia was similar to our study (29.0% and 8.0% respectively) [23~~17~~]. Poor nutritional status may also cause anemia [17], which may be reflected in the association of anemia with lower BMI in this urban Rwandan population.

The higher hemoglobin levels in the Rwandan women, with and without HIV infection, than elsewhere may be attributable to the higher altitude of Rwanda, a mountainous country, with an ~~average of~~elevation ~~1,700 meters~~ above sea level of 1,500 meters in Kigali, the capital city and site of this study ~~and over 2,500 meters in some parts of the country~~ [24~~2~~]. The high hemoglobin observed in this cohort of Rwandan women may be due to acclimatization to higher altitude ensuring that women living in high altitude regions of Rwanda have similar physiologic adaptations as those living at lower altitude levels [25~~3~~]. High altitude adaptations to fall in

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partial pressure of oxygen reduces the driving pressure needed for diffusion of oxygen across the alveolar-capillary barrier, and thus a fall in arterial partial pressure of oxygen. This results in reduction of oxygen delivery to body tissues and thus potential cellular hypoxia and organ dysfunction [264]. Thus, living in higher altitude may have resulted in a fall of arterial oxygen content and reduced oxygen tissue delivery. This may have resulted in participants' adaptation to ensure restoration of arterial oxygen saturation, which increases hemoglobin concentration in individuals who habitually reside in high altitudes areas, a principle used by endurance athletes [275].

We have reported higher prevalence of neutropenia in the HIV-infected than uninfected Rwandan women, a common finding in sub-Saharan Africa [2019,21,-281]. Neutropenia may be due to HIV suppression of bone marrow resulting in abnormal granulopoesis. Antigranulocyte antibodies have been described in HIV-infected persons [296], and neutropenia observed in HIV-infected adults may be attributed to decreased production of granulocyte colony-stimulating factor [3027]. It should be noted that one study from Nigeria among HIV-infected persons found a mean WBC count that was higher than the mean values for HIV-infected women in our study [210]. This difference could be attributed to different stages of HIV illnesses in the study populations and the fact that participants in our study were ART-naïve, which was not true for the Nigerian study.

Neutropenia observed in HIV-infected women in our study was of higher prevalence in women with CD4+ lymphocyte count <200 cells/μL. Similarly, the Women's Interagency HIV Study found baseline neutropenia, defined as <2000 cells/mm^3-, in 44% of women participants, and a longitudinal analysis found that worsening HIV disease was associated with subsequent neutropenia [1028]. Neutropenia in an Ivory Coast study was observed in 21% of HIV-infected patients starting co-trimoxazole prophylaxis, but low-grade neutropenia was not associated with adverse clinical consequences [3129] as is also the case in other sSub-Saharan African countries. Neutropenia in our study wais independently associated with low CD4+ lymphocyte count, and this suggests that the stage of HIV-infection is an important determinant to pre-treatment neutropenia.

We observed a higher prevalence of thrombocytopenia (platelets ≤ 125.0 x 10^3 /μL) in HIV-infected compared to HIV uninfected women, but no association between thrombocytopenia and CD4 count within HIV-infected women. In developed countries, thrombocytopenia is generally infrequent in healthy asymptomatic HIV-infected patients, and is associated with very advanced HIV disease and co-morbidities [320]. However, thrombocytopenia has been shown to be one of the common hematological abnormalities in patients before HAART initiation in sub-Saharan countries [28]10]. Although HIV-infected women in our study were HAART-naïve, the majority were asymptomatic and few reported WHO stage IV illness, and as noted may not have had advanced HIV disease.

Our study has some limitations. Its cross-sectional design makes it structurally impossible to determine temporal direction or causality. ~~In addition, CD4 cells are a component of WBC, and thus neutropenia could be influencing low CD4 count rather than vice-versa.~~ Secondly, all participants in this study were women, and as hemoglobin levels differ between men and women, our findings cannot be extrapolated to men. ~~Thirdly, the higher altitude of Rwanda as a mountainous country may have influenced hemoglobin levels in our participants.~~ Finally, the small number of women with WBC<2.0 resulted in inadequate power to assess predictors of neutropenia. It is possible, or even likely, that the large odds ratios for the associations of co-trimoxazole use (OR=5.69 CI=0.63, 51.45) and CD4 count<200 cells/μl (OR=7.18 CI=0.78, 65.82) with neutropenia would be significant with a larger sample size.

In conclusion, we observed high prevalence of anemia in HIV-infected and uninfected Rwandan women. Anemia was more common in the HIV-infected than uninfected women, especially those with greater disease progression as indicated by lower CD4 cell counts. Neutropenia and thrombocytopenia were more common in the HIV-infected than uninfected Rwandan women. As anemia and neutropenia are the most common hematologic abnormalities in HAART-naïve HIV-infected women, it is important to routinely assess these parameters for timely and adequate clinical management.

Acknowledgements:
We acknowledge RWISA participants for their valuable time and commitment, and particularly acknowledge all research staff for their contribution to this study.

Funding sources:
All authors acknowledge support from the AIDS International Training and Research Program *(Fogarty International Center, NIH D43-TW001403.) This study was supported by supplements from the National Institute of Allergy and Infectious Diseases to the Bronx/Manhattan Women's Interagency HIV Study (WIHS), which is funded by the National Institute of Allergy and Infectious (UO1-AI-35004). The study was also supported in part by the Center for AIDS Research of the Albert Einstein College of Medicine and Montefiore Medical Center funded by the National Institutes of Health (NIH AI-51519) and by the National Institute of Diabetes and Digestive and Kidney Disease (DK54615), and the Chicago WIHS (U01-AI-34993).*

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Table 1: Socio-demographic and hematologic characteristics by HIV status and CD4 cell count

	AMONG ALL WOMEN N (%)			*AMONG HIV+ WOMEN ONLY N (%)*			
Characteristics	**HIV-negative (N=226)**	**HIV-positive (N=710)**	***p*-value**	**HIV+ CD4>350 cells/µl (N=197)**	**HIV+ CD4 200-349 cells/µl (N=268)**	**HIV+ CD4 <200 cells/µl (N=245)**	***p*-value**
Age/years N (%)							
<30	34 (15%)	158 (22%)	<0.001	51 (26%)	50 (19%)	57 (23%)	0.35
30-40	59 (26%)	393 (55%)		107 (54%)	151 (56%)	135 (55%)	
40+	133 (59%)	159 (22%)		39 (20%)	67 (25%)	53 (22%)	
Income (Rwf)							
<10,000	92 (45%)	251 (36%)	0.02	66 (34%)	98 (37%)	87 (37%)	0.41
10,000- 35,000	79 (39%)	347 (50%)		105 (54%)	131 (50%)	111 (47%)	
>35,000	33 (16%)	97 (14%)		22 (11%)	35 (13%)	40 (17%)	
Level of education N (%)							
No schooling	67 (32%)	156 (22%)	0.02	46 (24%)	58 (22%)	52 (22%)	0.89
Some primary school	69 (33%)	269 (38%)		78 (40%)	99 (37%)	92 (38%)	
Secondary or University	76 (36%)	277 (39%)		71 (36%)	110 (41%)	96 (40%)	
Marital status N (%)							
Legally married/partner	80 (38%)	256 (36%)	0.004	86 (44%)	97 (36%)	73 (30%)	0.034
Widowed	108 (51%)	296 (42%)		71 (36%)	118 (44%)	107 (44%)	
Other	25 (12%)	153 (22%)		38 (19%)	53 (20%)	62 (26%)	
Body Mass Index (kg/m^2)							
Mean ±SD	(21.3±3.8%)	(21.6±3.9%)	0.22	(21.9±3.9%)	(21.9±3.9%)	(21.1±3.7%)	0.15
Alcohol use N (%)	56 (28%)	144 (21%)	0.04	39 (21%)	53 (21%)	52 (22%)	0.98
Smoking N (%)							
Yes	7 (3%)	18 (3%)	0.58	4 (2%)	7 (3%)	7 (3%)	0.86
WHO stage 4 N (%)							
Yes	~~32 (14%)~~Not applicable	288 (41%)	~~<0.001~~	59 (30%)	105 (39%)	124 (51%)	<0.00
Co-trimoxazole/Dapsone use in prior year N (%)							
Yes	41 (19%)	612 (87%)	<0.001	145 (75%)	247 (92%)	220 (91%)	<0.001
Employed N (%)							
Yes	51 (25%)	171 (25%)	0.99	50 (26%)	63 (24%)	58 (25%)	084
Hemoglobin (g/dl,)							
N	223	669	<0.001	180	251	238	<0.001
Mean ±SD	14.3±1.4	13.1±1.6		13.5±1.3	13.1±1.7	12.7±1.7	
Anemia N (%)	14 (6.3%)	137 (20.5%)		15 (7.6%)	43 (16.0%)	79 (32.2%)	<0.001
Marked anemia N (%)	0	28 (4.2%)	<0.001	4 (1.1%)	6 (2.4%)	20 (8.4%)	<0.001
White blood cell count, X 10^3 cells/mm^3							
N	223	670		181	251	238	
Mean ±SD	4.5±1.4	3.7±1.4	<0.001	4.3±1.6	3.8±1.3	3.3±1.4	<0.001
Neutropenia N (%)							
<2000 cells/mm^3	1 (0.45%)	28 (4.2%)	0.006	2 (1.1%)	6 (2.4%)	20 (8.4%)	<0.001
<1000 cells/mm^3	1 (0.45%)	1 (0.15%)					
Platelet count (X10^3/mm^3)							
N	208	654	0.05	179	246	229	0.55
Mean ±SD	231.8±84.5	223.2±109.0		225.9±106.7	222.9±109.9	221.4±110.3	
Thrombocytopenia	18 (8.6%)	88 (13.5%)	0.0547	24 (13.4%)	36 (14.6%)	31 (13.5%)	0.92

SD=Standard Deviation; WHO=World Health Organization; Anemia is defined as hemoglobin<12.0 g/dL; Marked anemia is defined as hemoglobin<10.0 g/dL; Thrombocytopenia is defined as platelet counts <125.0 X 10^3 /mm^3 . The HIV+ and HIV- women were compared using chi-square (X^2) test, and similarly CD4+ cell count categories within HIV+ women were compared the X^2 test. Rwf=Rwandan Francs

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Table 2: Multivariate Logistic Models with Forward Selection[1] for all women

Outcomes	Characteristics	OR (95% C.I.)	p-value
Hemoglobin ~~(g/dl)~~ < 12 g/dl (Anemia)			
	CD4 > 350 vs HIV-	1.47 (0.68, 3.21)	0.33
	CD4 200-350 vs HIV-	3.59 (1.89, 6.83)	< 0.001
	CD4 <200 vs HIV-	8.09 (4.37, 14.97)	<0.001
	Income 10-35 K vs <10 K	0.68 (0.45, 1.03)	0.07
	Income >35 K vs <10 K	0.94 (0.52, 1.70)	0.85
	BMI (per kg/m^2)	0.87 (0.82, 0.93)	<0.001
WBC < 2000 (Neutropenia)			
	CD4 > 350 vs HIV-	1.03 (0.08, 13.15)	0.98
	CD4 200-350 vs HIV-	1.86 (0.18, 18.85)	0.60
	CD4 <200 vs HIV-	7.18 (0.78, 65.82)	0.08
	Co-trimoxazole or dapsone use	5.69 (0.63, 51.45)	0.12
Platelet count (X 10^3 / mm^3) < 125 (Thromobocytopenia)			
	CD4 > 350 vs HIV-	2.62 (1.19, 5.78)	0.02
	CD4 200-350 vs HIV-	3.14 (1.41, 7.01)	0.005
	CD4 <200 vs HIV-	2.48 (1.09, 5.64)	0.03
	Age Per 5-year	1.20 (1.03, 1.39)	0.01
	Widowed vs married/partner	0.49 (0.29, 0.80)	0.005
	Other vs married/partner	0.59 (0.32, 1.07)	0.08
	BMI (per kg/m^2)	0.95 (0.89, 1.00)	0.06
	Co-trimoxazole or dapsone use	0.73 (0.40, 1.32)	0.30

Among all non-hemaetologic~~al~~ Variables in Table 1 and CD4 count category, p to enter = 0.20; Income is in Rwandan Francs;

Table 3: Prevalence of anemia, neutropenia and thrombocytopenia in HIV-infected women in 5 studies

Study (N)	Anemia			Neutropenia		Thrombocytopenia
	Hb<12.0	Hb<11.0	Hb<10.0	WBC<2000	WBC<1000	Platelets<125,000
RWISA (710) [Rwanda]	20.3%		4.9%	4.2%	0.1%	13.5%
WIHS* (2059) [North America]	37.0%		7.2%	44%	7%	14.6%
Uganda** (123)		23.6%				
APS*** (2197) [North America]	32.3%		6.8%			
PEARLS**** [Africa, Asia and Americas]					~15%	

*WIHS=Women's Interagency HIV Study[10, 18, 28]; **Uganda[20, 19]; ***APS=Anemia Prevalence Study[22, 21]; ****PEARLS=Prospective Evaluation of ART in Resource Limited Settings[28, 10]

Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women: a cross-sectional study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001600.R2
Article Type:	Research
Date Submitted by the Author:	26-Sep-2012
Complete List of Authors:	MUNYAZESA, Elisaphane; Rwanda Biomedical Center (RBC), National Reference Division, Department of Laboratory Networks Emile, Ivan; Rwanda Biomedical Center (RBC), National Reference Division, Department of Laboratory Networks Mutimura, Eugene; Women's Equity in Access to Care and Treatment, Research and Scientific Capacity Building Hoover, Donald; The State University of New Jersey, Department of Statistics Shi, Qiuhu; School of Health Sciences and Practice, New York Medical College McGinn, Aileen; Albert Einstein College of Medicine and Montefiore Medical College Center, Musiime, Stephenson; Rwanda Biomedical Center (RBC), King Faisal Hospital, Kigali Muhairwe, Fred; Byumba District Hospital, North Province, Rutagengwa, Alfred; Nyamata District Hospital, Bugesera, Eastern Province Dusingize, Jean; Women's Equity in Access to Care and Treatment, Research and Scientific Capacity Building Anastos, Kathryn; Albert Einstein College of Medicine and Montefiore Medical Center, Bronx,
Primary Subject Heading:	Infectious diseases
Secondary Subject Heading:	Haematology (incl blood transfusion), Infectious diseases
Keywords:	HIV & AIDS < INFECTIOUS DISEASES, Public health < INFECTIOUS DISEASES, Tropical medicine < INFECTIOUS DISEASES

Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women: a cross-sectional study

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Text: 2389 words
Abstract: 300 words
Tables: 5
Short Title: Hematological parameters in HIV+ Rwandan women

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Abstract

OBJECTIVES: Although hematologic abnormalities are common manifestations of HIV infection, few studies on hematologic parameters in HIV-infected persons have been undertaken in sub Saharan Africa. The authors assessed factors associated with hematological parameters in HIV-infected antiretroviral-naïve and HIV-uninfected Rwandan women.

STUDY DESIGN: cross-sectional analysis of a longitudinal cohort.

SETTING: Community-based women's associations.

PARTICIPANTS: 710 HIV-infected (HIV+) antiretroviral-naïve and 226 HIV-uninfected (HIV-) women from the Rwanda Women's Interassociation Study Assessment. Hematological parameters categorized as (abnormal vs. normal) were compared by HIV status and among HIV+ women by CD4+ count category using proportions. Multivariate logistic regression models using forward selection were fit.

RESULTS: Prevalence of anemia [hemoglobin (Hb) <12.0 g/dl] was higher in the HIV+ group (20.5% vs 6.3%; p<0.001), and increased with lower CD4 counts: ≥350 (7.6% %), 200-349 (16.0%%) and <200 cells/mm^3 (32.2%%). Marked anemia (Hb <10.0 g/dl) was found in 4.2% of HIV+ and none of the HIV- women (p<0.001), and was highest in HIV+ women with CD4+ <200 cells/mm^3 (8.4%). The HIV+ were more likely than HIV- women (4.2 vs. 0.5% respectively, p=0.002) to have moderate neutropenia with WBC <2.0 $X10^3$ cells/mm^3 and 8.4% of HIV+ women with CD4+ <200 cells/mm^3 had moderate neutropenia. In multivariate logistic regression analysis, BMI (OR 0.87 per kg/m^2, 95% CI 0.82-0.93; p<0.001), CD4 200-350 vs HIV- (OR 3.59, 95% CI 1.89-6.83; p<0.001) and CD4 <200 cells/mm^3 vs. HIV- (OR 8.09, 95% CI 4.37-14.97; <0.001) had large independent associations with anemia. There were large independent associations of CD4 <200 cells/mm^3 vs. HIV- (OR 7.18, 95% CI 0.78-65.82; p=0.081) and co-trimoxazole and/or Dapsone use (OR 5.69, 95% CI 0.63-51.45; p=0.122) with moderate neutropenia.

CONCLUSIONS: Anemia was more common than neutropenia or thrombocytopenia in the HIV-infected Rwandan women. Future comparisons of hematological parameters in HIV infected patients before and after antiretroviral therapy initiation are warranted.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a systemic disorder caused by the human immunodeficiency virus (HIV), and characterized by severe impairment and progressive damage of both cellular and humoral immune responses. Besides immunological complications of HIV disease [1], hematological abnormalities have been documented as strong independent predictors of morbidity and mortality in HIV-infected individuals [2]. HIV replicates not only in CD4+ lymphocyte cells, but also in macrophages and dendritic cells [1, 3, 4]. Such replication is followed by immune system depression, which can lead to life-threatening opportunistic infections. Hematological complications such as mild to severe anemia are associated with HIV disease progression and subsequent reduced survival [5].

Although numerous complications occur in HIV-infected patients [2, 6, 7], the most common hematologic abnormalities are anemia and neutropenia [6]. Anemia and neutropenia are generally caused by inadequate blood cell production because of bone marrow suppression by HIV infection mediated by abnormal cytokine expression and alteration of the bone marrow microenvironment [5, 8]. Anemia in HIV-infected persons is associated with CD4 cell depletion and progression to AIDS [9] and is one of the strongest predictors of HIV mortality and poor responses to antiretroviral therapy [2]. 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]. Thrombocytopenia is characterized by platelet counts below 125 x 10^3/mm^3, and also frequently occurs in HIV-infected patients [11-13]. Hematologic parameters mainly anemia and leukopenia in HIV-infected antiretroviral therapy (ART)-naïve patients result in poor ART treatment outcome and otherwise strongly predict mortality [2,14,15].

Although hematologic abnormalities are common manifestations of HIV infection and AIDS, and may have considerable impact on patients' wellbeing, treatment and care, few studies on hematologic parameters in HIV-infected persons have been undertaken in sub-Saharan Africa. Such information for HIV-infected adults in Rwanda may help to inform treatment of HIV-infected individuals in this region. We therefore assessed hematological parameters in HIV-infected antiretroviral-naïve and HIV-uninfected Rwandan women.

MATERIALS AND METHODS

Study Design

Participants were from the Rwanda Women's Interassociation Study and Assessment (RWISA), a prospective observational cohort study on the effectiveness and toxicity of antiretroviral therapy (ART) that enrolled 710 HIV-infected and 226 uninfected women in 2005. Participants from RWISA were recruited from the Women's Equity in Access to Care and Treatment (WE-ACTx) clinical site in Kigali, community-based organizations, associations of people living with HIV/AIDS, and HIV Health Center clinical care sites in Kigali. Volunteers were included if they were ART naïve except for possible exposure to single-dose nevirapine to prevent mother to child HIV transmission; were ≥25 years of age, had resided in Rwanda in 1994, and if HIV negative would be willing to undergo voluntary counseling and testing for HIV at 6-month intervals. All participants provided information on medical history, demographic characteristics, psychosocial history, experience of trauma during the 1994 Rwandan genocide, and symptoms of depression and post-traumatic stress. This also includes symptoms and diagnoses that define World Health Organization (WHO) Stage-IV HIV illness. A physical assessment was performed and specimens were taken for CD4 cell count, full blood count and other laboratory studies. Written informed consent was obtained from each participant in the local language (Kinyarwanda) in accordance with this study's protocols and procedures approved by the Rwanda National Ethics Committee and the Institutional Review Board of Montefiore Medical Center, Bronx, NY, USA. Details of the RWISA study procedures and informed consent process including video and individual discussion have been previously described [16].

Laboratory data

CD4 T lymphocytes counts were determined using the Becton Dickinson (BD) FASCount system (Becton Dickinson, Singapore) at the National Reference Laboratory, Kigali, Rwanda, and full blood count analyses were performed at King Faisal Hospital in Kigali using CELL-DYN 1800 automated blood analyzer (Abbott). A modified Methemoglobin method was used for the colorimetric determination of hemoglobin. A portion of the lysed, diluted sample from the WBC Mixing Chamber was used for hemoglobin measurement. Blood samples were sent to the central laboratory within 2 hours after collection where HIV-1 status and CD4 count were assessed. Blood samples were tested for HIV using the Abbott's Combo HIV Test. White blood

cell count and platelet counts were performed using automated hematological analyzer (micro Cobas; Hoffman La Roche, Basel, Switzerland).

Statistical analysis

Leukocyte values, hemoglobin levels and platelet counts were analyzed as continuous variables and compared by HIV status and CD4 cell count category (<200, 200-349 and ≥ 350/mm^3) in HIV positive women. Anemia and marked anemia were defined as Hb<12.0[17] and <10.0 mg/dl respectively[18,19], and neutropenia was examined at two thresholds: white blood cell count<2000 and <1000 cells/ mm^3 while thrombocytopenia was defined as platelets<125.0 X 10^3 /mm^3 [17]. Unadjusted statistical comparisons between i) categorical predictors and categorical outcomes were made using chi-square tests, and ii) categorical predictors and continuous outcomes were made using t-tests and ANOVA. Multivariate logistic regression models with anemia, neutropenia and thrombocytopenia as outcomes were fit using forward selection and a P to enter of 0.2. Statistical analyses were performed using STATA version 11.0 and SAS 9.1.3.

RESULTS

Demographic Characteristics

All 936 (226 HIV- and 710 HIV+) women who participated in the RWISA study were included in this analysis. Table 1 presents characteristics of the HIV- women and the HIV+ women by CD4 count category: ≥350, 200-349 and < 200 cells/mm^3. HIV-uninfected, compared to HIV+ women were older (59% vs. 22% over 40 years, respectively, p<0.001), and more likely to be widowed (51% vs. 42%, p=0.001). Less than half (41%) of HIV-infected women reported a prior WHO stage IV condition. Use of dapsone or co-trimoxazole in the prior 12 months was reported by 87% of HIV+ and 19% of HIV-negative women (<0.001).

Comparisons of Hematologic Parameters By HIV Status and CD4 Level in HIV+ Women

Anemia: Anemia was more common in HIV+ than HIV- women (20.5% vs 6.3% respectively; p<0.001), and among HIV+ women the prevalence of anemia was higher in the lower CD4 count categories: ≥350 (7.6%), 200-349 (16.0%) and <200 (32.2%) cells/mm^3 (Table 1). Marked anemia, defined as Hb<10.0 g/dL, was found in 4.2% of HIV+ and none of the HIV- women (p<0.001), again with the highest prevalence in HIV+ women with CD4<200 cells/mm^3 (8.4%).

Neutropenia: Mean (± standard deviation) white blood cell count was lower in HIV+ than HIV- negative women (3.7±1.4 vs 4.5±1.4 X 10^3 cells/$mm^{3)}$; and among HIV+ women decreased from 4.3±1.6 in those with CD4 ≥350/mm^3 to 3.3±1.4 X 10^3 cells/mm^3 in women with CD4<200/ mm^3 p<0.001). Neutropenia, defined as WBC<2,000 X 10^3 cells/mm^3, was more common in HIV+ than HIV- women (4.2 vs. 0.5%, p=0.006) and most prevalent in HIV+ women with CD4<200 cells/mm^3 (8.4%, p<0.001). Only one HIV+ and one HIV- woman had profound neutropenia, defined as WBC<1,000 X 10^3 cells/mm^3.

Thrombocytopenia: Mean platelet count was lower in the HIV+ compared to HIV negative women: 223.2 ± 109.0 x10^3/mm^3 vs. 231.8± 84.5 x10^3/mm^3 respectively, p=0.051 with minimal differences in platelet count by category of CD4+ lymphocyte count in HIV+ women (p=0.55). Thrombocytopenia was more common in HIV+ compared to HIV- women: (13.5% vs. 8.6%, p=0.047), with no significant differences among the CD4 groups in the HIV+ women (=0.92).

General Univariate / Multivariate Associations With Hematologic Outcomes

Tables 2A, 2B and 2C present the results of univariate and multivariate logistic regression analyses with forward selection for all participants with; anemia (Hb <12.0 vs. ≥12.0 g/dL, neutropenia (WBC <2,000 vs. ≥ 2000 cells/mm^3) and thrombocytopenia (platelets < 125 vs. ≥125 /mm^3), respectively, as outcomes. We summarize here the results of the multivariate models. Body mass index [OR 0.87 per kg/m^2, 95% confidence interval (CI) 0.82-0.93; p<0.001], CD4 200-350 cells/mm^3 vs. HIV- (OR 3.59, 95% CI 1.88-6.83; p<0.001) and CD4 < 200 cells/mm^3 vs HIV- (OR 8.09, 95% CI 4.37-14.97; <0.001) had large independent associations with anemia (Table 2A). Income had some independent association with anemia, but the trend and statistical significance across categories were not consistent. There were large independent associations of CD4 <200 cells/mm^3 vs. HIV- (OR 7.18, 95% CI 0.78-65.82; p=0.08) and co-trimoxazole/dapsone use (OR 5.69, 95% CI 0.63-51.45; p=0.12) with neutropenia (Table 2B). Finally, CD4>350 vs. HIV- (OR 2.18, 95% CI 1.10-4.32; p=0.03), CD4 200-350 vs. HIV- (OR 2.36, 95% CI 1.25-4.56; p=0.008) and CD4 < 200 cells/mm^3 vs HIV- (OR 1.95, 95% CI 1.01-3.79; p=0.05) had large independent associations with thrombocytopenia (Table 2C). Higher body mass index and not being married were negatively associated with thrombocytopenia. 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. While use of

co-trimoxazole/dapsone had large univariate associations with anemia and neutropenia, these were confounded by HIV infection / low CD4 and diminished after inclusion of this variable.

DISCUSSION

We assessed hemoglobin levels, white blood cell counts and platelet counts in HIV-infected antiretroviral naïve and uninfected Rwandan women, and found that greater anemia, neutropenia and thrombocytopenia were all associated with HIV-positive serostatus. While anemia and neutropenia in HIV-infected women were strongly associated with lower CD4+ cell counts, thrombocytopenia was not. To that end we have compared the prevalence of abnormal hematologic parameters we observed in HIV positive women with those seen in 5 studies in sub-Saharan Africa and Western World (Table 3).

Most notably, although our findings indicated that the HIV-infected women had lower mean hemoglobin and were more likely to have anemia or marked anemia than HIV-negative women, the proportions of HIV infected (as well as uninfected) women with anemia here were lower than those from prior published studies of women in sub-Saharan Africa and Western Countries [18,20-22]. For example, the mean hemoglobin observed here of 13.1 g/dL for HIV+ and 14.5 g/dL for HIV-uninfected women were each about a full point higher in both the HIV-infected and the HIV-uninfected women compared to 2056 HIV-infected (Hb 12.3 g/dL) and 569 HIV-uninfected (Hb 13.0 g/dL) participants in the Women's Interagency HIV Study (WIHS) [18]. Lower hemoglobin levels in HIV-infected than uninfected individuals are nearly universally observed, and our finding is similar to prior studies from sub-Saharan Africa, e.g. HIV-infected women were more likely to be anemic than HIV negative women (23.6% vs. 12.8%; p=0.031) in a Ugandan study [20]. In a recent Rwandan study of 200 HIV-infected (of whom 50 were on ART) and 50 uninfected women, the prevalence of anemia was similar to our study (29.0% and 8.0% respectively) [23]. Poor nutritional status may also cause anemia [17], which may be reflected in the association of anemia with lower BMI in this urban Rwandan population.

The higher hemoglobin levels in the Rwandan women, with and without HIV infection, than elsewhere may be attributable to the higher altitude of Rwanda, a mountainous country, with an elevation above sea level of 1,500 meters in Kigali, the capital city and site of this study [24]. The high hemoglobin observed in this cohort of Rwandan women may be due to acclimatization to higher altitude ensuring that women living in high altitude regions of Rwanda have similar physiologic adaptations as those living at lower altitude levels [25]. High altitude adaptations to fall in partial pressure of oxygen reduces the driving pressure needed for diffusion of oxygen

across the alveolar-capillary barrier, and thus a fall in arterial partial pressure of oxygen. This results in reduction of oxygen delivery to body tissues and thus potential cellular hypoxia and organ dysfunction [26]. Thus, living in higher altitude may have resulted in a fall of arterial oxygen content and reduced oxygen tissue delivery. This may have resulted in participants' adaptation to ensure restoration of arterial oxygen saturation, which increases hemoglobin concentration in individuals who habitually reside in high altitudes areas, a principle used by endurance athletes [27].

We have reported higher prevalence of neutropenia in the HIV-infected than uninfected Rwandan women, a common finding in sub-Saharan Africa [20,21,28]. Neutropenia may be due to HIV suppression of bone marrow resulting in abnormal granulopoesis. Antigranulocyte antibodies have been described in HIV-infected persons [29], and neutropenia observed in HIV-infected adults may be attributed to decreased production of granulocyte colony-stimulating factor [30]. It should be noted that one study from Nigeria among HIV-infected persons found a mean WBC count that was higher than the mean values for HIV-infected women in our study [21]. This difference could be attributed to different stages of HIV illness in the study populations and the fact that participants in our study were ART-naïve, which was not true for the Nigerian study.

Neutropenia observed in HIV-infected women in our study was of higher prevalence in women with CD4+ lymphocyte count <200 cells/μL. Similarly, the Women's Interagency HIV Study found baseline neutropenia, defined as <2000 cells/mm^3, in 44% of women participants and a longitudinal analysis found that worsening HIV disease was associated with subsequent neutropenia [10]. Neutropenia in an Ivory Coast study was observed in 21% of HIV-infected patients starting co-trimoxazole prophylaxis, but low-grade neutropenia was not associated with adverse clinical consequences [31] as is also the case in other sub-Saharan African countries. Neutropenia in our study was independently associated with low CD4+ lymphocyte count, and this suggests that the stage of HIV-infection is an important determinant to pre-treatment neutropenia.

We observed a higher prevalence of thrombocytopenia (platelets ≤ 125.0 x 10^3 /μL) in HIV-infected compared to HIV uninfected women, but no association between thrombocytopenia and

CD4 count within HIV-infected women. In developed countries, thrombocytopenia is generally infrequent in healthy asymptomatic HIV-infected patients, and is associated with very advanced HIV disease and co-morbidities [32]. However, thrombocytopenia has been shown to be one of the common hematological abnormalities in patients before HAART initiation in sub-Saharan countries [28]. Although HIV-infected women in our study were HAART-naïve, the majority were asymptomatic and few reported WHO stage IV illness, and as noted may not have had advanced HIV disease.

Our study has some limitations. Its non-randomized cross-sectional design makes it structurally impossible to determine temporal direction or causality including inability of multivariate models to adjust for all confounding. Secondly, all participants in this study were women, and as hemoglobin levels differ between men and women, our findings cannot be extrapolated to men. Finally, the small number of women with WBC<2.0 cells/mm^3 resulted in inadequate power to assess predictors of neutropenia. It is possible, or even likely, that the large odds ratios for the associations of co-trimoxazole use (OR=5.69 CI=0.63, 51.45) and CD4 count<200 cells/mm^3 (OR=7.18 CI=0.78, 65.82) with neutropenia would be significant with a larger sample size.

In conclusion, we observed high prevalence of anemia in HIV-infected and uninfected Rwandan women. Anemia was more common in the HIV-infected than uninfected women, especially those with greater disease progression as indicated by lower CD4 cell counts. Neutropenia and thrombocytopenia were more common in the HIV-infected than uninfected Rwandan women. As anemia and neutropenia are the most common hematologic abnormalities in HAART-naïve HIV-infected women, it is important to routinely assess these parameters for timely and adequate clinical management.

Acknowledgements:
We acknowledge RWISA participants for their valuable time and commitment, and particularly acknowledge all research staff for their contribution to this study.

Funding sources:
All authors acknowledge support from the AIDS International Training and Research Program *(Fogarty International Center, NIH D43-TW001403.) This study was supported by supplements from the National Institute of Allergy and Infectious Diseases to the Bronx/Manhattan Women's Interagency HIV Study (WIHS), which is funded by the National Institute of Allergy and Infectious (UO1-AI-35004). The study was also supported in part by the Center for AIDS*

Research of the Albert Einstein College of Medicine and Montefiore Medical Center funded by the National Institutes of Health (NIH AI-51519) and by the National Institute of Diabetes and Digestive and Kidney Disease (DK54615), and the Chicago WIHS (U01-AI-34993).

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Assessment of Hematological Parameters in HIV-Infected and Uninfected Rwandan Women: a cross-sectional study

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Conflict of interest: None

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SETTING: Community-based women's associations.

PARTICIPANTS: 710 HIV-infected (HIV+) antiretroviral-naïve and 226 HIV-uninfected (HIV-) women from the Rwanda Women's Interassociation Study Assessment. Hematological parameters categorized as (abnormal vs. normal) were compared by HIV status and among HIV+ women by CD4+ count category using proportions. Multivariate logistic regression models using forward selection were fit.

RESULTS: Prevalence of anemia [hemoglobin (Hb) <12.0 g/dl] was higher in the HIV+ group (20.5% vs 6.3%; p<0.001), and increased with lower CD4 counts: ≥350 (7.6% %), 200-349 (16.0%%) and <200 cells/mm^3 (32.2%%). Marked anemia (Hb <10.0 g/dl) was found in 4.2% of HIV+ and none of the HIV- women (p<0.001), and was highest in HIV+ women with CD4+ <200 cells/mm^3 (8.4%). The HIV+ were more likely than HIV- women (4.2 vs. 0.5% respectively, p=0.002) to have moderate neutropenia with WBC <2.0 X10^3 cells/mm^3 and 8.4% of HIV+ women with CD4+ <200 cells/mm^3 had moderate neutropenia. In multivariate logistic regression analysis, BMI (OR 0.87 per kg/m^2, 95% CI 0.82-0.93; p<0.001), CD4 200-350 vs HIV- (OR 3.59, 95% CI 1.89-6.83; p<0.001) and CD4 <200 cells/mm^3 vs. HIV- (OR 8.09, 95% CI 4.37-14.97; <0.001) had large independent associations with anemia. There were large independent associations of CD4 <200 cells/mm^3 vs. HIV- (OR 7.18, 95% CI 0.78-65.82; p=0.081) and co-trimoxazole and/or Dapsone use (OR 5.69, 95% CI 0.63-51.45; p=0.122) with moderate neutropenia.

CONCLUSIONS: Anemia was more common than neutropenia or thrombocytopenia in the HIV-infected Rwandan women. Future comparisons of hematological parameters in HIV infected patients before and after antiretroviral therapy initiation are warranted.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a systemic disorder caused by the human immunodeficiency virus (HIV), and characterized by severe impairment and progressive damage of both cellular and humoral immune responses. Besides immunological complications of HIV disease [1], hematological abnormalities have been documented as strong independent predictors of morbidity and mortality in HIV-infected individuals [2]. HIV replicates not only in CD4+ lymphocyte cells, but also in macrophages and dendritic cells [1, 3, 4]. Such replication is followed by immune system depression, which can lead to life-threatening opportunistic infections. Hematological complications such as mild to severe anemia are associated with HIV disease progression and subsequent reduced survival [5].

Although numerous complications occur in HIV-infected patients [2, 6, 7], the most common hematologic abnormalities are anemia and neutropenia [6]. Anemia and neutropenia are generally caused by inadequate blood cell production because of bone marrow suppression by HIV infection mediated by abnormal cytokine expression and alteration of the bone marrow microenvironment [5, 8]. Anemia in HIV-infected persons is associated with CD4 cell depletion and progression to AIDS [9] and is one of the strongest predictors of HIV mortality and poor responses to antiretroviral therapy [2]. 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]. Thrombocytopenia is characterized by platelet counts below 125 x 10^3/mm^3, and also frequently occurs in HIV-infected patients [11-13]. Hematologic parameters mainly anemia and leukopenia in HIV-infected antiretroviral therapy (ART)-naïve patients result in poor ART treatment outcome and otherwise strongly predict mortality [2,14,15].

Although hematologic abnormalities are common manifestations of HIV infection and AIDS, and may have considerable impact on patients' wellbeing, treatment and care, few studies on hematologic parameters in HIV-infected persons have been undertaken in sub-Saharan Africa. Such information for HIV-infected adults in Rwanda may help to inform treatment of HIV-infected individuals in this region. We therefore assessed hematological parameters in HIV-infected antiretroviral-naïve and HIV-uninfected Rwandan women.

MATERIALS AND METHODS

Study Design

Participants were from the Rwanda Women's Interassociation Study and Assessment (RWISA), a prospective observational cohort study on the effectiveness and toxicity of antiretroviral therapy (ART) that enrolled 710 HIV-infected and 226 uninfected women in 2005. Participants from RWISA were recruited from the Women's Equity in Access to Care and Treatment (WE-ACTx) clinical site in Kigali, community-based organizations, associations of people living with HIV/AIDS, and HIV Health Center clinical care sites in Kigali. Volunteers were included if they were ART naïve except for possible exposure to single-dose nevirapine to prevent mother to child HIV transmission; were ≥25 years of age, had resided in Rwanda in 1994, and if HIV negative would be willing to undergo voluntary counseling and testing for HIV at 6-month intervals. All participants provided information on medical history, demographic characteristics, psychosocial history, experience of trauma during the 1994 Rwandan genocide, and symptoms of depression and post-traumatic stress. This also includes symptoms and diagnoses that define World Health Organization (WHO) Stage-IV HIV illness. A physical assessment was performed and specimens were taken for CD4 cell count, full blood count and other laboratory studies. Written informed consent was obtained from each participant in the local language (Kinyarwanda) in accordance with this study's protocols and procedures approved by the Rwanda National Ethics Committee and the Institutional Review Board of Montefiore Medical Center, Bronx, NY, USA. Details of the RWISA study procedures and informed consent process including video and individual discussion have been previously described [16].

Laboratory data

CD4 T lymphocytes counts were determined using the Becton Dickinson (BD) FASCount system (Becton Dickinson, Singapore) at the National Reference Laboratory, Kigali, Rwanda, and full blood count analyses were performed at King Faisal Hospital in Kigali using CELL-DYN 1800 automated blood analyzer (Abbott). A modified Methemoglobin method was used for the colorimetric determination of hemoglobin. A portion of the lysed, diluted sample from the WBC Mixing Chamber was used for hemoglobin measurement. Blood samples were sent to the central laboratory within 2 hours after collection where HIV-1 status and CD4 count were assessed. Blood samples were tested for HIV using the Abbott's Combo HIV Test. White blood

cell count and platelet counts were performed using automated hematological analyzer (micro Cobas; Hoffman La Roche, Basel, Switzerland).

Statistical analysis

Leukocyte values, hemoglobin levels and platelet counts were analyzed as continuous variables and compared by HIV status and CD4 cell count category (<200, 200-349 and ≥ 350/mm^3) in HIV positive women. Anemia and marked anemia were defined as Hb<12.0[17] and <10.0 mg/dl respectively[18,19], and neutropenia was examined at two thresholds: white blood cell count<2000 and <1000 cells/ mm^3 while thrombocytopenia was defined as platelets<125.0 X 10^3 /mm^3 [17]. Unadjusted statistical comparisons between i) categorical predictors and categorical outcomes were made using chi-square tests, and ii) categorical predictors and continuous outcomes were made using t-tests and ANOVA. Multivariate logistic regression models with anemia, neutropenia and thrombocytopenia as outcomes were fit using forward selection and a P to enter of 0.2. Statistical analyses were performed using STATA version 11.0 and SAS 9.1.3.

RESULTS

Demographic Characteristics

All 936 (226 HIV- and 710 HIV+) women who participated in the RWISA study were included in this analysis. Table 1 presents characteristics of the HIV- women and the HIV+ women by CD4 count category: ≥350, 200-349 and < 200 cells/mm^3. HIV-uninfected, compared to HIV+ women were older (59% vs. 22% over 40 years, respectively, p<0.001), and more likely to be widowed (51% vs. 42%, p=0.001). Less than half (41%) of HIV-infected women reported a prior WHO stage IV condition. Use of dapsone or co-trimoxazole in the prior 12 months was reported by 87% of HIV+ and 19% of HIV-negative women (<0.001).

~~Univariate Analysis~~Comparisons of Hematologic Parameters By HIV Status and CD4 Level in HIV+ Women

Anemia: Anemia was more common in HIV+ than HIV- women (20.5% vs 6.3% respectively; p<0.001), and among HIV+ women the prevalence of anemia was higher in the lower CD4 count categories: ≥350 (7.6%), 200-349 (16.0%) and <200 (32.2%) cells/mm^3 (Table 1). Marked anemia, defined as Hb<10.0 g/dL, was found in 4.2% of HIV+ and none of the HIV- women (p<0.001), again with the highest prevalence in HIV+ women with CD4<200 cells/mm^3 (8.4%).

Neutropenia: Mean (± standard deviation) white blood cell count was lower in HIV+ than HIV- negative women (3.7±1.4 vs 4.5±1.4 X 10^3 cells/$mm^{3)}$; and among HIV+ women decreased from 4.3±1.6 in those with CD4 ≥350/mm^3 to 3.3±1.4 X 10^3 cells/mm^3 in women with CD4<200/ mm^3 p<0.001). Neutropenia, defined as WBC<2,000 X 10^3 cells/mm^3, was more common in HIV+ than HIV- women (4.2 vs. 0.5%, p=0.006) and most prevalent in HIV+ women with CD4<200 cells/mm^3 (8.4%, p<0.001). Only one HIV+ and one HIV- woman had profound neutropenia, defined as WBC<1,000 X 10^3 cells/mm^3.

Thrombocytopenia: Mean platelet count was lower in the HIV+ compared to HIV negative women: 223.2 ± 109.0 x10^3/mm^3 vs. 231.8± 84.5 x10^3/mm^3 respectively, p=0.051 with minimal differences in platelet count by category of CD4+ lymphocyte count in HIV+ women (p=0.55). Thrombocytopenia was more common in HIV+ compared to HIV- women: (13.5% vs. 8.6%, p=0.047), with no significant differences among the CD4 groups in the HIV+ women (=0.92).

General Univariate / Multivariate ~~Analysis~~Associations With Hematologic Outcomes

Tables 2A~~a~~, 2B~~b~~ and 2C~~c, respectively, present~~ ~~illustrates~~ the results of univariate and multivariate logistic regression analyses with forward selection for all participants with; anemia (Hb <12.0 vs. ≥12.0 g/dL, neutropenia (WBC <2,000 vs. ≥ 2000 cells/mm^3) and thrombocytopenia (platelets < 125 vs. ≥125 /mm^3), respectively, ~~as~~ outcomes. We summarize here the results of the multivariate models. Body mass index [OR 0.87 per kg/~~m~~m^2, 95% confidence interval (CI) 0.82-0.93; p<0.001], CD4 200-350 cells/mm^3 vs. HIV- (OR 3.59, 95% CI 1.88-6.83; p<0.001) and CD4 < 200 cells/mm^3 vs HIV- (OR 8.09, 95% CI 4.37-14.97; <0.001) had large independent associations with anemia (Table 2A~~a~~). Income had some independent association with anemia, but the trend and statistical significance across categories were not consistent. There were large independent associations of CD4 <200 cells/mm^3 vs. HIV- (OR 7.18, 95% CI 0.78-65.82; p=0.08) and co-trimoxazole/dapsone use (OR 5.69, 95% CI 0.63-51.45; p=0.12) with neutropenia (Table 2B~~b~~). Finally, CD4>350 -vs. HIV- (OR 2.18~~62~~, 95% CI 1.1~~09~~-~~45~~.32~~78~~; p=0.0~~32~~), CD4 200-350 vs. HIV- (OR 2.36~~3.14~~, 95% CI 1.25~~41~~-4.56~~7.01~~; p=0.00~~85~~) and CD4 < 200 cells/mm^3 vs HIV- (OR 1.95~~2.48~~, 95% CI 1.0~~19~~-3.79~~5.64~~; p=0.0~~53~~) had large independent associations with thrombocytopenia (Table 2C~~c~~). Higher body mass index and not being married were negatively associated with thrombocytopenia. 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

assoctiatinoassociation of HIV with these outcomes. While use of -co-trimoxazole/dapsone had large univariate associations with anemia and neutropenia, these were confounded by HIV infection / low CD4 and dieminished after inclusion of this variable.

DISCUSSION

We assessed hemoglobin levels, white blood cell counts and platelet counts in HIV-infected antiretroviral naïve and uninfected Rwandan women, and found that greater anemia, neutropenia and thrombocytopenia were all associated with HIV-positive serostatus. While anemia and neutropenia in HIV-infected women were strongly associated with lower CD4+ cell counts, thrombocytopenia was not. To that end we have compared the prevalence of abnormal hematologic parameters we observed in HIV positive women with those seen in 5 studies in sub-Saharan Africa and Western World (Table 3).

Most notably, although our findings indicated that the HIV-infected women had lower mean hemoglobin and were more likely to have anemia or marked anemia than HIV-negative women, the proportions of HIV infected (as well as uninfected) women with anemia here were lower than those from prior published studies of women in sub-Saharan Africa and Western Countries [18,20-22]. For example, the mean hemoglobin observed here of 13.1 g/dL for HIV+ and 14.5 g/dL for HIV-uninfected women were each about a full point higher in both the HIV-infected and the HIV-uninfected women compared to 2056 HIV-infected (Hb 12.3 g/dL) and 569 HIV-uninfected (Hb 13.0 g/dL) participants in the Women's Interagency HIV Study (WIHS) [18]. Lower hemoglobin levels in HIV-infected than uninfected individuals are nearly universally observed, and our finding is similar to prior studies from sub-Saharan Africa, e.g. HIV-infected women were more likely to be anemic than HIV negative women (23.6% vs. 12.8%; p=0.031) in a Ugandan study [20]. In a recent Rwandan study of 200 HIV-infected (of whom 50 were on ART) and 50 uninfected women, the prevalence of anemia was similar to our study (29.0% and 8.0% respectively) [23]. Poor nutritional status may also cause anemia [17], which may be reflected in the association of anemia with lower BMI in this urban Rwandan population.

The higher hemoglobin levels in the Rwandan women, with and without HIV infection, than elsewhere may be attributable to the higher altitude of Rwanda, a mountainous country, with an elevation above sea level of 1,500 meters in Kigali, the capital city and site of this study [24]. The high hemoglobin observed in this cohort of Rwandan women may be due to acclimatization to higher altitude ensuring that women living in high altitude regions of Rwanda have similar physiologic adaptations as those living at lower altitude levels [25]. High altitude adaptations to fall in partial pressure of oxygen reduces the driving pressure needed for diffusion of oxygen

across the alveolar-capillary barrier, and thus a fall in arterial partial pressure of oxygen. This results in reduction of oxygen delivery to body tissues and thus potential cellular hypoxia and organ dysfunction [26]. Thus, living in higher altitude may have resulted in a fall of arterial oxygen content and reduced oxygen tissue delivery. This may have resulted in participants' adaptation to ensure restoration of arterial oxygen saturation, which increases hemoglobin concentration in individuals who habitually reside in high altitudes areas, a principle used by endurance athletes [27].

We have reported higher prevalence of neutropenia in the HIV-infected than uninfected Rwandan women, a common finding in sub-Saharan Africa [20,21,28]. Neutropenia may be due to HIV suppression of bone marrow resulting in abnormal granulopoesis. Antigranulocyte antibodies have been described in HIV-infected persons [29], and neutropenia observed in HIV-infected adults may be attributed to decreased production of granulocyte colony-stimulating factor [30]. It should be noted that one study from Nigeria among HIV-infected persons found a mean WBC count that was higher than the mean values for HIV-infected women in our study [21]. This difference could be attributed to different stages of HIV illness in the study populations and the fact that participants in our study were ART-naïve, which was not true for the Nigerian study.

Neutropenia observed in HIV-infected women in our study was of higher prevalence in women with CD4+ lymphocyte count <200 cells/μL. Similarly, the Women's Interagency HIV Study found baseline neutropenia, defined as <2000 cells/mm^3, in 44% of women participants and a longitudinal analysis found that worsening HIV disease was associated with subsequent neutropenia [10]. Neutropenia in an Ivory Coast study was observed in 21% of HIV-infected patients starting co-trimoxazole prophylaxis, but low-grade neutropenia was not associated with adverse clinical consequences [31] as is also the case in other sub-Saharan African countries. Neutropenia in our study was independently associated with low CD4+ lymphocyte count, and this suggests that the stage of HIV-infection is an important determinant to pre-treatment neutropenia.

We observed a higher prevalence of thrombocytopenia (platelets ≤ 125.0 x 10^3 /μL) in HIV-infected compared to HIV uninfected women, but no association between thrombocytopenia and

CD4 count within HIV-infected women. In developed countries, thrombocytopenia is generally infrequent in healthy asymptomatic HIV-infected patients, and is associated with very advanced HIV disease and co-morbidities [32]. However, thrombocytopenia has been shown to be one of the common hematological abnormalities in patients before HAART initiation in sub-Saharan countries [28]. Although HIV-infected women in our study were HAART-naïve, the majority were asymptomatic and few reported WHO stage IV illness, and as noted may not have had advanced HIV disease.

Our study has some limitations. It's non-randomized cross-sectional design makes it structurally impossible to determine temporal direction or causality including inability of multivariate models to adjust for all confounding. Secondly, all participants in this study were women, and as hemoglobin levels differ between men and women, our findings cannot be extrapolated to men. Finally, the small number of women with WBC<2.0 cells/mm^3 resulted in inadequate power to assess predictors of neutropenia. It is possible, or even likely, that the large odds ratios for the associations of co-trimoxazole use (OR=5.69 CI=0.63, 51.45) and CD4 count<200 cells/mm^3 ~~cells/µl~~ (OR=7.18 CI=0.78, 65.82) with neutropenia would be significant with a larger sample size.

In conclusion, we observed high prevalence of anemia in HIV-infected and uninfected Rwandan women. Anemia was more common in the HIV-infected than uninfected women, especially those with greater disease progression as indicated by lower CD4 cell counts. Neutropenia and thrombocytopenia were more common in the HIV-infected than uninfected Rwandan women. As anemia and neutropenia are the most common hematologic abnormalities in HAART-naïve HIV-infected women, it is important to routinely assess these parameters for timely and adequate clinical management.

Acknowledgements:
We acknowledge RWISA participants for their valuable time and commitment, and particularly acknowledge all research staff for their contribution to this study.

Funding sources:
All authors acknowledge support from the AIDS International Training and Research Program *(Fogarty International Center, NIH D43-TW001403.) This study was supported by supplements from the National Institute of Allergy and Infectious Diseases to the Bronx/Manhattan Women's*

Interagency HIV Study (WIHS), which is funded by the National Institute of Allergy and Infectious (UO1-AI-35004). The study was also supported in part by the Center for AIDS Research of the Albert Einstein College of Medicine and Montefiore Medical Center funded by the National Institutes of Health (NIH AI-51519) and by the National Institute of Diabetes and Digestive and Kidney Disease (DK54615), and the Chicago WIHS (U01-AI-34993).

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Table 1: Socio-demographic and hematologic characteristics by HIV status and CD4 cell count

	AMONG ALL WOMEN N (%)			*AMONG HIV+ WOMEN ONLY N (%)*			
Characteristics	**HIV-negative (N=226)**	**HIV-positive (N=710)**	***p*-value**	**HIV+ CD4>350 cells/µl (N=197)**	**HIV+ CD4 200-349 cells/µl (N=268)**	**HIV+ CD4 <200 cells/µl (N=245)**	***p*-value**
Age/years N (%)							
<30	34 (15%)	158 (22%)	<0.001	51 (26%)	50 (19%)	57 (23%)	0.35
30-40	59 (26%)	393 (55%)		107 (54%)	151 (56%)	135 (55%)	
40+	133 (59%)	159 (22%)		39 (20%)	67 (25%)	53 (22%)	
Income (Rwf)							
<10,000	92 (45%)	251 (36%)	0.02	66 (34%)	98 (37%)	87 (37%)	0.41
10,000- 35,000	79 (39%)	347 (50%)		105 (54%)	131 (50%)	111 (47%)	
>35,000	33 (16%)	97 (14%)		22 (11%)	35 (13%)	40 (17%)	
Level of education N (%)							
No schooling	67 (32%)	156 (22%)	0.02	46 (24%)	58 (22%)	52 (22%)	0.89
Some primary school	69 (33%)	269 (38%)		78 (40%)	99 (37%)	92 (38%)	
Secondary or University	76 (36%)	277 (39%)		71 (36%)	110 (41%)	96 (40%)	
Marital status N (%)							
Legally married/partner	80 (38%)	256 (36%)	0.004	86 (44%)	97 (36%)	73 (30%)	0.034
Widowed	108 (51%)	296 (42%)		71 (36%)	118 (44%)	107 (44%)	
Other	25 (12%)	153 (22%)		38 (19%)	53 (20%)	62 (26%)	
Body Mass Index (kg/m^2)							
Mean ±SD	(21.3±3.8%)	(21.6±3.9%)	0.22	(21.9±3.9%)	(21.9±3.9%)	(21.1±3.7%)	0.15
Alcohol use N (%)	56 (28%)	144 (21%)	0.04	39 (21%)	53 (21%)	52 (22%)	0.98
Smoking N (%)							
Yes	7 (3%)	18 (3%)	0.58	4 (2%)	7 (3%)	7 (3%)	0.86
WHO stage 4 N (%)							
Yes	Not applicable	288 (41%)		59 (30%)	105 (39%)	124 (51%)	<0.001
Co-trimoxazole/Dapsone use in prior year N (%)							
Yes	41 (19%)	612 (87%)	<0.001	145 (75%)	247 (92%)	220 (91%)	<0.001
Employed N (%)							
Yes	51 (25%)	171 (25%)	0.99	50 (26%)	63 (24%)	58 (25%)	084
Hemoglobin (g/dl,)							
N	223	669	<0.001	180	251	238	<0.001
Mean ±SD	14.3±1.4	13.1±1.6		13.5±1.3	13.1±1.7	12.7±1.7	
Anemia N (%)	14 (6.3%)	137 (20.5%)		15 (7.6%)	43 (16.0%)	79 (32.2%)	<0.001
Marked anemia N (%)	0	28 (4.2%)	<0.001	4 (1.1%)	6 (2.4%)	20 (8.4%)	<0.001
White blood cell count, X 10^3 cells/mm^3							
N	223	670		181	251	238	
Mean ±SD	4.5±1.4	3.7±1.4	<0.001	4.3±1.6	3.8±1.3	3.3±1.4	<0.001
Neutropenia N (%)							
<2000 cells/mm^3	1 (0.45%)	28 (4.2%)	0.006	2 (1.1%)	6 (2.4%)	20 (8.4%)	<0.001
<1000 cells/mm^3	1 (0.45%)	1 (0.15%)					
Platelet count (X10^3/mm$^{3)}$							
N	208	654	0.05	179	246	229	0.55
Mean ±SD	231.8±84.5	223.2±109.0		225.9±106.7	222.9±109.9	221.4±110.3	
Thrombocytopenia	18 (8.6%)	88 (13.5%)	0.0547	24 (13.4%)	36 (14.6%)	31 (13.5%)	0.92

SD=Standard Deviation; WHO=World Health Organization; Anemia is defined as hemoglobin<12.0 g/dL; Marked anemia is defined as hemoglobin<10.0 g/dL; Thrombocytopenia is defined as platelet counts <125.0 X 10^3 /mm^3 . The HIV+ and HIV- women were compared using chi-square (X^2) test, and similarly CD4+ cell count categories within HIV+ women were compared the X^2 test. Rwf=Rwandan Francs

Table 2.A: Logistic Regression Analyses for modeling anemia, Hemoglobin (g/dl) <12 vs ≥ 12.

	Univariate Model		Multivariate Model[1]	
Variable	**OR (95% C.I.)**	**p-value**	**OR (95% C.I.)**	***p*-value**
HIV status, HIV+ vs. HIV-	3.84 (2.17, 6.82)	<0.001	NA[2]	
HIV+, CD4 >350 vs. HIV-	1.27 (0.59, 2.75)	0.54	1.47 (0.68, 3.21)	0.33
HIV+, CD4 200-350 vs. HIV-	3.09 (1.64, 5.81)	<0.001	3.59 (1.88, 6.83)	<0.001
HIV+, CD4 < 200 vs. HIV-	7.42 (4.05, 13.58)	<0.001	8.09 (4.37, 14.97)	<0.001
Age (years), 30-40 vs. <30	0.84 (0.54, 1.31)	0.44		
Age (years), 40+ vs. <30	0.65 (0.40, 1.06)	0.08		
10-35k Rwf vs. < 10k	0.67(0.46, 0.98)	0.04	0.68 (0.45, 1.03)	0.07
>35k Rwf vs. < 10k	0.80 (0.47,1.38)	0.43	0.94 (0.52, 1.70)	0.85
Some primary school	0.96 (0.61, 1.51)	0.87		
Secondary or university	1.00 (0.65,1.56)	0.98		
BMI (per kg/m^2) Per unit	0.87 (0.82, 0.92)	<0.001	0.87 (0.82, 0.93)	<0.001
Alcohol 1-4 drinks/week	0.97 (0.42, 2.25)	0.95		
Alcohol > 4 drinks/week	1.09 (0.68, 175)	0.71		
Co-trimoxazole or dapsone use	2.45 (1.54, 3.89)	0.0002		
WHO stage 4 illness Yes vs. No	2.40 (1.68, 3.43)	<0.001		
Widowed vs. married/ partner	0.99 (0.67, 1.47)	0.96		
Other vs. married/ partner	1.29 (0.80, 2.07)	0.30		
Smoking vs. not smoking	0.98 (0.33, 2.92)	0.98		
Employed vs. not employed	1.07 (0.71, 1.63)	0.73		

1. By Stepwise selection with overall p=0.05 to enter and p=0.10 to remain for the entire variable. Only the variables selected into the final model are included in the Table; **2.**HIV+ status was fit into the multivariate model by CD4 category with HIV- as baseline.

Table 2.B: Logistic Regression Analyses for modeling neutropenia WBC (cells/mm^3) <2000 vs ≥ 2000

	Univariate Model		Multivariate Model[1,2]	
Variable	**OR (95% C.I.)**	**p-value**	**OR (95% C.I.)**	***p*-value**
HIV status, HIV+ vs. HIV-	9.68 (1.31, 71.58)	0.03	NA[2]	
HIV+, CD4 >350 vs. HIV-	2.51 (0.23, 27.89)	0.45	1.03 (0.08, 13.15)	0.98
HIV+, CD4 200-350 vs. HIV-	5.44 (0.68, 45.51)	0.12	1.86 (0.78, 65.82)	0.60
HIV+, CD4 < 200 vs. HIV-	20.37 (2.71, 153.1)	0.003	7.18 (0.78, 65.82)	0.08
Age (years), 30-40 vs. <30	0.77 (0.33, 1.77)	0.54		
Age (years), 40+ vs. <30	0.28 (0.08, 0.92)	0.04		
10-35k Rwf vs. < 10k	1.07 (0.47, 2.42)	0.87		
>35k Rwf vs. < 10k	1.41 (0.48, 4.14)	0.53		
Some primary school	1.05 (0.39, 2.80)	0.92		
Secondary or university	1.20 (0.46, 3.09)	0.71		
BMI (per kg/m^2) Per unit	0.96 (0.86, 1.06)	0.40		
Alcohol 1-4 drinks/week	0.71 (0.09, 5.43)	0.74		
Alcohol > 4 drinks/week	1.07 (0.40, 2.89)	0.89		
WHO stage 4 illness Yes vs. No	3.36 (1.57, 7.21)	0.002		
Co-trimoxazole or dapsone use	12.18 (1.65, 90.0)	0.014	5.69 (0.63, 52.5)	0.12
Widowed vs married/ partner	1.15 (0.50, 2.66)	0.74		
Other vs married/ partner	1.19 (0.43, 3.34)	0.74		
Smoking vs. not smoking	1.28 (0.17, 9.83)	0.81		
Employed vs. not employed	0.36 (0.11, 1.22)	0.10		

1. By Stepwise selection with overall p=0.05 to enter and p=0.10 to remain for the entire variable. Only the variables selected into the final model are included in the Table**; 2.** HIV+ status was fit into the multivariate model by CD4 category with HIV- as baseline

Table 2.C: Logistic Regression Analyses for thrombocytopenia, platelets (mm^3) <125 vs ≥ 125.

	Univariate Model		Multivariate Model[1]	
Variable	**OR (95% C.I.)**	**p-value**	**OR (95% C.I.)**	***p*-value**
HIV status, HIV+ vs. HIV-	1.64 (0.96, 2.80)	0.07	NA[2]	
HIV+, CD4 >350 vs. HIV-	1.66 (0.87, 3.16)	0.13	2.18 (1.10, 4.32)	0.03
HIV+, CD4 200-350 vs. HIV-	1.81 (0.99, 3.29)	0.05	2.36 (1.25, 4.45)	0.008
HIV+, CD4 < 200 vs. HIV-	1.47 (0.79, 2.75)	0.23	1.95 (1.01, 3.79)	0.05
Age (years), 30-40 vs. <30	1.33 (0.74, 2.37)	0.34	1.29 (0.72, 2.33)	0.40
Age (years), 40+ vs. <30	1.61 (0.88, 2.95)	0.12	2.32 (1.20, 4.50)	0.01
10-35k Rwf vs. < 10k	0.89 (0.58, 1.38)	0.61		
>35k Rwf vs. < 10k	0.74 (0.38, 1.45)	0.38		
Some primary school	0.66 (0.40, 1.11)	0.12		
Secondary or university	0.77 (0.47, 1.26)	0.29		
BMI (per kg/m^2) Per unit	0.96 (0.90, 1.01)	0.12	0.95 (0.89, 1.00)	0.06
Alcohol 1-4 drinks/week	0.17 (0.02, 1.22)	0.08		
Alcohol > 4 drinks/week	0.87 (0.49, 1.53)	0.62		
WHO stage 4 illness vs. No	0.78 (0.50, 1.22)	0.28		
Co-trimoxazole or dapsone use	1.10 (0.69, 1.74)	0.69		
Widowed vs. married/ partner	0.63 (0.40, 0.98)	0.04	0.49 (0.29, 0.80)	0.005
Other vs. married/ partner	0.62 (0.34, 1.10)	0.10	0.59 (0.32, 1.07)	0.08
Smoking vs. not smoking	0.71 (0.16, 3.09)	0.65		
Employed vs. not employed	1.15 (0.71, 1.86)	0.57		

1. By Stepwise selection with overall p=0.05 to enter and p=0.10 to remain for the entire variable. Only the variables selected into the final model are included in the Table**; 2.** HIV+ status was fit into the multivariate model by CD4 category with HIV- as baseline.

Table 3: Prevalence of anemia, neutropenia and thrombocytopenia in HIV-infected women in 5 studies

Study (N)	Anemia			Neutropenia		Thrombocytopenia
	Hb<12.0	Hb<11.0	Hb<10.0	WBC<2000	WBC<1000	Platelets<125,000
RWISA (710) [Rwanda]	20.3%		4.9%	4.2%	0.1%	13.5%
WIHS* (2059) [North America]	37.0%		7.2%	44%	7%	14.6%
Uganda** (123)		23.6%				
APS*** (2197) [North America]	32.3%		6.8%			
PEARLS**** [Africa, Asia and Americas]					~15%	

*WIHS=Women's Interagency HIV Study[10, 18]; **Uganda[20]; ***APS=Anemia Prevalence Study[22]; ****PEARLS=Prospective Evaluation of ART in Resource Limited Settings[28]

RWANDA BIOMEDICAL CENTER

A Healthy People. A Wealthy Nation

National Reference Laboratory (NRL) Division

Kigali, September 26th, 2012
Ref. No: NRL/2012

Mr. Richards Sands
The Managing Editor, BMJ Open
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Dear Editor:

We thank you and the reviewers for the careful and complete review of our revised manuscript. I am attaching a further revised manuscript and our responses to the reviewer's comments below.

When making the changes to the review we noticed that we had modeled age as a continuous variable in Table 2 while it had been categorical in Table 1. We believe that it is important to be consistent and the categorical was more correct so we have changed age to categorical in Table 2, which resulted in changes to coefficients of other variables in the multivariate model for thrombocytopenia Table 2c. We apologize for not having noticed this problem earlier.

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 modification[1-7]. 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 unstable[5]. It also creates other problems with collinearity that can destabilize estimates[6]. Other fallacies of examination of effect modification occur from logistic models if the underlying association is not multiplicative in the odds ratio[7].

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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
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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.”

Again, we thank the editor and reviewer for their time and commitment.

Sincerely,

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