BMJ Open

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Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-020432
Article Type:	Protocol
Date Submitted by the Author:	04-Nov-2017
Complete List of Authors:	Murenzi, Gad; Rwanda Military Hospital Dusingize, Jean; Regional Alliance for Sustainable Development , Research and Scientific Capacity Building Rurangwa, Theogene; Rwanda Military Hospital Sinayobye, Jean d'Amour; Regional Alliance for Sustainable Development, ; Women's Equity in Access to Care & Treatment (WE-ACTx), Munyaneza, Athanase; Rwanda Military Hospital Murangwa, Anthere; Rwanda Military Hospital Zawadi, Thierry; Rwanda Military Hospital Hebert, Tiffany; Yeshiva University Albert Einstein College of Medicine Mugenzi, Pacifique; Rwanda Military Hospital Adedimeji, Adebola; Yeshiva University Albert Einstein College of Medicine Mutesa, Leon; Rwanda Military Hospital; University of Rwanda College of Medicine and Health Sciences Anastos, Kathryn; Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, Castle, Philip; Yeshiva University Albert Einstein College of Medicine,
Keywords:	human papillomavirus (HPV), cervical cancer, HIV & AIDS < INFECTIOUS DISEASES, GYNAECOLOGY, cervical intraepithelial neoplasia

A Study of Cervical Cancer Screening Technologies in Human Immunodeficiency Virus-Infected Women Living in Rwanda

Gad Murenzi, MD*[1], Jean-Claude Dusingize, MD, MS[1], Theogene Rurangwa, MD, MMed[1], Jean d'Amour Sinayobye, MD, MS[1], Athanase Munyaneza, RN[1], Anthere Murangwa, MS[1], Thierry Zawadi, MD[1], Tiffany Hebert, MD[2], Pacifique Mugenzi, MD[1], MMed, Adebola Adedimeji, PhD, MPH[2], Leon Mutesa, MD, PhD[1,3], Kathryn Anastos, MD[2], Philip E. Castle, PhD, MPH[2,4]

[1]Rwanda Military Hospital, Kigali, Rwanda; [2]Albert Einstein College of Medicine, Bronx, NY, USA; [3]University of Rwanda, Kigali, Rwanda; [4]Global Coalition Against Cervical Cancer, Arlington, VA, USA.

*Correspondence: gadcollins@gmail.com, +250788589085

Disclosures: This research study has received HPV tests for reduced or no cost from Cepheid, Arbor Vita Corporation, and Roche.

Funding: This study was funded by NCI/NIH Grant 5U54CA19016304 and by a grant from the Prevent Cancer Foundation.

Abstract

Introduction. The optimal method(s) for screening human immunodeficiency virus-infected women, especially for those living in sub-Saharan Africa, for cervical precancer and early cancer has yet to be established.

Methods and analysis. A convenience sample of >5,000 Rwandan women, aged 30-54 years, living with HIV infection will be enrolled into a cross-sectional study of cervical cancer screening strategies. Eligible and consenting women will be enrolled into the study, complete a short risk-factor questionnaire, and screened for high-risk human papillomavirus (hrHPV) using the Xpert HPV assay (Cepheid, Sunnyvale, CA, USA), unaided visual inspection after acetic acid (VIA), and aided VIA using the EVA system (Mobile ODT, Tel Aviv, Israel). Women positive for hrHPV or by VIA will undergo colposcopy, which will include the collection of two cervical specimens prior to undergoing a 4-quadrant microbiopsy protocol. The colposcopy-collected specimens will be tested by dual immunocytochemical staining for $p16^{INK4a}$ and Ki-67 (CINtec® PLUS Cytology, Ventana, Tucson, AZ, USA) and for E6 or E7 for 8 hrHPV genotypes (HPV16, 18, 31, 33, 35, 45, 52, and 58) using the next-generation AV Avantage hrHPV E6/E7 test (Arbor Vita Corporation, Freemont, CA, USA). Women with local pathology diagnosis of cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or more severe (CIN2+) or pathology-review diagnosis of CIN grade 3 or more severe (CIN3+) will receive treatment. Clinical performance and cost-effectiveness (e.g., sensitivity, specificity, and predictive values) of different screening strategies and algorithms will be evaluated.

Ethics and dissemination. The protocol has been approved by local and institutional review boards for human subjects research. Results will be disseminated to the scientific community through peer-reviewed publication and to the Rwandan stakeholders through an external advisory panel.

Strengths and Weaknesses

- We will enroll a very large sample size of HIV-infected women living in Rwanda who otherwise would probably not get cervical cancer screening.
- We will employ rigorous disease ascertainment protocols to minimize misclassification.
- Some tests, such as the Xpert HPV and the AV Avantage HPV E6/E7 assays, will be done on site in Rwanda using technologies that could feasibly be deployed there.
- A weakness of the study is that it will not be feasible to include cervical cytology as a comparator due to financial and logistical constraints.

Introduction

Invasive cervical cancer (ICC) remains a significant cause of morbidity and mortality globally. Approximately 530,000 cases of and 270,000 deaths due to ICC occur annually, making it the 4th most common malignancy and cause of cancer-related death in women globally.[1] In many high-income countries (HIC), cervical cancer rates have declined by 50% or more[2] due to the introduction of effective, high-coverage Pap testing (cervical cytology)-based screening programs that include timely follow-up of screen positives, treatment of women with precursor lesions, and management of cancers. Now, almost 90% of ICC and ICC-related deaths occur in low- and middle income countries (LMICs) due to a lack of resources and healthcare infrastructure needed to provide preventive services.

ICC and ICC-related mortality rates are particularly high in Sub-Saharan Africa, which also has the highest rates of HIV infection in the world. Now, over 12 million HIV-infected (HIV[+]) women in Sub-Saharan Africa are living longer because of anti-retroviral therapy, thus increasing their likelihood of dying from ICC.[3] However, many of these women are already exposed to human papillomavirus (HPV), the viral cause of cervical cancer, and will not benefit from or be targeted for prophylactic HPV vaccination. Thus, cervical cancer screening is needed for the foreseeable future.

However, setting up effective cytology for cervical cancer screening is expensive and requires a complex clinical and lab infrastructure that generally does not exist in LMICs.[4;5] Moreover, it is now well understood that cytology has only a low- to moderate one-time sensitivity for precursor lesions and therefore must be done repeatedly over many years to reduce cancer risk. Alternative

strategies to address the cervical cancer burden in LMICs, especially in SSA, must be developed and validated.

Persistent cervical infections by high-risk HPV (hrHPV) types cause virtually all ICC and its immediate precursor lesions, e.g. cervical intraepithelial neoplasia grade 3 (CIN3) and adenocarcinoma *in situ* (AIS) everywhere in the world.[6;7] hrHPV causes most anal and vaginal cancer and a significant proportion of vulvar, penile, and oropharyngeal cancers.[8] HPV16 is the most important causal type, responsible for ~60% of ICC.[9] HPV18 is the next most important, responsible for 10-15% of ICC, including 30-40% of adenocarcinoma of the cervix[9], which is on the rise in Western Countries.[10;11] Together, HPV16 and HPV18 account for ~70% of ICC, and the same 15 hrHPV types account for ~99% of ICC everywhere in the world.[9]

There is now overwhelming evidence to suggest that testing for hrHPV is more sensitive, albeit less specific, than high-quality cytology for identifying women with cervical precancer.[12-16] One-time hrHPV testing can reduce the risk of ICC incidence by approximately 40% in 6.5 years compared to cytology screening[16], and ICC mortality by approximately 40% (approximately 50% overall) in 8 years compared to cytology.[17] Importantly, a negative hrHPV test provides superior reassurance against CIN3+ [18] and against ICC[16;17], permitting safe extension of screening intervals.

The World Health Organization released cervical cancer screening and treatment guidelines in 2013, recommending two evidence-based approaches to ICC screening[19]: (I) Use either hrHPV testing or visual inspection after acetic acid (VIA), which involves the inspection of the cervix with a speculum in place and following the application of dilute acetic acid to help identify potential CIN by its characteristic white coloring in the presence of acetic acid (acetowhite), as

alternative initial screening tests instead of Pap, and (II) immediately treat those who screen positive using the screening test, rather than require diagnostic verification through colposcopy and biopsy. This approach is commonly called screen-and-treat (S&T), and is increasingly thought to be more amenable to LMIC settings.

However, hrHPV testing is also a much more effective screen than VIA[17], which on a large-scale appears to only down-stage cancer rather than prevent it.[20] Thus, the recent American Society for Clinical Oncology (ASC) resource-stratified guidelines for secondary cervical cancer prevention[21;22] emphasize that hrHPV testing is the preferred choice for screening, with VIA only being used until hrHPV testing becomes available, and that HIV-infected women, because of their higher risk, should be screened twice as frequently as the general (HIV-uninfected) population.

Recent data in HIV[+] women living in the U.S. suggest that hrHPV testing may have clinical utility similar to that in HIV-negative (HIV[-]) women. Several observational studies have shown that an extended screening interval is safe in HIV[+] women who test hrHPV and Pap negative as it is for HIV[-] women.[23;24] In a study of women enrolled in Women's Interagency Health Study (WIHS) in 2002, HIV[+] and HIV[-] women who tested hrHPV and Pap negative were at a similarly low risk of cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or more severe (CIN2+) histology over a 5-year follow-up.[23] In addition, no cases of histologically confirmed CIN2+ were diagnosed in the follow-up of hrHPV- and Pap-negative HIV[+] women aged 30-64 years who underwent routine three-year hrHPV and cytology cotesting at Kaiser Permanente Northern California.[24] Thus, both studies found very high negative predictive values (NPV) >99% in HIV[+] women who test hrHPV negative. However, how hrHPV testing can

best be used to screen HIV[+] women living in Sub-Saharan Africa to prevent cervical cancer remains to be determined.

Recent studies[25-28] in HIV[+] women living in Sub-Saharan Africa have compared hrHPV, VIA, and/or Pap for the detection of cervical precancer/cancer. The results can be summarized as follows: 1) hrHPV detection was more sensitive but less specific than VIA and 2) surprisingly, cytology was equally or more sensitive but less specific than VIA and 2) surprisingly, cytology was equally or more sensitive but less specific (vs. the converse) than hrHPV testing. Results and conclusions are varied, leaving unanswered the question of what screening strategy in HIV[+] women living in Sub-Saharan Africa has the greatest effectiveness and cost effectiveness.

Regardless of the screening method, most screen-positive women who go to colposcopy or are treated immediately without diagnostic verification do not have cervical precancer and cancer (positive predictive value [PPV] for screening tests are typically 10%-20%). In places like Sub-Saharan Africa that lack necessary infrastructure and personnel such as pathologists[29], excessive referral to colposcopy is problematic. Although WHO recommendations for S&T will hopefully overcome this bottleneck and increase the number of women living in LMICs who get screened, many countries may not adopt current S&T strategies because of concerns of low specificity and overtreatment, resulting in increased costs, unnecessary patient discomfort and concern, and wasting valuable healthcare resources that could otherwise be used to expand access to screening. Thus, methods to increase the accuracy of screening by reducing the numbers of women having colposcopy and biopsy or getting treated immediately in this context are highly desirable as they will likely increase the uptake of cervical cancer screening.

In order to improve the specificity of screening tests, secondary tests (biomarkers) are used following a screen-positive result, with women who test positive for the triage undergoing further management (e.g., colposcopy or immediate treatment) and those who test negative typically being deferred to further evaluation in 6-18 months to allow hrHPV infections to clear. There are several very promising biomarkers that might be used to improve the specificity and positive predictive value (PPV) of the screening tests.[24] Given that HIV-infected women are more likely to test hrHPV positive[30-34], it is important to validate a triage strategy of using a secondary biomarker that sensitively and specifically rules-in women with cervical precancer and cancer among the HIV-positive, hrHPV-positive women.

We are therefore conducting a cervical cancer screening study of >5,000 Rwandan women, aged 30-54 years, living with HIV infection. We will evaluate different screening tests (hrHPV DNA and VIA) and different triage tests and biomarkers for screen-positive women (E6/E7 oncoprotein detection, p16INK4a immunocytochemistry, and hrHPV viral methylation). Screen-positive women undergo a rigorous colposcopic evaluation with multiple biopsies taken and the biopsies will undergo pathology review, to minimize the misclassification of endpoints. The primary objective of the study is to determine and compare clinical performance (Sensitivity (Se), Specificity (Sp), PPV, and NPV) and cost-effectiveness for identifying HIV[+] women with CIN3+ and CIN2+ of different cervical cancer screening and management algorithms.

Methods and Analysis

Study design and population: We are recruiting those women receiving care in health centers (HC) and various hospitals operated by the Ministry of Health or Rwanda Military Hospital during 2016-18 (**Table 1**). Sites were selected in collaboration with Rwanda Biomedical Center

(RBC), which calculated estimated numbers of potentially eligible women using data from the HIV database (OpenMRS-Open Medical Records System; http://openmrs.org/). Sites were also selected from all provinces to ensure geographic representation. The total is the number of 30-54 year old women receiving care at all selected sites and our study population is an estimate of the women who will actually be eligible according to all inclusion and exclusion criteria.

Inclusion criteria include 1) living and receiving HIV care in Rwanda, 2) aged 30-54 years, 3) confirmed HIV+, 4) no prior cervical cancer screening, 5) no history of ICC, and 6) willing, able and competent to provide written, informed consent. Exclusion criteria, in addition to not meeting the inclusion criteria, include 1) pregnant, 2) signs of abnormal, non-menstrual bleeding suggestive of ICC, 3) without a cervix due to hysterectomy, and 4) not sufficiently healthy to participate in a research study based on the judgment of the clinicians. Excluded women will be advised to seek routine cervical cancer screening through government programs.

Prior to initiation of enrollment at a specific clinic, the local HIV-care provider team identifies potentially eligible women at their routine clinic visits and offer them enrollment. Women indicating interest in the study are then registered by our research nurses using the eligibility criteria checklist. We enroll women at one site until all the eligible women at that site are screened for cervical cancer. Our team of at least two research nurses schedules 12 to 15 women three to four days a week by calling them and confirming appointments over the telephone. We have two teams in the field meaning that we run two cervical cancer screening clinics simultaneously.

Enrollment Visit: The study participant flow is summarized in **Figure 1.** During their appointment for screening, women are educated on cervical cancer risk factors, mainly HPV

infection, and why they are more at risk to develop cervical cancer than HIV-uninfected women. They are also allowed to ask questions before they commit to participating in the study. Women are asked to provide informed, written consent to participate in the study using a printed out consent form. Those who provide consent complete a short nurse-administered questionnaire on cervical cancer risk factors and sociodemographic characteristics using a data capture screen in Microsoft Access.

Enrolled women then undergo a pelvic exam, with VIA and a single cervical exfoliated ("Pap") specimen collected and placed into 20 ml PreservCyt (Hologic, Bedford, MA, USA) which is then sent to the lab at RMH for hrHPV testing. Finally, a portable colposcope (http://www.mobileodt.com/; MobleODT, Tel Aviv, Israel) is used for digital cervicography (~VIA with magnification) and the image is captured and saved for quality control, research, and to develop a digital library.

Colposcopy Visit: Screen-positive women are called using a telephone as soon as the hrHPV result is available and provided colposcopy. All women receiving colposcopy will have two additional specimens collected, one into PreservCyt for the evaluation of other molecular biomarkers (genotype-specific hrHPV viral methylation and load, and p16/Ki-67 immunocytochemistry CINtec® PLUS Cytology Kit [Roche, Tucson, AZ, USA]) and a second using a dry swab for HPV16, 18, 31, 33, 35, 45, 52, and 58 E6/E7 oncoprotein detection by the next generation lateral flow hrHPV oncoprotein test from Arbor Vita Corporation (Fremont, CA, USA) included in this study as a triage for screen-positive women to identify those women who are at higher risk of having CIN3+. The residual PreservCyt specimens from both the screening and colposcopy visits will be stored at -20ºC, creating a biobank in Rwanda for future retrospective evaluations of promising new biomarkers and tests.

After specimen collection, a colposcopic evaluation of the cervix is done and a modified version of the 4-quadrant microbiopsy procedure is performed.[35] Compared to the standard biopsy, the microbiopsy protocol improves disease ascertainment and reduces biases by selecting on the most obvious acetowhite lesions while removing less tissue (~13 mm^2 for 4 microbiopsies vs. ~28mm^2 for 1 standard biopsy). Modifications to the standard 4-quadrant microbiopsy procedure are: 1) endocervical curettage is taken only for those women whose squamocolumnar junction is not entirely visible and the lesion extends into the endocervical canal; and 2) standard biopsies of very large lesions can be taken to increase the likelihood that the most severe area is biopsied.

Pathology: Biopsies are processed in a single cassette so that a single slide has a section from all biopsies taken. Biopsies read by a local pathologist at RMH and Dr. Hebert or another pathologist at Montefiore Medical Center, Bronx, NY, USA. Women receiving a diagnosis of CIN2+ by the local pathologist or CIN3+ diagnosis by consensus review will be referred for treatment. A slide with biopsies also will undergo p16 immunohistochemistry (IHC) using the CINtec® Histology Kit (Roche).

Endpoints: The primary scientific endpoint of the study will be histologically confirmed, consensus CIN3+. The secondary, clinical endpoint will be histologically confirmed ≥+ diagnosed by the Rwandan pathologist. Additional endpoints using pathology review and p16 IHC will be used but not for evaluating the performance of p16 immunocytochemistry due to the possibility of p16-related autocorrelation.

Treatment: Women diagnosed with CIN2+ will be referred for treatment. Those precancerous lesions will be treated by ablation if they meet WHO criteria for cryotherapy.[36] Those who do not meet those criteria will undergo an excision procedure (e.g., loop electrosurgical excision

procedure [LEEP] or cold-knife cone [CKC]) or, in the case of an ICC diagnosis, referred for cancer management. Screen-positive women with <CIN2 will be advised to seek re-screening in a year through the existing healthcare system.

Data sources: Data are collected from the following sources:

1. A nurse-administered questionnaire on sociodemographic characteristics and cervical cancer risk factors including age at first sexual intercourse, number of sexual partners, smoking, contraception, parity and socioeconomic status.

2. Pelvic exam, VIA, Mobile ODT and colposcopy data capture forms

3. Medical record data on HIV status (e.g., (http://www.who.int/hiv/pub/guidelines/HIVstaging150307.pdf), CD4 count, viral load, antiretroviral therapy (ART) regimen(s)), care, and dates.

Laboratory Testing: The following laboratory tests will be performed:

Xpert HPV Testing—cervical Pap specimens in PreservCyt will be sent to the RMH laboratory in Kigali, Rwanda for hrHPV DNA testing using the Xpert HPV test (Cepheid, Sunnyvale, CA, USA).[37-42] The Xpert HPV Assay is a new, qualitative, real-time PCR assay for the detection of hrHPV DNA. The Xpert HPV Assay includes simultaneous detection of 14 hrHPV types, hydroxymethylbilane synthase (HMBS), and an internal Probe Check Control (PCC). The 14 targeted hrHPV types are detected in 5 fluorescent channels: 1) HPV16, 2) HPV18 and hrHPV 45 (HPV18/45), 3) HPV31, 33, 35, 52, and 58, 4) HPV51 and HPV59, and 5) HPV39, 56, 66, and 68. HMBS (fluorescent channel 6) verifies specimen adequacy.

Specimens are mixed and a 1-mL pre-aliquot is removed using a disposable pipette and placed in the testing cartridge per the manufacturer's instructions. Unsatisfactory results due to insufficient cellular content are re-run. If the second test is also unsatisfactory, the final result will be unsatisfactory but women are referred to colposcopy for safety.

HPV Viral Methylation—To identify single hrHPV type infections, we will select single-channel positives from the Xpert HPV assay. For those that are hrHPV positive for a channel other than HPV16, which is detected singly, we will test them to identify the single type infections using a standard protocol for PCR amplification using MY09/11 L1 consensus primers and hrHPV genotype detection using dot-hybridization for 39 individual type-specific probes and a mixture of probes for 10 other uncommon hrHPV types as previously described.[43;44] To isolate the DNA, ThinPrep specimens (1.5 mL) will be pelleted, re-suspended in STM, digested with Proteinase K, precipitated overnight in ammonium acetate ethanol at -20°C, washed, and suspended and stored in TE buffer.

The isolated DNA will then undergo bisulfite conversion.[45] Following bisulfite conversion and DNA purification and de-sulphonation, bisulfite-treated DNA will be used as template for Next-Gen Sequencing (NGS) (HiSeq2000, Illumina, San Diego, CA) using barcoded-type specific primers. Sequences for pads and barcodes are not found in the targeted genomic region. Use of padding and barcodes enables reads to be identified by amplicon (forward or reverse) or by sample during downstream bioinformatics analysis.[46]

All PCR products for NGS are pooled (by assay) and a single DNA band containing multiple amplicons from different samples (with unique barcodes) is isolated from a gel for NGS.[46] Briefly, equal concentrations of each barcoded PCR product (based on PCR band intensity) are

pooled and isolated. Upon confirmation of correct product size, all purified DNA pools are combined and submitted for library preparation and paired-end 100 base pair Illumina HiSeq2000 sequencing at the Einstein Genomics Core Facility.

Methylation status will be determined in the lab of Dr. Robert Burk at Albert Einstein College of Medicine (USA). Prior to determination of methylation status, de-multiplexing based on the unique barcodes is performed using in-house generated scripts to obtain paired-end NGS reads of each sample. Reads are aligned with hrHPV reference genome sequences by bowtie v0.12.9.[47] Methylation status of each CpG site is then determined by bismark v0.7.7[48] using the default quality score parameter set to Q30, and the formula of the methylation ratio of the number of C read by the number of C+T read.

E6/E7 Oncoprotein Testing—Dry swab specimens collected at the time of colposcopy will be tested for individual E6/E7 oncoproteins as previously described[49;50], according to the manufacturer's instructions, at the RMH laboratory in Kigali, Rwanda. The only deviation from the previous methods is that 3 lateral flow strips will be used to detect 8 hrHPV types in this study vs. 1 lateral flow strip used to detect 3 types in the study in China.

Analyses: We will evaluate combinations of the above mentioned screening strategies and tests to estimate the clinical performance (e.g., Se, Sp, PPV, and NPV) for the detection of consensus CIN3+ and community CIN2+. A log binomial model implemented with generalized estimating equations will be used to take into account correlation between different tests from the same subject. Note that these models are developed for the estimation and comparison of performance for two tests, but the model can be extended to allow more than two tests by including more indicator variables for test type.

Some analyses of biomarkers, such as viral methylation are restricted to hrHPV-positives. Comparisons of hrHPV viral methylation to other triage biomarkers will be restricted to the subset that gets tested for viral methylation as described.

Sample size calculations: We based our sample size on the ability to detect modest but minimally important differences in Se of 15%. We conservatively assume that the population prevalence of CIN3+ is 2% based on our past study in Rwanda.[33;51] We propose to enroll and have complete follow-up of at least 5,000 HIV[+] women. A sample size of 5,000 HIV[+] women with completed follow-up of the screen positives will yield 100 cases of CIN3+, which has at least 80% power (α=5%) to crudely detect a 15% difference in Se between two screening strategies for a range of 10%-25% discordance. With this sample size of 5,000 women, 4,900 will not have CIN3+; we will also have at least 90% power (α=5%) to detect a difference in Sp of 3% for discordance up to 40%. Finally, we will have 80% power (α=5%) to crudely detect an 8%, 10%, or 11% difference in PPV if the reference PPV is 10%, 20%, or 30%, respectively.[52]

Cost Effectiveness: We will conduct assessments of the costs and cost-effectiveness of the different combinations of screening and triage tests, i.e., algorithms, as well as those of the entire community-based screening "system." Costs measurement will be conducted using a micro-costing (ingredients) approach in which resource use throughout each step in the screening process will be tracked and unit costs for each of the resources will be applied to generate an average screening cost per woman to be compared against what the estimated costs are for a possible program based on hrHPV screening and VIA triage or VIA screening. For estimating costs of the screening system and scale-up of screening to 100,000 women in a month, analyses will distinguish financial costs, which reflect actual expenditures of the program, from economic

costs, including the value of donated and shared resources to more fully assess opportunity costs. Projections on budget impact and economic cost implications over time will be made under varying assumptions of screening uptake, follow-up compliance, and scenarios of changing disease burden.

Clinical outcomes will include true positive, true negative, false negative, and false positive test results, number of colposcopies, incident cancer, and cancer death. Cost-effectiveness will be measured as cost/CIN2+ detected, cost/CIN3+ detected, cost/invasive cancer prevented, cost/cancer death prevented, cost/life-year saved, and cost/quality-adjusted life year (QALY) saved; in addition, we will calculate harm/benefit ratios, using varying definitions of harms (colposcopies, false positive results) to benefits (cancers prevented, deaths prevented, life years and QALYs saved). Costs and effectiveness will be discounted at a 3% annual rate, with the rate varied from 0-5% in sensitivity analysis. For assessment of value-of-information (VOI), we will use net monetary benefits (NMB), defined as a function of the willingness-to-pay threshold (WTP) for different costs and outcomes as: $NMB=(WTP * Effectiveness) - Costs$.

Ethics and Dissemination

Ethics: This study protocol was reviewed and approved by the Rwanda National Ethics Committee (RNEC) as well as the Institutional Review Board for human subjects research at Albert Einstein College of Medicine.

Confidentiality measures and protection against potential risks: The risks for those participating in our study include:

- Collection of Pap specimens/cervical swabs involves a modest risk of bleeding which is typically very limited when it occurs. Testing positive for any test may cause psychological distress (anxiety).
- Colposcopy and excisional treatments induce vaginal bleeding and may incur pain, infection, and short-term psychological distress (anxiety). A diagnosis of CIN2 or more severe may cause psychological distress (anxiety). A diagnosis of invasive cervical cancer may cause severe psychological distress.
- Questions in the questionnaire, regarding sexual behavior and other matters of a personal nature, may cause anxiety and embarrassment. Participants are advised that they are free not to answer specific questions.
- There is also the risk of psycho-social stress which could occur if there was inadvertent disclosure of confidential medical or other personal information.

Protection against the risk of inadvertent disclosure of confidential information is addressed by the standard procedures at the Rwandan study site, including: (i) storing completed paper copies of questionnaires and other hard copy information (described above), identified by study number only, in a filing system separate from the name-address file of participants in the study; and (ii) only the designated local personnel have access to cross-reference the files; (iii) all paper files, including consent forms, will be maintained in locked cabinets in locked rooms, with access restricted to specific research personnel.

In addition, we will include the following security measures to protect the data:

- Controlled access to project data;

- A tracking system for data forms and activities;
- Date and time of stamping of all data records with electronic signatures;
- Audit trails to track all changes made to data records; and
- Data kept on password-protected computers and in locked rooms.

Potential Patient Benefits: There are potential direct benefits to study participants. HIV[+] women participating in the study are at very high risk of cervical cancer. They will be rigorously screened and evaluated, more effectively than the standard of care anywhere in the world. As a result, women with precancer who are at imminent risk of invasive cervical cancer will be diagnosed sooner and treated more effectively than women receiving routine care and thereby more likely averting the development of cervical cancer. Women with cervical cancer will be diagnosed earlier thereby reducing the morbidity and the risk of mortality caused by cervical cancer. Conversely, any pain, bleeding, or stress that might occur related to colposcopy or cervical swab are typically modest and well tolerated.

There are also substantial potential societal benefits related to the study due to its implications to improving cervical cancer screening practices and guidelines in HIV[+] women – changes in practice which might also benefit the study participants themselves, if and when these changes are enacted. There is a great need to identify more effective and practical methods for cervical cancer screening for HIV[+] women living in Africa, who are living longer than ever and are therefore at potentially greater risk of cervical cancer.

Dissemination: We plan to publish a series of scientific reports in peer-reviewed scientific journals. As building research capacity in Rwanda is a major goal of this research project, all investigators of the research team will be asked and supported to lead at least one analysis and one manuscript preparation, based on interests and expertise.

In addition, an external advisory panel (EAP) composed of leaders from the Rwanda Ministry of Health, University of Rwanda, and Rwanda medical community has been formed. The responsibilities of the EAP include providing advice on the conduct of the project and interpretation for and dissemination of the study results to Rwandan stakeholders. The latter is important for the adoption of evidence-based best practices for cervical cancer screening as warranted.

Contributor Statement:

KA, PEC, JCD, AA, and JDS conceived the original concept of the study and the interventions. GM, KA, JDS, and PEC drafted the protocol. PEC performed the sample size calculations, and PEC and GM will lead analysis of the results. GM, JDS, and Athanase Munyaneza are supporting patient recruitment. GM, TR, and Athanase Munyaneza are providing clinical care for patients. Anthere Murangwa and LM oversee laboratory testing, and TZ and TH are responsible for pathology. PM and LM oversee and administer the study activities at the clinical site in Rwanda. All authors (GM, JCD, TR, JDS, Athanase Munyaneza, Anthere Murangwa, TZ, TH, PM, AA, LM, KA, and PEC) contributed to the scientific design of the study and the protocol development, are involved in the implementation of the project, and have read and approved the final manuscript.

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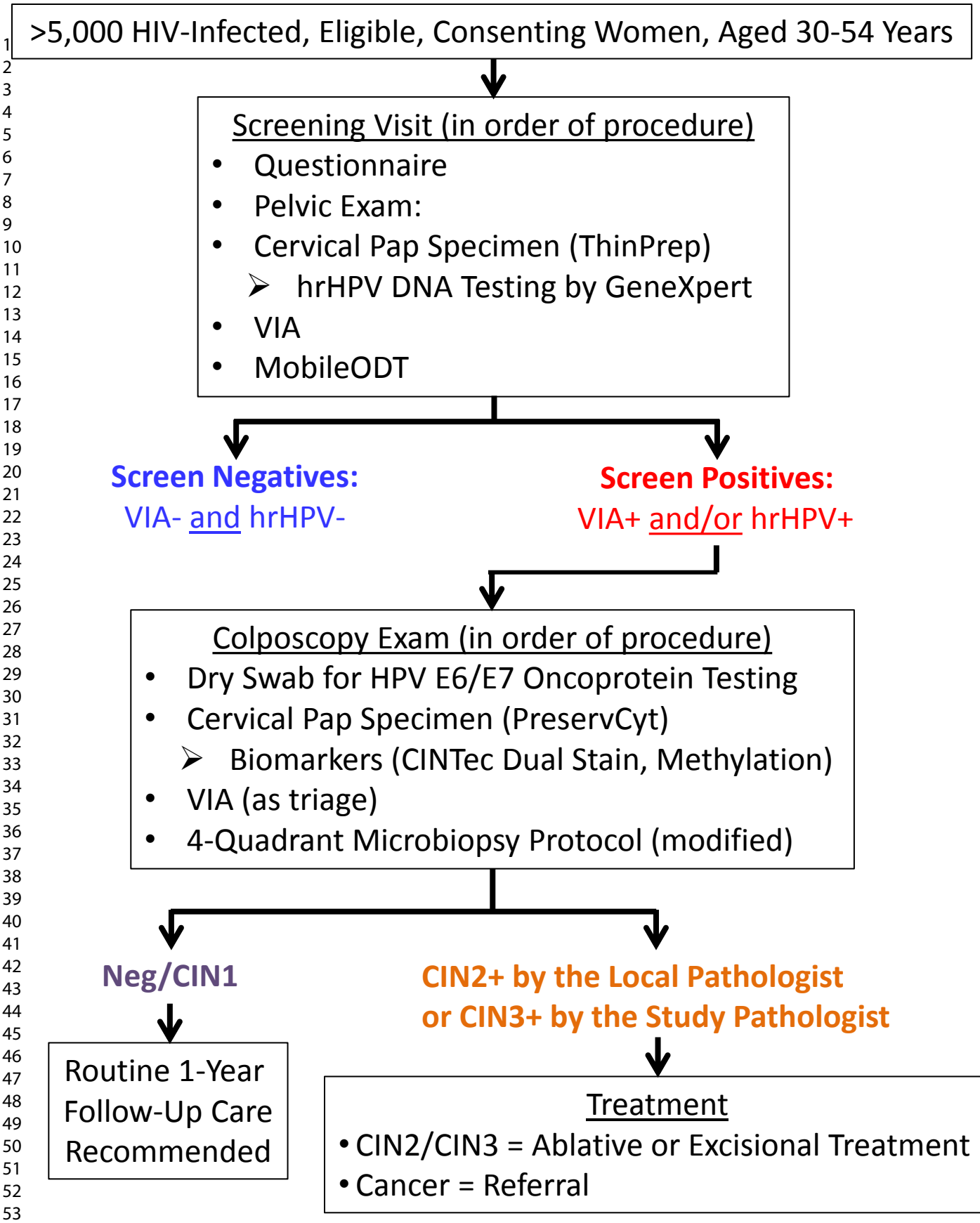
Table 1: Recruitment sites and estimated population to be recruited from each site.

Province	Site	Type of site	Potential participants per site (approximate)
Kigali	Cor-unum HC	Public Health Center	1,405
Kigali	Kimironko HC	Public Health Center	1,227
Kigali	Rwampara HC	Public Referral Hospital	1,098
Kigali	Kicukiro HC	Public Health Center	1,054
Kigali	Kacyiru HC	Public Health Center	905
Kigali	Gikondo HC	Public Health Center	925
Kigali	Rwanda Military Hospital	Public Referral Hospital	300
Kigali	WEACTx for Hope	Private HIV Clinic	500
Kigali	Busanza HC	Public Health Center	100
Kigali	Nyarugunga HC	Public Health Center	100
West	Gisenyi DH	Public District Hospital	810
Total			8,424

Figure 1 Study diagram



BMJ Open

A Study of Cervical Cancer Screening Technologies in Human Immunodeficiency Virus-Infected Women Living in Rwanda

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-020432.R1
Article Type:	Protocol
Date Submitted by the Author:	19-Mar-2018
Complete List of Authors:	Murenzi, Gad; Rwanda Military Hospital Dusingize, Jean; Regional Alliance for Sustainable Development , Research and Scientific Capacity Building Rurangwa, Theogene; Rwanda Military Hospital Sinayobye, Jean d'Amour; Regional Alliance for Sustainable Development, ; Women's Equity in Access to Care & Treatment (WE-ACTx), Munyaneza, Athanase; Rwanda Military Hospital Murangwa, Anthere; Rwanda Military Hospital Zawadi, Thierry; Rwanda Military Hospital Hebert, Tiffany; Yeshiva University Albert Einstein College of Medicine Mugenzi, Pacifique; Rwanda Military Hospital Adedimeji, Adebola; Yeshiva University Albert Einstein College of Medicine Mutesa, Leon; Rwanda Military Hospital; University of Rwanda College of Medicine and Health Sciences Anastos, Kathryn; Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, Castle, Philip; Yeshiva University Albert Einstein College of Medicine,
Primary Subject Heading:	Global health
Secondary Subject Heading:	Obstetrics and gynaecology, Infectious diseases, HIV/AIDS, Epidemiology, Diagnostics
Keywords:	human papillomavirus (HPV), cervical cancer, HIV & AIDS < INFECTIOUS DISEASES, GYNAECOLOGY, cervical intraepithelial neoplasia

A Study of Cervical Cancer Screening Technologies in Human Immunodeficiency Virus-Infected Women Living in Rwanda

Gad Murenzi, MD*[1], Jean-Claude Dusingize, MD, MS[1], Theogene Rurangwa, MD, MMed[1], Jean d'Amour Sinayobye, MD, MS[1], Athanase Munyaneza, RN[1], Anthere Murangwa, MS[1], Thierry Zawadi, MD[1], Tiffany Hebert, MD[2], Pacifique Mugenzi, MD[1], MMed, Adebola Adedimeji, PhD, MPH[2], Leon Mutesa, MD, PhD[1,3], Kathryn Anastos, MD[2], Philip E. Castle, PhD, MPH[2,4]

[1]Rwanda Military Hospital, Kigali, Rwanda; [2]Albert Einstein College of Medicine, Bronx, NY, USA; [3]University of Rwanda, Kigali, Rwanda; [4]Global Coalition Against Cervical Cancer, Arlington, VA, USA.

*Correspondence: gadcollins@gmail.com, +250788589085

Disclosures: This research study has received HPV tests for reduced or no cost from Cepheid, Arbor Vita Corporation, and Roche.

Funding: This study was funded by NCI/NIH Grant 5U54CA19016304 and by a grant from the Prevent Cancer Foundation.

Abstract

Introduction. The optimal method(s) for screening human immunodeficiency virus-infected women, especially for those living in sub-Saharan Africa, for cervical precancer and early cancer has yet to be established.

Methods and analysis. A convenience sample of >5,000 Rwandan women, aged 30-54 years, living with HIV infection will be enrolled into a cross-sectional study of cervical-cancer screening strategies. Eligible and consenting women will be enrolled into the study, complete a short risk-factor questionnaire, and screened for high-risk human papillomavirus (hrHPV) using the Xpert HPV assay (Cepheid, Sunnyvale, CA, USA), unaided visual inspection after acetic acid (VIA), and aided VIA using the EVA system (Mobile ODT, Tel Aviv, Israel). Women positive for hrHPV or by VIA will undergo colposcopy, which will include the collection of two cervical specimens prior to undergoing a 4-quadrant microbiopsy protocol. The colposcopy-collected specimens will be tested by dual immunocytochemical staining for $p16^{INK4a}$ and Ki-67 (CINtec® PLUS Cytology, Ventana, Tucson, AZ, USA) and for E6 or E7 for 8 hrHPV genotypes (HPV16, 18, 31, 33, 35, 45, 52, and 58) using the next-generation AV Avantage hrHPV E6/E7 test (Arbor Vita Corporation, Freemont, CA, USA). Women with local pathology diagnosis of cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or more severe (CIN2+) or pathology-review diagnosis of CIN grade 3 or more severe (CIN3+) will receive treatment. Clinical performance and cost-effectiveness (e.g., sensitivity, specificity, and predictive values) of different screening strategies and algorithms will be evaluated.

Ethics and dissemination. The protocol has been approved by local and institutional review boards for human subjects research. Results will be disseminated to the scientific community through peer-reviewed publication and to the Rwandan stakeholders through an external advisory panel.

Strengths and Weaknesses

- We will enroll a very large sample size of HIV-infected women living in Rwanda who otherwise would probably not get cervical-cancer screening.
- We will employ rigorous disease ascertainment protocols to minimize misclassification.
- Some tests, such as the Xpert HPV and the AV Avantage HPV E6/E7 assays, will be done on site in Rwanda using technologies that could feasibly be deployed there.
- A weakness of the study is that it will not be feasible to include cervical cytology as a comparator due to financial and logistical constraints.

Introduction

Invasive cervical cancer (ICC) remains a significant cause of morbidity and mortality globally. Approximately 530,000 cases of and 270,000 deaths due to ICC occur annually, making it the 4th most common malignancy and cause of cancer-related death in women globally.[1] In many high-income countries (HIC), cervical cancer rates have declined by 50% or more[2] due to the introduction of effective, high-coverage Pap testing (cervical cytology)-based screening programs that include timely follow-up of screen positives, treatment of women with precursor lesions, and management of cancers. Now, almost 90% of ICC and ICC-related deaths occur in low- and middle income countries (LMICs) due to a lack of resources and healthcare infrastructure needed to provide preventive services.

ICC and ICC-related mortality rates are particularly high in Sub-Saharan Africa, which also has the highest rates of HIV infection in the world. Now, over 12 million HIV-infected (HIV[+]) women in Sub-Saharan Africa are living longer because of anti-retroviral therapy, thus increasing their likelihood of dying from ICC.[3] However, many of these women are already exposed to human papillomavirus (HPV), the viral cause of cervical cancer, and will not benefit from or be targeted for prophylactic HPV vaccination. Thus, cervical-cancer screening is needed for the foreseeable future.

However, setting up effective cytology for cervical-cancer screening is expensive and requires a complex clinical and lab infrastructure that generally does not exist in LMICs.[4;5] Moreover, it is now well understood that cytology has only a low- to moderate one-time sensitivity for precursor lesions and therefore must be done repeatedly over many years to reduce cancer risk. Alternative

strategies to address the cervical cancer burden in LMICs, especially in SSA, must be developed and validated.

Persistent cervical infections by high-risk HPV (hrHPV) types cause virtually all ICC and its immediate precursor lesions, e.g. cervical intraepithelial neoplasia grade 3 (CIN3) and adenocarcinoma *in situ* (AIS) everywhere in the world.[6;7] hrHPV causes most anal and vaginal cancer and a significant proportion of vulvar, penile, and oropharyngeal cancers.[8] HPV16 is the most important causal type, responsible for ~60% of ICC.[9] HPV18 is the next most important, responsible for 10-15% of ICC, including 30-40% of adenocarcinoma of the cervix[9], which is on the rise in Western Countries.[10;11] Together, HPV16 and HPV18 account for ~70% of ICC, and the same 15 hrHPV types account for ~99% of ICC everywhere in the world.[9]

There is now overwhelming evidence to suggest that testing for hrHPV is more sensitive, albeit less specific, than high-quality cytology for identifying women with cervical precancer.[12-16] One-time hrHPV testing can reduce the risk of ICC incidence by approximately 40% in 6.5 years compared to cytology screening[16], and ICC mortality by approximately 40% (approximately 50% overall) in 8 years compared to cytology.[17] Importantly, a negative hrHPV test provides superior reassurance against CIN3+ [18] and against ICC[16;17], permitting safe extension of screening intervals.

The World Health Organization released cervical-cancer screening and treatment guidelines in 2013, recommending two evidence-based approaches to cervical-cancer screening[19]: (I) Use either hrHPV testing or visual inspection after acetic acid (VIA), which involves the inspection of the cervix with a speculum in place and following the application of dilute acetic acid to help identify potential CIN by its characteristic white coloring in the presence of acetic acid

(acetowhite), as alternative initial screening tests instead of Pap, and (II) immediately treat those who screen positive using the screening test, rather than require diagnostic verification through colposcopy and biopsy. This approach is commonly called screen-and-treat (S&T), and is increasingly thought to be more amenable to LMIC settings.

However, hrHPV testing is also a much more effective screen than VIA[17], which on a large-scale appears to only down-stage cancer rather than prevent it.[20] Thus, the recent American Society for Clinical Oncology (ASC) resource-stratified guidelines for secondary cervical cancer prevention[21;22] emphasize that hrHPV testing is the preferred choice for screening, with VIA only being used until hrHPV testing becomes available, and that HIV-infected women, because of their higher risk, should be screened twice as frequently as the general (HIV-uninfected) population.

Recent data in HIV[+] women living in the U.S. suggest that hrHPV testing may have clinical utility similar to that in HIV-negative (HIV[-]) women. Several observational studies have shown that an extended screening interval is safe in HIV[+] women who test hrHPV and Pap negative as it is for HIV[-] women.[23;24] In a study of women enrolled in Women's Interagency Health Study (WIHS) in 2002, HIV[+] and HIV[-] women who tested hrHPV and Pap negative were at a similarly low risk of cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or more severe (CIN2+) histology over a 5-year follow-up.[23] In addition, no cases of histologically confirmed CIN2+ were diagnosed in the follow-up of hrHPV- and Pap-negative HIV[+] women aged 30-64 years who underwent routine three-year hrHPV and cytology cotesting at Kaiser Permanente Northern California.[24] Thus, both studies found very high negative predictive values (NPV) >99% in HIV[+] women who test hrHPV negative. However, how hrHPV testing can

best be used to screen HIV[+] women living in Sub-Saharan Africa to prevent cervical cancer remains to be determined.

Recent studies[25-28] in HIV[+] women living in Sub-Saharan Africa have compared hrHPV, VIA, and/or Pap for the detection of cervical precancer/cancer. The results can be summarized as follows: 1) hrHPV detection was more sensitive but less specific than VIA; 2) surprisingly, cytology was equally or more sensitive but less specific than VIA; and 3) surprisingly, cytology was equally or more sensitive but less specific (vs. the converse) than hrHPV testing. Results and conclusions are varied, leaving unanswered the question of what screening strategy in HIV[+] women living in Sub-Saharan Africa has the greatest effectiveness and cost effectiveness.

Regardless of the screening method, most screen-positive women who go to colposcopy or are treated immediately without diagnostic verification do not have cervical precancer and cancer (positive predictive value [PPV] for screening tests are typically 10%-20%). In places like Sub-Saharan Africa that lack necessary infrastructure and personnel such as pathologists[29], excessive referral to colposcopy is problematic. Although WHO recommendations for S&T will hopefully overcome this bottleneck and increase the number of women living in LMICs who get screened, many countries may not adopt current S&T strategies because of concerns of low specificity and overtreatment, resulting in increased costs, unnecessary patient discomfort and concern, and wasting valuable healthcare resources that could otherwise be used to expand access to screening. Thus, methods to increase the accuracy of screening by reducing the numbers of women having colposcopy and biopsy or getting treated immediately in this context are highly desirable as they will likely increase the uptake of cervical-cancer screening.

In order to improve the specificity of screening tests, secondary tests (biomarkers) are used following a screen-positive result, with women who test positive for the triage undergoing further management (e.g., colposcopy or immediate treatment) and those who test negative typically being deferred to further evaluation in 6-18 months to allow hrHPV infections to clear. There are several very promising biomarkers that might be used to improve the specificity and positive predictive value (PPV) of the screening tests.[24] Given that HIV-infected women are more likely to test hrHPV positive[30-34], it is important to validate a triage strategy of using a secondary biomarker that sensitively and specifically rules-in women with cervical precancer and cancer among the HIV-positive, hrHPV-positive women.

We are therefore conducting a cervical-cancer screening study of >5,000 Rwandan women, aged 30-54 years, living with HIV infection. We will evaluate different screening tests (hrHPV DNA and VIA), those recommended by the WHO for cervical-cancer screening[19], and different triage tests and biomarkers for screen-positive women (E6/E7 oncoprotein detection, p16INK4a immunocytochemistry, and hrHPV viral methylation). Screen-positive women undergo a rigorous colposcopic evaluation with multiple biopsies taken and the biopsies will undergo pathology review, to minimize the misclassification of endpoints. The primary objective of the study is to determine and compare clinical performance (Sensitivity (Se), Specificity (Sp), PPV, and NPV) and cost-effectiveness for identifying HIV[+] women with CIN3+ and CIN2+ of different cervical-cancer screening and management algorithms.

Methods and Analysis

Study design and population: We are recruiting those women receiving care for confirmed HIV infection at health centers (HC) and various hospitals operated by the Ministry of Health or

Rwanda Military Hospital during 2016-18 (**Table 1**). Sites were selected in collaboration with Rwanda Biomedical Center (RBC), which calculate estimated numbers of potentially eligible women using data from the HIV database (OpenMRS-Open Medical Records System; http://openmrs.org/). Sites were also selected from all provinces to ensure geographic representation. The total of 8,424 is the estimated number of women eligible for the study according to the inclusion/exclusion criteria (see below) at the beginning for the study. From each site, a convenience sample of women were recruited to participate.

Inclusion criteria include 1) living and receiving HIV care in Rwanda, 2) aged 30-54 years, 3) confirmed HIV+ based on medical records, 4) no prior cervical-cancer screening, 5) no history of ICC, and 6) willing, able and competent to provide written, informed consent. We extended age range beyond that of age range (30-49 years) recommended by the WHO for cervical-cancer screening[19] because there is limited evidence for the optimal upper age for cervical-cancer screening of HIV-infected women. *Exclusion criteria*, in addition to not meeting the inclusion criteria, include 1) pregnant, 2) signs of abnormal, non-menstrual bleeding suggestive of ICC, 3) without a cervix due to hysterectomy, and 4) not sufficiently healthy to participate in a research study based on the judgment of the clinicians. Excluded women are advised to seek routine cervical-cancer screening through government programs.

Prior to initiation of enrollment at a specific clinic, the local HIV-care provider team identifies potentially eligible women at their routine clinic visits and offers them enrollment. Women indicating interest in the study are then registered by our research nurses using the eligibility criteria checklist. Women at one site are enrolled until all the eligible and willing women at that site are screened for cervical cancer. The study team of at least two research nurses schedules 12 to 15 women three to four days a week by calling them and confirming appointments over the

telephone. Two teams of nurses are in the field, meaning that two cervical-cancer screening clinics can be run simultaneously.

Enrollment Visit: Enrollment visits, including pelvic exams with VIA and specimen collection, are done entirely by a team of two study nurses. During their enrollment visit, women are educated on cervical cancer risk factors, mainly HPV infection, and why they are more at risk to develop cervical cancer than HIV-uninfected women. They are also allowed to ask questions before they commit to participating in the study. Women are asked to provide informed, written consent to participate in the study using a printed out consent form. Those who provide consent complete a short nurse-administered questionnaire (**Appendix I**) on cervical cancer risk factors and sociodemographic characteristics using a data capture screen in Microsoft Access. The questionnaire collects information on basic sociodemographics, factors associated with acquiring HPV (e.g., marital status and recent and lifetime number of sexual partners), factors associated with increased risk of progression of hrHPV infection to precancer and cancer (e.g., smoking and other tobacco use, parity, and oral and other contraceptive use), and other infections common in Rwanda such as malaria[35] and tuberculosis[36] that have been previously reported to be associated with precancer among hrHPV-infected women. The questionnaire was not pretested.

Enrolled women then undergo a pelvic exam, with VIA and a single cervical exfoliated ("Pap") specimen collected and placed into 20 ml PreservCyt (Hologic, Bedford, MA, USA) which is then sent to the lab at RMH for hrHPV testing. Finally, a portable colposcope (http://www.mobileodt.com/; MobleODT, Tel Aviv, Israel) is used for digital cervicography (~VIA with magnification) and the image is captured and saved for quality control, research, and to develop a digital library.

Colposcopy Visit: Screen-positive women (women who test hrHPV and/or VIA positive) are called using a telephone as soon as the hrHPV result is available and invited to return for colposcopy within one month. All women receiving colposcopy will have two additional specimens collected, one into PreservCyt for the evaluation of other molecular biomarkers (genotype-specific hrHPV viral methylation and load, and p16/Ki-67 immunocytochemistry CINtec® PLUS Cytology Kit [Roche, Tucson, AZ, USA]) and a second using a dry swab for HPV16, 18, 31, 33, 35, 45, 52, and 58 E6/E7 oncoprotein detection by the next generation lateral flow hrHPV oncoprotein test from Arbor Vita Corporation (Fremont, CA, USA) included in this study as a triage for screen-positive women to identify those women who are at higher risk of having CIN3+. The residual PreservCyt specimens from both the screening and colposcopy visits will be stored at -20ºC, creating a biobank in Rwanda for future retrospective evaluations of promising new biomarkers and tests.

After specimen collection, a colposcopic evaluation of the cervix is done and a modified version of the 4-quadrant microbiopsy procedure is performed.[37] Compared to the standard biopsy, the microbiopsy protocol improves disease ascertainment and reduces biases by selecting on the most obvious acetowhite lesions while removing less tissue (~13 mm^2 for 4 microbiopsies vs. ~28mm^2 for 1 standard biopsy). Modifications to the standard 4-quadrant microbiopsy procedure are: 1) endocervical curettage is taken only for those women whose squamocolumnar junction is not entirely visible and the lesion extends into the endocervical canal; and 2) standard biopsies of very large lesions can be taken to increase the likelihood that the most severe area is biopsied.

Pathology: Biopsies are processed in a single cassette so that a single slide has a section from all biopsies taken. Biopsies read by a local pathologist at RMH and Dr. Hebert or another pathologist at Montefiore Medical Center, Bronx, NY, USA. Women receiving a diagnosis of

CIN2+ by the Rwandan pathologist (T.Z.) or, as a safety precaution, CIN3+ diagnosis by Montefiore pathologist (T.H.) will receive treatment[38]: 1) CIN2, CIN3, or AIS will be referred to study doctors to undergo an excision procedure (e.g., loop electrosurgical excision procedure [LEEP] or cold-knife cone [CKC]) and 2) ICC will be referred to RMH Hospital for care. Women with <CIN2 will be advised to seek re-screening in a year.

A slide with biopsies also will undergo p16 immunohistochemistry (IHC) using the CINtec® Histology Kit (Roche) for research purposes only.

Endpoints: The primary scientific endpoint of the study will be histologically confirmed, consensus CIN2+ i.e., both pathologists diagnosed CIN2+ (without adjudication). The secondary, clinical endpoint will be histologically confirmed CIN2+ diagnosed by the Rwandan pathologist. Additional endpoints using pathology review and p16 IHC will be used but not for evaluating the performance of p16 immunocytochemistry due to the possibility of p16-related autocorrelation.

Treatment: Women diagnosed with CIN2+ will be referred for treatment. Those precancerous lesions will be treated by ablation if they meet WHO criteria for cryotherapy.[39] Those who do not meet those criteria will undergo an excision procedure (e.g., loop electrosurgical excision procedure [LEEP] or cold-knife cone [CKC]) or, in the case of an ICC diagnosis, referred for cancer management. Screen-positive women with <CIN2 will be advised to seek re-screening in a year through the existing healthcare system.

Data sources: Data are collected from the following sources:

1. A nurse-administered questionnaire on sociodemographic characteristics and cervical cancer risk factors including age at first sexual intercourse, number of sexual partners, smoking, contraception, parity and socioeconomic status.

2. Pelvic exam, VIA, Mobile ODT and colposcopy data capture forms

3. Medical record data on HIV status (e.g., (http://www.who.int/hiv/pub/guidelines/HIVstaging150307.pdf), CD4 count, viral load, antiretroviral therapy (ART) regimen(s)), care, and dates.

Laboratory Testing: The following laboratory tests will be performed:

Xpert HPV Testing—cervical Pap specimens in PreservCyt will be sent to the RMH laboratory in Kigali, Rwanda for hrHPV DNA testing using the Xpert HPV test (Cepheid, Sunnyvale, CA, USA).[40-45] The Xpert HPV Assay is a new, qualitative, real-time PCR assay for the detection of hrHPV DNA. The Xpert HPV Assay includes simultaneous detection of 14 hrHPV types, hydroxymethylbilane synthase (HMBS), and an internal Probe Check Control (PCC). The 14 targeted hrHPV types are detected in 5 fluorescent channels: 1) HPV16, 2) HPV18 and hrHPV 45 (HPV18/45), 3) HPV31, 33, 35, 52, and 58, 4) HPV51 and HPV59, and 5) HPV39, 56, 66, and 68. HMBS (fluorescent channel 6) verifies specimen adequacy.

Specimens are mixed and a 1-mL pre-aliquot is removed using a disposable pipette and placed in the testing cartridge per the manufacturer's instructions. Unsatisfactory results due to insufficient cellular content are re-run. If the second test is also unsatisfactory, the final result will be unsatisfactory but women are referred to colposcopy for safety.

HPV Viral Methylation—To identify single hrHPV type infections, we will select single-channel positives from the Xpert HPV assay. For those that are hrHPV positive for a channel other than HPV16, which is detected singly, we will test them to identify the single type infections using a standard protocol for PCR amplification using MY09/11 L1 consensus primers and hrHPV genotype detection using dot-hybridization for 39 individual type-specific probes and a mixture of probes for 10 other uncommon hrHPV types as previously described.[46;47] To isolate the DNA, ThinPrep specimens (1.5 mL) will be pelleted, re-suspended in STM, digested with Proteinase K, precipitated overnight in ammonium acetate ethanol at -20°C, washed, and suspended and stored in TE buffer.

The isolated DNA will then undergo bisulfite conversion.[48] Following bisulfite conversion and DNA purification and de-sulphonation, bisulfite-treated DNA will be used as template for Next-Gen Sequencing (NGS) (HiSeq2000, Illumina, San Diego, CA) using barcoded-type specific primers. Sequences for pads and barcodes are not found in the targeted genomic region. Use of padding and barcodes enables reads to be identified by amplicon (forward or reverse) or by sample during downstream bioinformatics analysis.[49]

All PCR products for NGS are pooled (by assay) and a single DNA band containing multiple amplicons from different samples (with unique barcodes) is isolated from a gel for NGS.[49] Briefly, equal concentrations of each barcoded PCR product (based on PCR band intensity) are pooled and isolated. Upon confirmation of correct product size, all purified DNA pools are combined and submitted for library preparation and paired-end 100 base pair Illumina HiSeq2000 sequencing at the Einstein Genomics Core Facility.

Methylation status will be determined in the lab of Dr. Robert Burk at Albert Einstein College of Medicine (USA). Prior to determination of methylation status, de-multiplexing based on the unique barcodes is performed using in-house generated scripts to obtain paired-end NGS reads of each sample. Reads are aligned with hrHPV reference genome sequences by bowtie v0.12.9.[50] Methylation status of each CpG site is then determined by bismark v0.7.7[51] using the default quality score parameter set to Q30, and the formula of the methylation ratio of the number of C read by the number of C+T read.

E6/E7 Oncoprotein Testing—Dry swab specimens collected at the time of colposcopy will be tested for individual E6/E7 oncoproteins as previously described[52;53], according to the manufacturer's instructions, at the RMH laboratory in Kigali, Rwanda. The E6/E7 oncoprotein test uses three lateral flow strips to detect 8 hrHPV types whereas the E6 oncoprotein test used a single lateral flow strip to detect 3 hrHPV types.

Analyses: We will evaluate combinations of the above mentioned screening strategies and tests to estimate the clinical performance (e.g., Se, Sp, PPV, and NPV) for the detection of consensus CIN3+ and community CIN2+. A log binomial model implemented with generalized estimating equations will be used to take into account correlation between different tests from the same subject. Note that these models are developed for the estimation and comparison of performance for two tests, but the model can be extended to allow more than two tests by including more indicator variables for test type.

Some analyses of biomarkers, such as viral methylation are restricted to hrHPV-positives. Comparisons of hrHPV viral methylation to other triage biomarkers will be restricted to the subset that gets tested for viral methylation as described.

Sample size calculations: We based our sample size on the ability to detect modest but minimally important differences in Se of 15%. We conservatively assume that the population prevalence of CIN3+ is 2% based on our past study in Rwanda.[33;35] We propose to enroll and have complete follow-up of at least 5,000 HIV[+] women. A sample size of 5,000 HIV[+] women with completed follow-up of the screen positives will yield 100 cases of CIN3+, which has at least 80% power (α=5%) to crudely detect a 15% difference in Se between two screening strategies for a range of 10%-25% discordance. With this sample size of 5,000 women, 4,900 will not have CIN3+; we will also have at least 90% power (α=5%) to detect a difference in Sp of 3% for discordance up to 40%. Finally, we will have 80% power (α=5%) to crudely detect an 8%, 10%, or 11% difference in PPV if the reference PPV is 10%, 20%, or 30%, respectively.[54]

Cost Effectiveness: We will conduct assessments of the costs and cost-effectiveness of the different combinations of screening and triage tests, i.e., algorithms, as well as those of the entire community-based screening "system." Costs measurement will be conducted using a micro-costing (ingredients) approach in which resource use throughout each step in the screening process will be tracked and unit costs for each of the resources will be applied to generate an average screening cost per woman to be compared against what the estimated costs are for a possible program based on hrHPV screening and VIA triage or VIA screening. For estimating costs of the screening system and scale-up of screening to 100,000 women in a month, analyses will distinguish financial costs, which reflect actual expenditures of the program, from economic costs, including the value of donated and shared resources to more fully assess opportunity costs. Projections on budget impact and economic cost implications over time will be made under varying assumptions of screening uptake, follow-up compliance, and scenarios of changing disease burden.

Clinical outcomes will include true positive, true negative, false negative, and false positive test results, number of colposcopies, incident cancer, and cancer death. Cost-effectiveness will be measured as cost/CIN2+ detected, cost/CIN3+ detected, cost/invasive cancer prevented, cost/cancer death prevented, cost/life-year saved, and cost/quality-adjusted life year (QALY) saved; in addition, we will calculate harm/benefit ratios, using varying definitions of harms (colposcopies, false positive results) to benefits (cancers prevented, deaths prevented, life years and QALYs saved). Costs and effectiveness will be discounted at a 3% annual rate, with the rate varied from 0-5% in sensitivity analysis. For assessment of value-of-information (VOI), we will use net monetary benefits (NMB), defined as a function of the willingness-to-pay threshold (WTP) for different costs and outcomes as: $NMB=(WTP * Effectiveness) - Costs$.

Patient and Public Involvement

- *How was the development of the research question and outcome measures informed by patients' priorities, experience, and preferences?* There was no patient engagement in the development of the study.

- *How did you involve patients in the design of this study?* There was no patient engagement in the design of the study.

- *Were patients involved in the recruitment to and conduct of the study?* There was no patient involvement in the recruitment and conduct of the study.

- *How will the results be disseminated to study participants?* Participants will receive their results directly since it is related to their care.

- *For randomized controlled trials, was the burden of the intervention assessed by patients themselves*? Not applicable

- *Patient advisers should also be thanked in the contributorship statement/acknowledgements*. Not applicable

Ethics and Dissemination

Ethics: This study protocol was reviewed and approved by the Rwanda National Ethics Committee (RNEC) as well as the Institutional Review Board for human subjects research at Albert Einstein College of Medicine.

Confidentiality measures and protection against potential risks: The risks for those participating in our study include:

- Collection of Pap specimens/cervical swabs involves a modest risk of bleeding which is typically very limited when it occurs. Testing positive for any test may cause psychological distress (anxiety).

- Colposcopy and excisional treatments induce vaginal bleeding and may incur pain, infection, and short-term psychological distress (anxiety). A diagnosis of CIN2 or more severe may cause psychological distress (anxiety). A diagnosis of invasive cervical cancer may cause severe psychological distress.

- Questions in the questionnaire, regarding sexual behavior and other matters of a personal nature, may cause anxiety and embarrassment. Participants are advised that they are free not to answer specific questions.

- There is also the risk of psycho-social stress which could occur if there was inadvertent disclosure of confidential medical or other personal information.

Protection against the risk of inadvertent disclosure of confidential information is addressed by the standard procedures at the Rwandan study site, including: (i) storing completed paper copies of questionnaires and other hard copy information (described above), identified by study number only, in a filing system separate from the name-address file of participants in the study; and (ii) only the designated local personnel have access to cross-reference the files; (iii) all paper files, including consent forms, will be maintained in locked cabinets in locked rooms, with access restricted to specific research personnel.

In addition, we will include the following security measures to protect the data:

- Controlled access to project data;
- A tracking system for data forms and activities;
- Date and time of stamping of all data records with electronic signatures;
- Audit trails to track all changes made to data records; and
- Data kept on password-protected computers and in locked rooms.

Potential Patient Benefits: There are potential direct benefits to study participants. HIV[+] women participating in the study are at very high risk of cervical cancer. They will be rigorously screened and evaluated, more effectively than the standard of care anywhere in the world. As a result, women with precancer who are at imminent risk of invasive cervical cancer will be diagnosed sooner and treated more effectively than women receiving routine care and thereby more likely averting the development of cervical cancer. Women with cervical cancer will be diagnosed earlier thereby reducing the morbidity and the risk of mortality caused by cervical

cancer. Conversely, any pain, bleeding, or stress that might occur related to colposcopy or cervical swab are typically modest and well tolerated.

There are also substantial potential societal benefits related to the study due to its implications to improving cervical-cancer screening practices and guidelines in HIV[+] women – changes in practice which might also benefit the study participants themselves, if and when these changes are enacted. There is a great need to identify more effective and practical methods for cervical-cancer screening for HIV[+] women living in Africa, who are living longer than ever and are therefore at potentially greater risk of cervical cancer.

Dissemination: We plan to publish a series of scientific reports in peer-reviewed scientific journals. As building research capacity in Rwanda is a major goal of this research project, all investigators of the research team will be asked and supported to lead at least one analysis and one manuscript preparation, based on interests and expertise.

In addition, an external advisory panel (EAP) composed of leaders from the Rwanda Ministry of Health, University of Rwanda, and Rwanda medical community has been formed. The responsibilities of the EAP include providing advice on the conduct of the project and interpretation for and dissemination of the study results to Rwandan stakeholders. The latter is important for the adoption of evidence-based best practices for cervical-cancer screening as warranted.

Limitations

There are several limitations to the study that bear mentioning. First, cervical cytology was not included in the study. There is limited cervical cytology services available locally and of unknown quality and it is unlikely that cytology will be widely available in Rwanda, making its

inclusion as a comparator test of limited value. Moreover, there are significant costs and logistical challenges in shipping PreservCyt specimens to and having cytology slides made and read in the U.S. Second, we did not conduct biopsies in screen-negative women, which would have allowed us to estimate absolute clinical performance. The burden of sending screen-negative women to colposcopy was deemed too great and it was impractical to send a sufficient numbers of screen-negative women to colposcopy to accurately estimate the false-negative disease (CIN3+) fraction. Thus, only relative clinical performance of the screening tests can be estimated from this study.

Contributor Statement:

KA, PEC, JCD, AA, and JDS conceived the original concept of the study and the interventions. GM, KA, JDS, and PEC drafted the protocol. PEC performed the sample size calculations, and PEC and GM will lead analysis of the results. GM, JDS, and Athanase Munyaneza are supporting patient recruitment. GM, TR, and Athanase Munyaneza are providing clinical care for patients. Anthere Murangwa and LM oversee laboratory testing, and TZ and TH are responsible for pathology. PM and LM oversee and administer the study activities at the clinical site in Rwanda. All authors (GM, JCD, TR, JDS, Athanase Munyaneza, Anthere Murangwa, TZ, TH, PM, AA, LM, KA, and PEC) contributed to the scientific design of the study and the protocol development, are involved in the implementation of the project, and have read and approved the final manuscript.

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Table 1: Recruitment sites and estimated eligible population.

Province	Site	Type of site	Potential participants per site (approximate)
Kigali	Cor-unum HC	Public Health Center	1,405
Kigali	Kimironko HC	Public Health Center	1,227
Kigali	Rwampara HC	Public Referral Hospital	1,098
Kigali	Kicukiro HC	Public Health Center	1,054
Kigali	Kacyiru HC	Public Health Center	905
Kigali	Gikondo HC	Public Health Center	925
Kigali	Rwanda Military Hospital	Public Referral Hospital	300
Kigali	WEACTx for Hope	Private HIV Clinic	500
Kigali	Busanza HC	Public Health Center	100
Kigali	Nyarugunga HC	Public Health Center	100
West	Gisenyi DH	Public District Hospital	810
Total			8,424

Appendix I. Study Questionnaire

A. Socio-demographics

1. **What is your date of birth? D D / M M / Y Y Y Y (enter all 0 if not remembered) (If date of birth or year of birth is given, skip to Question 3)**
2. **What is your age?** __ __ (Years)
3. **What is your marital status?**

☐ Married/Cohabiting
☐ Divorced
☐ Widowed
☐ Separated
☐ Single
☐ Choose not to answer

4. **Do you live in Kigali?**

☐ Yes
☐ No {Skip to Question A5}
☐ Choose not to answer {Skip to Question A5}

5. **In what area of Kigali City Province do you live?**

☐ Nyarugenge
☐ Kicukiro
☐ Gasabo urban (e.g., Kacyiru, Kimironko, Kinyinya, etc.)
☐ Gasabo rural (e.g., Nduba, Jabana, Gikomero, etc.)
☐ Do not know
☐ Choose not to answer

6. **What is your household income per month in FRW?**

☐ < 5,000
☐ 5,000 – 9,999
☐ 10,000 – 34,999
☐ 35,000 – 59,999

☐ 60,000 – 84,999
☐ 85,000 – 109,999
☐ 110,000 – 134,999
☐ 135,000 – 269,999
☐ 270,000 or more

7. **How many people live in your household?** __ __ {Enter 00 if refuse to answer}

8. **What is your occupation?**

☐ Employed by government, another institution, or company
☐ Self-employed (Small and medium enterprises)
☐ Self-employed (High income earnings)
☐ Farming (peasants)
☐ Unemployed/Does not work
☐ Other (specify) _____
☐ Choose not to answer {Skip to Question B1}

{The following questions are sensitive and personal in nature. Your answers will be kept confidential. You may choose not to answer certain questions. Answering any question is voluntary}

B. <u>Sexual Behaviors</u>

1. **How old were you when you first had sex?** __ __ (Years) {Enter 00 if refuse to answer}

2. **How many sexual partners have you had in your lifetime?**

☐ No Partners (Ineligible)
☐ 1 Partner
☐ 2-3 Partners
☐ 4-5 Partners
☐ 6-9 Partners
☐ 10 or more partners
☐ Choose not to answer

3. **How many sexual partners have you had in the last 6 months?**

☐ No Partners

☐ 1 Partner

☐ 2 or more Partners

☐ Choose not to answer

C. Parity

1. **What age did you have your first child? __ __** (Years) {Enter 00 if refuse to answer, Enter 99 if never pregnant} (if 00 or 99, skip to Question D1)

2. **How many live births have you had in your lifetime? __ __**

3. **Have you given birth in the last year?**

☐ Yes

☐ No

☐ Choose not to answer

D. Tobacco Use

1. **Have you ever smoked cigarettes?**

☐ Yes

☐ No (skip to Question D3)

☐ Choose not to answer (skip to Question D3)

2. **Do you currently smoke cigarettes?**

☐ Yes

☐ No

☐ Choose not to answer

3. **Have you ever chewed/used tobacco orally (Ubugoro)?**

☐ Yes

☐ No (skip to Question D5)

☐ Choose not to answer (skip to Question D5)

4. **Are you currently chewing/using tobacco orally (Ubugoro)?**

☐ Yes

☐ No

☐ Choose not to answer

5. Have you ever chewed/used tobacco orally (Tobacco leaves-Igikamba)?

☐ Yes
☐ No (skip to Question E1)
☐ Choose not to answer (skip to Question E1)

6. Are you currently chewing/using tobacco orally (Tobacco leaves-Igikamba)?

☐ Yes
☐ No
☐ Choose not to answer

E. Contraceptive Use

1. Have you ever used oral contraceptives?

☐ Yes
☐ No (Skip to Question E3)
☐ Choose not to answer (Skip to Question E3)

2. Do you currently use oral contraceptives?

☐ Yes
☐ No
☐ Choose not to answer

3. Have you ever used Depo Provera (contraceptive)?

☐ Yes
☐ No (Skip to Question E5)
☐ Choose not to answer (Skip to Question E5)

4. Do you currently use Depo Provera (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

5. Have you ever used Jadell (contraceptive)?

☐ Yes
☐ No (Skip to Question E7)

☐ Choose not to answer (Skip to Question E7)

6. Do you currently use Jadell (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

7. Have you ever used IUD (contraceptive)?

☐ Yes
☐ No (Skip to Question E9)
☐ Choose not to answer (Skip to Question E9)

8. Do you currently use IUD (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

9. Do use condoms as a contraceptive?

☐ Yes
☐ No (skip to Question F1)
☐ Choose not to answer (skip to Question F1)

10. Have you used condoms as a contraceptive in the last 6 months?

☐ Yes
☐ No
☐ Choose not to answer

F. Infections

1. Have you ever had Malaria

☐ Yes
☐ No (skip to Question F8)
☐ Choose not to answer (skip to Question F8)

2. How many times have you had Malaria? __ __

3. **What was the year that you last had Malaria? Y Y Y Y**

4. **Have you had your Malaria treated using drugs?**

☐ Yes
☐ No
☐ Choose not to answer

5. **If Yes, how was it treated?**

☐ Treated only with traditional medicine
☐ Treated only with drugs (e.g. Coartem)
☐ Treated with both (traditional and modern)
☐ Not treated
☐ Choose not to answer

6. **Was your last episode of Malaria treated using drugs?**

☐ Yes
☐ No
☐ Choose not to answer

7. **If Yes, how was it treated?**

☐ Treated only with traditional medicine
☐ Treated only with drugs (e.g. Coartem)
☐ Treated with both (traditional and modern)
☐ Not treated
☐ Choose not to answer

8. **Have you ever had Tuberculosis (TB)?**

☐ Yes
☐ No (skip to end)
☐ Choose not to answer (skip to end)

9. **If Yes, how many times have you had TB in your lifetime? __ __**

10. **What year did you last have Tuberculosis (TB)? Y Y Y Y**

END OF QUESTIONNAIRE

BMJ Open

A Study of Cervical Cancer Screening Technologies in Human Immunodeficiency Virus-Infected Women Living in Rwanda

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-020432.R2
Article Type:	Protocol
Date Submitted by the Author:	22-May-2018
Complete List of Authors:	Murenzi, Gad; Rwanda Military Hospital Dusingize, Jean; Regional Alliance for Sustainable Development , Research and Scientific Capacity Building Rurangwa, Theogene; Rwanda Military Hospital Sinayobye, Jean d'Amour; Regional Alliance for Sustainable Development, ; Women's Equity in Access to Care & Treatment (WE-ACTx), Munyaneza, Athanase; Rwanda Military Hospital Murangwa, Anthere; Rwanda Military Hospital Zawadi, Thierry; Rwanda Military Hospital Hebert, Tiffany; Yeshiva University Albert Einstein College of Medicine Mugenzi, Pacifique; Rwanda Military Hospital Adedimeji, Adebola; Yeshiva University Albert Einstein College of Medicine Mutesa, Leon; Rwanda Military Hospital; University of Rwanda College of Medicine and Health Sciences Anastos, Kathryn; Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, Castle, Philip; Yeshiva University Albert Einstein College of Medicine,
Primary Subject Heading:	Global health
Secondary Subject Heading:	Obstetrics and gynaecology, Infectious diseases, HIV/AIDS, Epidemiology, Diagnostics
Keywords:	human papillomavirus (HPV), cervical cancer, HIV & AIDS < INFECTIOUS DISEASES, GYNAECOLOGY, cervical intraepithelial neoplasia

A Study of Cervical Cancer Screening Technologies in Human Immunodeficiency Virus-Infected Women Living in Rwanda

Gad Murenzi, MD*[1], Jean-Claude Dusingize, MD, MS[1], Theogene Rurangwa, MD, MMed[1], Jean d'Amour Sinayobye, MD, MS[1], Athanase Munyaneza, RN[1], Anthere Murangwa, MS[1], Thierry Zawadi, MD[1], Tiffany Hebert, MD[2], Pacifique Mugenzi, MD[1], MMed, Adebola Adedimeji, PhD, MPH[2], Leon Mutesa, MD, PhD[1,3], Kathryn Anastos, MD[2], Philip E. Castle, PhD, MPH[2]

[1]Rwanda Military Hospital, Kigali, Rwanda; [2]Albert Einstein College of Medicine, Bronx, NY, USA; [3]University of Rwanda, Kigali, Rwanda

*Correspondence: gadcollins@gmail.com, +250788589085

Disclosures: This research study has received HPV tests for reduced or no cost from Cepheid, Arbor Vita Corporation, and Roche.

Funding: This study was funded by NCI/NIH Grant 5U54CA19016304 and by a grant from the Prevent Cancer Foundation.

Abstract

Introduction. The optimal method(s) for screening human immunodeficiency virus-infected women, especially for those living in sub-Saharan Africa, for cervical precancer and early cancer has yet to be established.

Methods and analysis. A convenience sample of >5,000 Rwandan women, ages 30-54 years and living with HIV infection, are being consented and enrolled into a cross-sectional study of cervical-cancer screening strategies. Participants are completing an administered short risk-factor questionnaire and being screened for high-risk human papillomavirus (hrHPV) using the Xpert HPV assay (Cepheid, Sunnyvale, CA, USA), unaided visual inspection after acetic acid (VIA), and aided VIA using the EVA system (Mobile ODT, Tel Aviv, Israel). Women positive for hrHPV and/or by VIA undergo colposcopy, which includes the collection of two cervical specimens prior to undergoing a 4-quadrant microbiopsy protocol. The colposcopy-collected specimens are being tested by dual immunocytochemical staining for $p16^{INK4a}$ and Ki-67 (CINtec® PLUS Cytology, Ventana, Tucson, AZ, USA) and for E6 or E7 for 8 hrHPV genotypes (HPV16, 18, 31, 33, 35, 45, 52, and 58) using the next-generation AV Avantage hrHPV E6/E7 test (Arbor Vita Corporation, Freemont, CA, USA). Women with local pathology diagnosis of cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or more severe (CIN2+) or pathology-review diagnosis of CIN grade 3 or more severe (CIN3+) receive treatment. Clinical performance and cost-effectiveness (e.g., sensitivity, specificity, and predictive values) of different screening strategies and algorithms will be evaluated.

Ethics and dissemination. The protocol was approved by local and institutional review boards for human subjects research. At the completion of the study, results will be disseminated to the scientific community through peer-reviewed publication and to the Rwandan stakeholders through an external advisory panel.

Strengths and Weaknesses

- We are enrolling a very large sample size of HIV-infected women living in Rwanda who otherwise would probably not get cervical-cancer screening.
- We are employing rigorous disease ascertainment protocols to minimize misclassification.
- Some tests, such as the Xpert HPV and the AV Avantage HPV E6/E7 assays, are being done on site in Rwanda using technologies that could feasibly be deployed there.
- A weakness of the study is that cervical cytology is not being included as a comparator due to financial and logistical constraints.

Introduction

Invasive cervical cancer (ICC) remains a significant cause of morbidity and mortality globally. Approximately 530,000 cases of and 270,000 deaths due to ICC occur annually, making it the 4th most common malignancy and cause of cancer-related death in women globally.[1] In many high-income countries (HIC), ICC rates have declined by 50% or more[2] due to the introduction of effective, high-coverage Pap testing (cervical cytology)-based screening programs that include timely follow-up of screen positives, treatment of women with precursor lesions, and management of cancers. Now, almost 90% of ICC and ICC-related deaths occur in low- and middle income countries (LMICs) due to a lack of resources and healthcare infrastructure needed to provide preventive services.

ICC and ICC-related mortality rates are particularly high in Sub-Saharan Africa, which also has the highest rates of HIV infection in the world. Now, over 12 million HIV-infected (HIV[+]) women in Sub-Saharan Africa are living longer because of anti-retroviral therapy, thus increasing their likelihood of dying from ICC.[3] However, many of these women are already exposed to human papillomavirus (HPV), the viral cause of ICC, and will not benefit from or be targeted for prophylactic HPV vaccination. Thus, cervical-cancer screening is needed for the foreseeable future.

However, setting up effective cytology for cervical-cancer screening is expensive and requires a complex clinical and lab infrastructure that generally does not exist in LMICs.[4;5] Moreover, it is now well understood that cytology has only a low- to moderate one-time sensitivity for precursor lesions and therefore must be done repeatedly over many years to reduce cancer risk. Alternative

strategies to address the ICC burden in LMICs, especially in SSA, must be developed and validated.

Persistent cervical infections by high-risk HPV (hrHPV) types cause virtually all ICC and its immediate precursor lesions, e.g. cervical intraepithelial neoplasia grade 3 (CIN3) and adenocarcinoma *in situ* (AIS) everywhere in the world.[6;7] hrHPV causes most anal and vaginal cancer and a significant proportion of vulvar, penile, and oropharyngeal cancers.[8] HPV16 is the most important causal type, responsible for ~60% of ICC.[9] HPV18 is the next most important, responsible for 10-15% of ICC, including 30-40% of adenocarcinoma of the cervix[9], which is on the rise in Western Countries.[10;11] Together, HPV16 and HPV18 account for ~70% of ICC, and the same 15 hrHPV types account for ~99% of ICC everywhere in the world.[9]

There is now overwhelming evidence to suggest that testing for hrHPV is more sensitive, albeit less specific, than high-quality cytology for identifying women with cervical precancer.[12-16] One-time hrHPV testing can reduce the risk of ICC incidence by approximately 40% in 6.5 years compared to cytology screening[16], and ICC mortality by approximately 40% (approximately 50% overall) in 8 years compared to cytology.[17] Importantly, a negative hrHPV test provides superior reassurance against CIN3+ [18] and against ICC[16;17], permitting safe extension of screening intervals.

The World Health Organization released cervical-cancer screening and treatment guidelines in 2013, recommending two evidence-based approaches to cervical-cancer screening[19]: (I) Use either hrHPV testing or visual inspection after acetic acid (VIA), which involves the inspection of the cervix with a speculum in place and following the application of dilute acetic acid to help identify potential CIN by its characteristic white coloring in the presence of acetic acid

(acetowhite), as alternative initial screening tests instead of Pap, and (II) immediately treat those who screen positive using the screening test, rather than require diagnostic verification through colposcopy and biopsy. This approach is commonly called screen-and-treat (S&T), and is increasingly thought to be more amenable to LMIC settings.

However, hrHPV testing is also a much more effective screen than VIA[17], which on a large-scale appears to only down-stage cancer rather than prevent it.[20] Thus, the recent American Society for Clinical Oncology (ASC) resource-stratified guidelines for secondary cervical-cancer prevention[21;22] emphasize that hrHPV testing is the preferred choice for screening, with VIA only being used until hrHPV testing becomes available, and that HIV-infected women, because of their higher risk, should be screened twice as frequently as the general (HIV-uninfected) population.

Recent data in HIV[+] women living in the U.S. suggest that hrHPV testing may have clinical utility similar to that in HIV-negative (HIV[-]) women. Several observational studies have shown that an extended screening interval is safe in HIV[+] women who test hrHPV and Pap negative as it is for HIV[-] women.[23;24] In a study of women enrolled in Women's Interagency Health Study (WIHS) in 2002, HIV[+] and HIV[-] women who tested hrHPV and Pap negative were at a similarly low risk of cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or more severe (CIN2+) histology over a 5-year follow-up.[23] In addition, no cases of histologically confirmed CIN2+ were diagnosed in the follow-up of hrHPV- and Pap-negative HIV[+] women aged 30-64 years who underwent routine three-year hrHPV and cytology cotesting at Kaiser Permanente Northern California.[24] Thus, both studies found very high negative predictive values (NPV) >99% in HIV[+] women who test hrHPV negative. However, how hrHPV testing can

best be used to screen HIV[+] women living in Sub-Saharan Africa to prevent ICC remains to be determined.

Recent studies[25-28] in HIV[+] women living in Sub-Saharan Africa have compared hrHPV, VIA, and/or Pap for the detection of cervical precancer/cancer. The results can be summarized as follows: 1) hrHPV detection was more sensitive but less specific than VIA; 2) surprisingly, cytology was equally or more sensitive but less specific than VIA; and 3) surprisingly, cytology was equally or more sensitive but less specific (vs. the converse) than hrHPV testing. Results and conclusions are varied, leaving unanswered the question of what screening strategy in HIV[+] women living in Sub-Saharan Africa has the greatest effectiveness and cost effectiveness.

Regardless of the screening method, most screen-positive women who go to colposcopy or are treated immediately without diagnostic verification do not have cervical precancer and cancer (positive predictive value [PPV] for screening tests are typically 10%-20%). In places like Sub-Saharan Africa that lack necessary infrastructure and personnel such as pathologists[29], excessive referral to colposcopy is problematic. Although WHO recommendations for S&T will hopefully overcome this bottleneck and increase the number of women living in LMICs who get screened, many countries may not adopt current S&T strategies because of concerns of low specificity and overtreatment, resulting in increased costs, unnecessary patient discomfort and concern, and wasting valuable healthcare resources that could otherwise be used to expand access to screening. Thus, methods to increase the accuracy of screening by reducing the numbers of women having colposcopy and biopsy or getting treated immediately in this context are highly desirable as they will likely increase the uptake of cervical-cancer screening.

In order to improve the specificity of screening tests, secondary tests (biomarkers) are used following a screen-positive result, with women who test positive for the triage undergoing further management (e.g., colposcopy or immediate treatment) and those who test negative typically being deferred to further evaluation in 6-18 months to allow hrHPV infections to clear. There are several very promising biomarkers that might be used to improve the specificity and positive predictive value (PPV) of the screening tests.[24] Given that HIV-infected women are more likely to test hrHPV positive[30-34], it is important to validate a triage strategy of using a secondary biomarker that sensitively and specifically rules-in women with cervical precancer and cancer among the HIV-positive, hrHPV-positive women.

We are therefore conducting a cervical-cancer screening study of >5,000 Rwandan women, ages 30-54 years, living with HIV infection. We are evaluating different screening tests (hrHPV DNA and VIA), those recommended by the WHO for cervical-cancer screening[19], and different triage tests and biomarkers for the management of screen-positive women (E6/E7 oncoprotein detection, p16INK4a immunocytochemistry, and hrHPV viral methylation). Screen-positive women undergo a rigorous colposcopic evaluation with multiple biopsies taken and the biopsies will undergo pathology review, to minimize the misclassification of endpoints. The primary objective of the study is to determine and compare clinical performance (Sensitivity (Se), Specificity (Sp), PPV, and NPV) and cost-effectiveness for identifying HIV[+] women with CIN3+ and CIN2+ of different cervical-cancer screening and management algorithms.

Methods and Analysis

Study design and population: We are recruiting those women receiving care for confirmed HIV infection at health centers (HC) and various hospitals operated by the Ministry of Health or

Rwanda Military Hospital during 2016-18 (**Table 1**). Sites were selected in collaboration with Rwanda Biomedical Center (RBC), which calculate estimated numbers of potentially eligible women using data from their HIV database (OpenMRS-Open Medical Records System; http://openmrs.org/). Sites were also selected from all provinces to ensure geographic representation. The total of 8,424 was the estimated number of women eligible for the study according to the inclusion/exclusion criteria (see below) at the beginning for the study in 2016. From each site, a convenience sample of women were/are being recruited to participate.

Inclusion criteria include 1) living and receiving HIV care in Rwanda, 2) ages 30-54 years, 3) confirmed HIV+ based on medical records, 4) no prior cervical-cancer screening, 5) no history of ICC, and 6) willing, able and competent to provide written, informed consent. We extended age range beyond that of age range (30-49 years) recommended by the WHO for cervical-cancer screening[19] because there is limited evidence for the optimal upper age for cervical-cancer screening of HIV-infected women. *Exclusion criteria*, in addition to not meeting the inclusion criteria, include 1) pregnant, 2) signs of abnormal, non-menstrual bleeding suggestive of ICC, 3) without a cervix due to hysterectomy, and 4) not sufficiently healthy to participate in a research study based on the judgment of the clinicians. Excluded women are being advised to seek routine cervical-cancer screening through government programs.

Prior to initiation of enrollment at a specific clinic, the local HIV-care provider team identifies potentially eligible women at their routine clinic visits and offers them enrollment. Women indicating interest in the study then are being registered by our research nurses using the eligibility criteria checklist. Women at one site are being enrolled until all the eligible and willing women at that site are screened. The study team of at least two research nurses schedules 12 to 15 women three to four days a week by calling them and confirming appointments over the

telephone. Two teams of nurses are in the field, meaning that two cervical-cancer screening clinics can be run simultaneously.

Enrollment Visit: The study participant flow is summarized in **Figure 1**. Enrollment visits, including pelvic exams with VIA and specimen collection, are being done entirely by a team of two study nurses. During their enrollment visit, women are being educated on cervical-cancer risk factors, mainly HPV infection, and why they are more at risk to develop ICC than HIV-uninfected women. They also are being allowed to ask questions before they commit to participating in the study. Women are then being asked to provide informed, written consent to participate in the study using a printed out consent form. Those who provide consent complete a short nurse-administered questionnaire (**Appendix I**) on cervical cancer risk factors and sociodemographic characteristics using a data capture screen in Microsoft Access. The questionnaire collects information on basic sociodemographics, factors associated with acquiring HPV (e.g., marital status and recent and lifetime number of sexual partners), factors associated with increased risk of progression of hrHPV infection to precancer and cancer (e.g., smoking and other tobacco use, parity, and oral and other contraceptive use), and other infections common in Rwanda such as malaria[35] and tuberculosis[36] that have been previously reported to be associated with precancer among hrHPV-infected women. The questionnaire was not pretested.

Enrolled women then undergo a pelvic exam, with VIA and a single cervical exfoliated ("Pap") specimen collected and placed into 20 ml PreservCyt (Hologic, Bedford, MA, USA) which is then being sent to the lab at RMH for hrHPV testing. Finally, a portable colposcope (http://www.mobileodt.com/; MobleODT, Tel Aviv, Israel) is being used for digital cervicography (comparable to VIA with magnification) and the image is being captured and saved for quality control, research, and to develop a digital library.

Colposcopy Visit: Screen-positive women (women who test hrHPV and/or VIA positive) are being called using a telephone as soon as the hrHPV result is available and being invited to return for colposcopy within one month. All screen-positive women receiving colposcopy will are having two additional specimens collected, one into PreservCyt for the evaluation of other molecular biomarkers (genotype-specific hrHPV viral methylation and load, and p16/Ki-67 immunocytochemistry CINtec® PLUS Cytology Kit [Roche, Tucson, AZ, USA]) and a second as a dry swab for HPV16, 18, 31, 33, 35, 45, 52, and 58 E6/E7 oncoprotein detection by the next generation lateral flow hrHPV oncoprotein test from Arbor Vita Corporation (Fremont, CA, USA) being included in this study as a triage for screen-positive women to identify those women who are at higher risk of having CIN3+. The residual PreservCyt specimens from both the screening and colposcopy visits are being stored at -20ºC, creating a biobank in Rwanda for future retrospective evaluations of promising new biomarkers and tests.

After specimen collection, a colposcopic evaluation of the cervix is being done with a modified version of the 4-quadrant microbiopsy procedure being performed.[37] Compared to the standard biopsy, the microbiopsy protocol improves disease ascertainment and reduces biases related to selecting the most visually obvious acetowhite lesions while removing less tissue (~13 mm^2 for 4 microbiopsies vs. ~28mm^2 for 1 standard biopsy). Modifications to the standard 4-quadrant microbiopsy procedure are: 1) endocervical curettage is being taken only for those women whose squamocolumnar junction is not entirely visible and the lesion extends into the endocervical canal; and 2) standard-size biopsies of very large lesions are being taken to increase the likelihood that the most severe area is being biopsied.

Pathology: Biopsies are being processed in a single cassette so that a single slide has a section from all biopsies taken. Biopsies are being read by a local pathologist at RMH and Dr. Hebert or

another pathologist at Montefiore Medical Center, Bronx, NY, USA. Women receiving a diagnosis of CIN2+ by the Rwandan pathologist (T.Z.) or, as a safety precaution, CIN3+ diagnosis by Montefiore pathologist (T.H.) are receiving treatment[38]: 1) CIN2, CIN3, or AIS are being referred to study doctors to undergo an excision procedure (e.g., loop electrosurgical excision procedure [LEEP] or cold-knife cone [CKC]) and 2) ICC are being referred to RMH Hospital for care. Women with <CIN2 are being advised to seek re-screening in a year.

A slide with biopsies also are undergoing p16 immunohistochemistry (IHC) using the CINtec® Histology Kit (Roche) for research purposes only.

Endpoints: The primary scientific endpoints of the study are histologically confirmed, consensus CIN2+ i.e., both pathologists diagnose CIN2+ (without adjudication) or CIN3+ by the study pathologist. The secondary, clinical endpoint is histologically confirmed CIN2+ diagnosed by the Rwandan pathologist. Additional endpoints using pathology review and p16 IHC are being used but not for evaluating the performance of p16 immunocytochemistry due to the possibility of p16-related autocorrelation.

Treatment: Women diagnosed with CIN2+ are being referred for treatment. Those precancerous lesions are being treated by ablation if they meet WHO criteria for cryotherapy.[39] Those who do not meet those criteria undergo an excision procedure (e.g., loop electrosurgical excision procedure [LEEP] or cold-knife cone [CKC]) or, in the case of an ICC diagnosis, referred for cancer management. Screen-positive women with <CIN2 are being advised to seek re-screening in a year through the existing healthcare system.

Data sources: Data are being collected from the following sources:

1. A nurse-administered questionnaire on sociodemographic characteristics and cervical cancer risk factors including age at first sexual intercourse, number of sexual partners, smoking, contraception, parity and socioeconomic status.

2. Pelvic exam, VIA, Mobile ODT and colposcopy data capture forms

3. Medical record data on HIV status (e.g., (http://www.who.int/hiv/pub/guidelines/HIVstaging150307.pdf), CD4 count, viral load, antiretroviral therapy (ART) regimen(s)), care, and dates.

Laboratory Testing: The following laboratory tests are being performed:

Xpert HPV Testing—cervical Pap specimens in PreservCyt are being sent to the RMH laboratory in Kigali, Rwanda for hrHPV DNA testing using the Xpert HPV test (Cepheid, Sunnyvale, CA, USA).[40-45] The Xpert HPV Assay is a new, qualitative, real-time PCR assay for the detection of hrHPV DNA. The Xpert HPV Assay includes simultaneous detection of 14 hrHPV types, hydroxymethylbilane synthase (HMBS), and an internal Probe Check Control (PCC). The 14 targeted hrHPV types are detected in 5 fluorescent channels: 1) HPV16, 2) HPV18 and hrHPV 45 (HPV18/45), 3) HPV31, 33, 35, 52, and 58, 4) HPV51 and HPV59, and 5) HPV39, 56, 66, and 68. HMBS (fluorescent channel 6) verifies specimen adequacy.

Specimens are being mixed and a 1-mL pre-aliquot is being removed using a disposable pipette and placed in the testing cartridge per the manufacturer's instructions. Unsatisfactory results due to insufficient cellular content are being re-tested. If the second test is also unsatisfactory, the final result are being recorded as unsatisfactory but women with unsatisfactory results are being referred to colposcopy for safety.

HPV Viral Methylation—We will conduct a retrospective analysis of HPV viral methylation and its association with CIN2+. To identify single hrHPV type infections, we will select single-channel positives from the Xpert HPV assay. For those that are hrHPV positive for a channel other than HPV16, which is detected singly, we will test them to identify the single type infections using a standard protocol for PCR amplification using MY09/11 L1 consensus primers and hrHPV genotype detection using dot-hybridization for 39 individual type-specific probes and a mixture of probes for 10 other uncommon hrHPV types as previously described.[46;47] To isolate the DNA, ThinPrep specimens (1.5 mL) will be pelleted, re-suspended in STM, digested with Proteinase K, precipitated overnight in ammonium acetate ethanol at -20°C, washed, and suspended and stored in TE buffer.

The isolated DNA then will undergo bisulfite conversion.[48] Following bisulfite conversion and DNA purification and de-sulphonation, bisulfite-treated DNA will be used as template for Next-Gen Sequencing (NGS) (HiSeq2000, Illumina, San Diego, CA) using barcoded-type specific primers. Sequences for pads and barcodes are not found in the targeted genomic region. Use of padding and barcodes will enable reads to be identified by amplicon (forward or reverse) or by sample during downstream bioinformatics analysis.[49]

All PCR products for NGS will be pooled (by assay) and a single DNA band containing multiple amplicons from different samples (with unique barcodes) will be isolated from a gel for NGS.[49] Briefly, equal concentrations of each barcoded PCR product (based on PCR band intensity) will be pooled and isolated. Upon confirmation of correct product size, all purified DNA pools will be combined and submitted for library preparation and paired-end 100 base pair Illumina HiSeq2000 sequencing at the Einstein Genomics Core Facility.

Methylation status are being determined in the lab of Dr. Robert Burk at Albert Einstein College of Medicine (USA). Prior to determination of methylation status, de-multiplexing based on the unique barcodes is being performed using in-house generated scripts to obtain paired-end NGS reads of each sample. Reads are being aligned with hrHPV reference genome sequences by bowtie v0.12.9.[50] Methylation status of each CpG site is then determined by bismark v0.7.7[51] using the default quality score parameter set to Q30, and the formula of the methylation ratio of the number of C read by the number of C+T read.

E6/E7 Oncoprotein Testing—Dry swab specimens, collected at the time of colposcopy, are being tested for individual E6/E7 oncoproteins as previously described[52;53], according to the manufacturer's instructions, at the RMH laboratory in Kigali, Rwanda. The E6/E7 oncoprotein test uses three lateral flow strips to detect 8 hrHPV types whereas the E6 oncoprotein test used a single lateral flow strip to detect 3 hrHPV types.

Analyses: We will evaluate combinations of the above mentioned screening strategies and tests to estimate the clinical performance (e.g., Se, Sp, PPV, and NPV) for the detection of consensus CIN3+ and community CIN2+. A log binomial model using generalized estimating equations will be used to take into account correlation between different tests from the same subject. Note while these models will be developed for the estimation and comparison of performance for two tests, the model can be extended to allow more than two tests by including more indicator variables for test type.

Some analyses of biomarkers, such as viral methylation will be restricted to hrHPV-positives. Comparisons of hrHPV viral methylation to other triage biomarkers will be restricted to the subset that gets tested for viral methylation as described.

Sample size calculations: We are basing our sample size on the ability to detect modest but minimally important differences in Se of 15%. We conservatively assume that the population prevalence of CIN3+ is 2% based on our past study in Rwanda.[33;35] We will enroll and have complete follow-up of at least 5,000 HIV[+] women. A sample size of 5,000 HIV[+] women with completed follow-up of the screen positives will yield 100 cases of CIN3+, which will have at least 80% power (α=5%) to crudely detect a 15% difference in Se between two screening strategies for a range of 10%-25% discordance. With this sample size of 5,000 women, 4,900 will not have CIN3+; we will also have at least 90% power (α=5%) to detect a difference in Sp of 3% for discordance up to 40%. Finally, we will have 80% power (α=5%) to crudely detect an 8%, 10%, or 11% difference in PPV if the reference PPV is 10%, 20%, or 30%, respectively.[54]

Cost Effectiveness: We will conduct assessments of the costs and cost-effectiveness of the different combinations of screening and triage tests, i.e., algorithms, as well as those of the entire community-based screening "system." Costs measurement will be conducted using a micro-costing (ingredients) approach in which resource use throughout each step in the screening process will be tracked and unit costs for each of the resources will be applied to generate an average screening cost per woman to be compared against what the estimated costs are for a possible program based on hrHPV screening and VIA triage or VIA screening. For estimating costs of the screening system and scale-up of screening to 100,000 women in a month, analyses will distinguish financial costs, which reflect actual expenditures of the program, from economic costs, including the value of donated and shared resources to more fully assess opportunity costs. Projections on budget impact and economic cost implications over time will be made under varying assumptions of screening uptake, follow-up compliance, and scenarios of changing disease burden.

Clinical outcomes will include true positive, true negative, false negative, and false positive test results, number of colposcopies, incident cancer, and cancer death. Cost-effectiveness will be measured as cost/CIN2+ detected, cost/CIN3+ detected, cost/invasive cancer prevented, cost/cancer death prevented, cost/life-year saved, and cost/quality-adjusted life year (QALY) saved; in addition, we will calculate harm/benefit ratios, using varying definitions of harms (colposcopies, false positive results) to benefits (cancers prevented, deaths prevented, life years and QALYs saved). Costs and effectiveness will be discounted at a 3% annual rate, with the rate varied from 0-5% in sensitivity analysis. For assessment of value-of-information (VOI), we will use net monetary benefits (NMB), defined as a function of the willingness-to-pay threshold (WTP) for different costs and outcomes as: $NMB=(WTP * Effectiveness) - Costs$.

Patient and Public Involvement

There was no patient engagement in the development or design of the study, recruitment, and the conduct of the study. Participants are receiving their results directly since it is related to their care. As this was not a randomized controlled trial, the burden of the intervention was not assessed by patients themselves. There were no patient advisors to acknowledge.

Ethics and Dissemination

Ethics: This study protocol was reviewed and approved by the Rwanda National Ethics Committee (RNEC) as well as the Institutional Review Board for human subjects research at Albert Einstein College of Medicine.

Confidentiality measures and protection against potential risks: The risks for those participating in our study include:

- Collection of Pap specimens/cervical swabs involves a modest risk of bleeding which is typically very limited when it occurs. Testing positive for any test may cause psychological distress (anxiety).
- Colposcopy and excisional treatments induce vaginal bleeding and may incur pain, infection, and short-term psychological distress (anxiety). A diagnosis of CIN2 or more severe may cause psychological distress (anxiety). A diagnosis of ICC may cause severe psychological distress.
- Questions in the questionnaire, regarding sexual behavior and other matters of a personal nature, may cause anxiety and embarrassment. Participants are advised that they are free not to answer specific questions.
- There is also the risk of psycho-social stress which could occur if there was inadvertent disclosure of confidential medical or other personal information.

Protection against the risk of inadvertent disclosure of confidential information is being addressed by the standard procedures at the Rwandan study site, including: (i) storing completed paper copies of questionnaires and other hard copy information (described above), identified by study number only, in a filing system separate from the name-address file of participants in the study; and (ii) only the designated local personnel have access to cross-reference the files; (iii) all paper files, including consent forms, are being maintained in locked cabinets in locked rooms, with access restricted to specific research personnel.

In addition, we will include the following security measures to protect the data:

- Controlled access to project data;

- A tracking system for data forms and activities;
- Date and time of stamping of all data records with electronic signatures;
- Audit trails to track all changes made to data records; and
- Data kept on password-protected computers and in locked rooms.

Potential Patient Benefits: There are potential direct benefits to study participants. HIV[+] women participating in the study are at very high risk of ICC. They are being rigorously screened and evaluated, more effectively than the standard of care anywhere in the world. As a result of the study, women with precancer who are at imminent risk of ICC are being diagnosed sooner and treated more effectively than women receiving routine care and thereby more likely averting the development of ICC. Women with cervical cancer are being diagnosed earlier thereby reducing the morbidity and the risk of mortality caused by ICC. Conversely, any pain, bleeding, or stress that might occur related to colposcopy or cervical swab are typically modest and well tolerated.

There are also substantial potential societal benefits related to the study due to its implications to improving cervical-cancer screening practices and guidelines in HIV[+] women – changes in practice which might also benefit the study participants themselves, if and when these changes are enacted. There is a great need to identify more effective and practical methods for cervical-cancer screening for HIV[+] women living in Africa, who are living longer than ever and are therefore at potentially greater risk of ICC.

Dissemination: We plan to publish a series of scientific reports in peer-reviewed scientific journals. As building research capacity in Rwanda is a major goal of this research project, all investigators of the research team are being asked and supported to lead at least one analysis and one manuscript preparation, based on interests and expertise.

In addition, an external advisory panel (EAP) composed of leaders from the Rwanda Ministry of Health, University of Rwanda, and Rwanda medical community has been formed. The responsibilities of the EAP include providing advice on the conduct of the project and interpretation for and dissemination of the study results to Rwandan stakeholders. The latter is important for the adoption of evidence-based best practices for cervical-cancer screening as warranted.

Limitations

There are several limitations to the study that bear mentioning. First, cervical cytology was not included in the study. There is limited cervical cytology services available locally and of unknown quality and it is unlikely that cytology are being widely available in Rwanda, making its inclusion as a comparator test of limited value. Moreover, there are significant costs and logistical challenges in shipping PreservCyt specimens to and having cytology slides made and read in the U.S. Second, we did not conduct biopsies in screen-negative women, which would have allowed us to estimate absolute clinical performance. The burden of sending screen-negative women to colposcopy was deemed too great and it was impractical to send a sufficient numbers of screen-negative women to colposcopy to accurately estimate the false-negative disease (CIN3+) fraction. Thus, only relative clinical performance of the screening tests can be estimated from this study.

Contributor Statement:

KA, PEC, JCD, AA, and JDS conceived the original concept of the study and the interventions. GM, KA, JDS, and PEC drafted the protocol. PEC performed the sample size calculations, and PEC and GM will lead analysis of the results. GM, JDS, and Athanase Munyaneza are supporting patient recruitment. GM, TR, and Athanase Munyaneza are providing clinical care for patients. Anthere Murangwa and LM oversee laboratory testing, and TZ and TH are responsible for pathology. PM and LM oversee and administer the study activities at the clinical site in Rwanda. All authors (GM, JCD, TR, JDS, Athanase Munyaneza, Anthere Murangwa, TZ, TH, PM, AA, LM, KA, and PEC) contributed to the scientific design of the study and the protocol development, are involved in the implementation of the project, and have read and approved the final manuscript.

Figure Legends:

Figure 1. Study Design

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Table 1: Recruitment sites and estimated eligible population.

Province	Site	Type of site	Potential participants per site (approximate)
Kigali	Cor-unum HC	Public Health Center	1,405
Kigali	Kimironko HC	Public Health Center	1,227
Kigali	Rwampara HC	Public Referral Hospital	1,098
Kigali	Kicukiro HC	Public Health Center	1,054
Kigali	Kacyiru HC	Public Health Center	905
Kigali	Gikondo HC	Public Health Center	925
Kigali	Rwanda Military Hospital	Public Referral Hospital	300
Kigali	WEACTx for Hope	Private HIV Clinic	500
Kigali	Busanza HC	Public Health Center	100
Kigali	Nyarugunga HC	Public Health Center	100
West	Gisenyi DH	Public District Hospital	810
Total			8,424

Figure 1. Study diagram

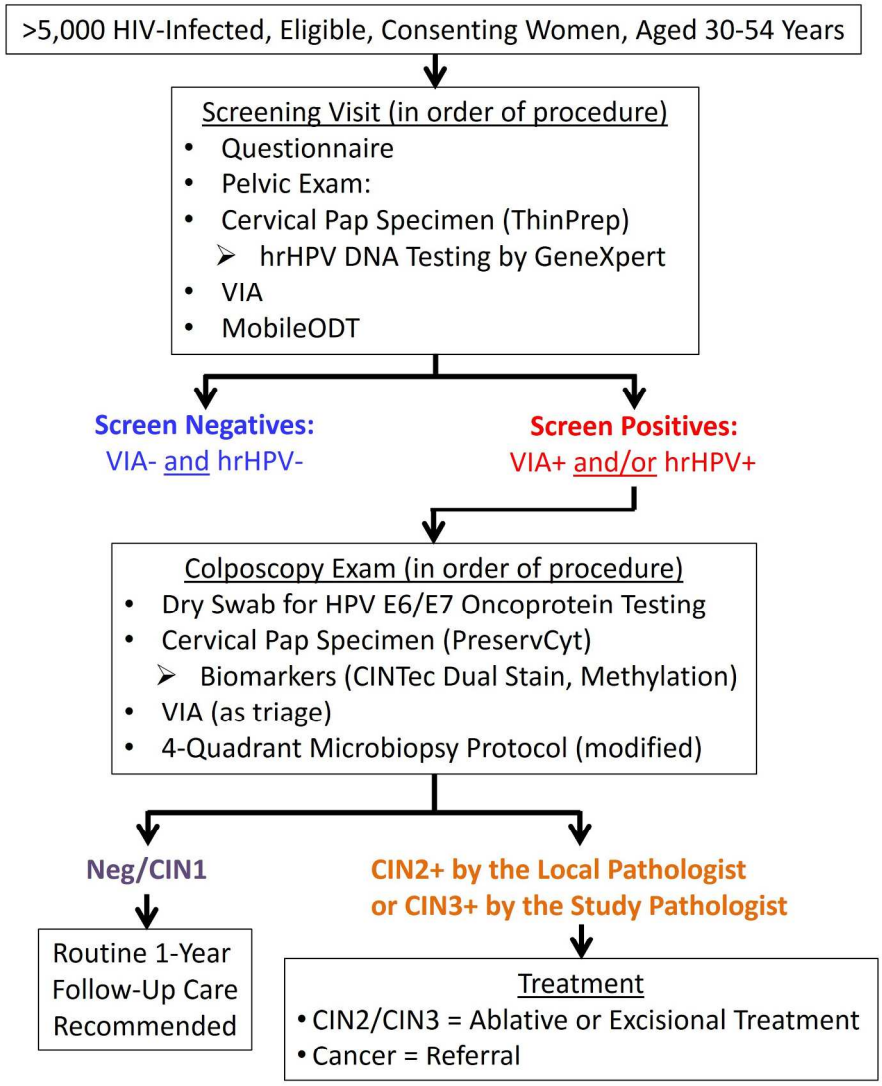

Figure 1. Study Design

190x254mm (300 x 300 DPI)

Appendix I. Study Questionnaire

A. Socio-demographics

1. **What is your date of birth? D D / M M / Y Y Y Y (enter all 0 if not remembered) (If date of birth or year of birth is given, skip to Question 3)**
2. **What is your age?** __ __ (Years)
3. **What is your marital status?**

☐ Married/Cohabiting
☐ Divorced
☐ Widowed
☐ Separated
☐ Single
☐ Choose not to answer

4. **Do you live in Kigali?**

☐ Yes
☐ No {Skip to Question A5}
☐ Choose not to answer {Skip to Question A5}

5. **In what area of Kigali City Province do you live?**

☐ Nyarugenge
☐ Kicukiro
☐ Gasabo urban (e.g., Kacyiru, Kimironko, Kinyinya, etc.)
☐ Gasabo rural (e.g., Nduba, Jabana, Gikomero, etc.)
☐ Do not know
☐ Choose not to answer

6. **What is your household income per month in FRW?**

☐ < 5,000
☐ 5,000 – 9,999
☐ 10,000 – 34,999
☐ 35,000 – 59,999

☐ 60,000 – 84,999
☐ 85,000 – 109,999
☐ 110,000 – 134,999
☐ 135,000 – 269,999
☐ 270,000 or more

7. **How many people live in your household?** __ __ {Enter 00 if refuse to answer}

8. **What is your occupation?**

☐ Employed by government, another institution, or company
☐ Self-employed (Small and medium enterprises)
☐ Self-employed (High income earnings)
☐ Farming (peasants)
☐ Unemployed/Does not work
☐ Other (specify) _____
☐ Choose not to answer {Skip to Question B1}

{The following questions are sensitive and personal in nature. Your answers will be kept confidential. You may choose not to answer certain questions. Answering any question is voluntary}

B. <u>Sexual Behaviors</u>

1. **How old were you when you first had sex?** __ __ (Years) {Enter 00 if refuse to answer}

2. **How many sexual partners have you had in your lifetime?**

☐ No Partners (Ineligible)
☐ 1 Partner
☐ 2-3 Partners
☐ 4-5 Partners
☐ 6-9 Partners
☐ 10 or more partners
☐ Choose not to answer

3. **How many sexual partners have you had in the last 6 months?**

☐ No Partners

☐ 1 Partner

☐ 2 or more Partners

☐ Choose not to answer

C. Parity

1. **What age did you have your first child? __ __** (Years) {Enter 00 if refuse to answer, Enter 99 if never pregnant} (if 00 or 99, skip to Question D1)

2. **How many live births have you had in your lifetime? __ __**

3. **Have you given birth in the last year?**

☐ Yes

☐ No

☐ Choose not to answer

D. Tobacco Use

1. **Have you ever smoked cigarettes?**

☐ Yes

☐ No (skip to Question D3)

☐ Choose not to answer (skip to Question D3)

2. **Do you currently smoke cigarettes?**

☐ Yes

☐ No

☐ Choose not to answer

3. **Have you ever chewed/used tobacco orally (Ubugoro)?**

☐ Yes

☐ No (skip to Question D5)

☐ Choose not to answer (skip to Question D5)

4. **Are you currently chewing/using tobacco orally (Ubugoro)?**

☐ Yes

☐ No

☐ Choose not to answer

5. Have you ever chewed/used tobacco orally (Tobacco leaves-Igikamba)?

☐ Yes
☐ No (skip to Question E1)
☐ Choose not to answer (skip to Question E1)

6. Are you currently chewing/using tobacco orally (Tobacco leaves-Igikamba)?

☐ Yes
☐ No
☐ Choose not to answer

E. Contraceptive Use

1. Have you ever used oral contraceptives?

☐ Yes
☐ No (Skip to Question E3)
☐ Choose not to answer (Skip to Question E3)

2. Do you currently use oral contraceptives?

☐ Yes
☐ No
☐ Choose not to answer

3. Have you ever used Depo Provera (contraceptive)?

☐ Yes
☐ No (Skip to Question E5)
☐ Choose not to answer (Skip to Question E5)

4. Do you currently use Depo Provera (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

5. Have you ever used Jadell (contraceptive)?

☐ Yes
☐ No (Skip to Question E7)

☐ Choose not to answer (Skip to Question E7)

6. Do you currently use Jadell (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

7. Have you ever used IUD (contraceptive)?

☐ Yes
☐ No (Skip to Question E9)
☐ Choose not to answer (Skip to Question E9)

8. Do you currently use IUD (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

9. Do use condoms as a contraceptive?

☐ Yes
☐ No (skip to Question F1)
☐ Choose not to answer (skip to Question F1)

10. Have you used condoms as a contraceptive in the last 6 months?

☐ Yes
☐ No
☐ Choose not to answer

F. Infections

1. Have you ever had Malaria

☐ Yes
☐ No (skip to Question F8)
☐ Choose not to answer (skip to Question F8)

2. How many times have you had Malaria? __ __

3. **What was the year that you last had Malaria? Y Y Y Y**

4. **Have you had your Malaria treated using drugs?**

☐ Yes
☐ No
☐ Choose not to answer

5. **If Yes, how was it treated?**

☐ Treated only with traditional medicine
☐ Treated only with drugs (e.g. Coartem)
☐ Treated with both (traditional and modern)
☐ Not treated
☐ Choose not to answer

6. **Was your last episode of Malaria treated using drugs?**

☐ Yes
☐ No
☐ Choose not to answer

7. **If Yes, how was it treated?**

☐ Treated only with traditional medicine
☐ Treated only with drugs (e.g. Coartem)
☐ Treated with both (traditional and modern)
☐ Not treated
☐ Choose not to answer

8. **Have you ever had Tuberculosis (TB)?**

☐ Yes
☐ No (skip to end)
☐ Choose not to answer (skip to end)

9. **If Yes, how many times have you had TB in your lifetime? __ __**

10. **What year did you last have Tuberculosis (TB)? Y Y Y Y**

END OF QUESTIONNAIRE

BMJ Open

A Protocol for the Study of Cervical-Cancer Screening Technologies in Human Immunodeficiency Virus-Infected Women Living in Rwanda

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2017-020432.R3
Article Type:	Protocol
Date Submitted by the Author:	19-Jun-2018
Complete List of Authors:	Murenzi, Gad; Rwanda Military Hospital Dusingize, Jean; Regional Alliance for Sustainable Development , Research and Scientific Capacity Building Rurangwa, Theogene; Rwanda Military Hospital Sinayobye, Jean d'Amour; Regional Alliance for Sustainable Development, ; Women's Equity in Access to Care & Treatment (WE-ACTx), Munyaneza, Athanase; Rwanda Military Hospital Murangwa, Anthere; Rwanda Military Hospital Zawadi, Thierry; Rwanda Military Hospital Hebert, Tiffany; Yeshiva University Albert Einstein College of Medicine Mugenzi, Pacifique; Rwanda Military Hospital Adedimeji, Adebola; Yeshiva University Albert Einstein College of Medicine Mutesa, Leon; Rwanda Military Hospital; University of Rwanda College of Medicine and Health Sciences Anastos, Kathryn; Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, Castle, Philip; Yeshiva University Albert Einstein College of Medicine,
Primary Subject Heading:	Global health
Secondary Subject Heading:	Obstetrics and gynaecology, Infectious diseases, HIV/AIDS, Epidemiology, Diagnostics
Keywords:	human papillomavirus (HPV), cervical cancer, HIV & AIDS < INFECTIOUS DISEASES, GYNAECOLOGY, cervical intraepithelial neoplasia

A Protocol for the Study of Cervical-Cancer Screening Technologies in Human Immunodeficiency Virus-Infected Women Living in Rwanda

Gad Murenzi, MD*[1], Jean-Claude Dusingize, MD, MS[1], Theogene Rurangwa, MD, MMed[1], Jean d'Amour Sinayobye, MD, MS[1], Athanase Munyaneza, RN[1], Anthere Murangwa, MS[1], Thierry Zawadi, MD[1], Tiffany Hebert, MD[2], Pacifique Mugenzi, MD[1], MMed, Adebola Adedimeji, PhD, MPH[2], Leon Mutesa, MD, PhD[1,3], Kathryn Anastos, MD[2], Philip E. Castle, PhD, MPH[2]

[1]Rwanda Military Hospital, Kigali, Rwanda; [2]Albert Einstein College of Medicine, Bronx, NY, USA; [3]University of Rwanda, Kigali, Rwanda

*Correspondence: gadcollins@gmail.com, +250788589085

Disclosures: This research study has received HPV tests for reduced or no cost from Cepheid, Arbor Vita Corporation, and Roche.

Funding: This study was funded by NCI/NIH Grant 5U54CA19016304 and by a grant from the Prevent Cancer Foundation.

Abstract

Introduction. The optimal method(s) for screening human immunodeficiency virus-infected women, especially for those living in sub-Saharan Africa, for cervical precancer and early cancer has yet to be established.

Methods and analysis. A convenience sample of >5,000 Rwandan women, ages 30-54 years and living with HIV infection, are being consented and enrolled into a cross-sectional study of cervical-cancer screening strategies. Participants are completing an administered short risk-factor questionnaire and being screened for high-risk human papillomavirus (hrHPV) using the Xpert HPV assay (Cepheid, Sunnyvale, CA, USA), unaided visual inspection after acetic acid (VIA), and aided VIA using the EVA system (Mobile ODT, Tel Aviv, Israel). Women positive for hrHPV and/or by VIA undergo colposcopy, which includes the collection of two cervical specimens prior to undergoing a 4-quadrant microbiopsy protocol. The colposcopy-collected specimens are being tested by dual immunocytochemical staining for $p16^{INK4a}$ and Ki-67 (CINtec® PLUS Cytology, Ventana, Tucson, AZ, USA) and for E6 or E7 for 8 hrHPV genotypes (HPV16, 18, 31, 33, 35, 45, 52, and 58) using the next-generation AV Avantage hrHPV E6/E7 test (Arbor Vita Corporation, Freemont, CA, USA). Women with local pathology diagnosis of cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or more severe (CIN2+) or pathology-review diagnosis of CIN grade 3 or more severe (CIN3+) receive treatment. Clinical performance and cost-effectiveness (e.g., sensitivity, specificity, and predictive values) of different screening strategies and algorithms will be evaluated.

Ethics and dissemination. The protocol was approved by local and institutional review boards for human subjects research. At the completion of the study, results will be disseminated to the scientific community through peer-reviewed publication and to the Rwandan stakeholders through an external advisory panel.

Strengths and Weaknesses

- We are enrolling a very large sample size of HIV-infected women living in Rwanda who otherwise would probably not get cervical-cancer screening.
- We are employing rigorous disease ascertainment protocols to minimize misclassification.
- Some tests, such as the Xpert HPV and the AV Avantage HPV E6/E7 assays, are being done on site in Rwanda using technologies that could feasibly be deployed there.
- A weakness of the study is that cervical cytology is not being included as a comparator due to financial and logistical constraints.

Introduction

Invasive cervical cancer (ICC) remains a significant cause of morbidity and mortality globally. Approximately 530,000 cases of and 270,000 deaths due to ICC occur annually, making it the 4th most common malignancy and cause of cancer-related death in women globally.[1] In many high-income countries (HIC), ICC rates have declined by 50% or more[2] due to the introduction of effective, high-coverage Pap testing (cervical cytology)-based screening programs that include timely follow-up of screen positives, treatment of women with precursor lesions, and management of cancers. Now, almost 90% of ICC and ICC-related deaths occur in low- and middle income countries (LMICs) due to a lack of resources and healthcare infrastructure needed to provide preventive services.

ICC and ICC-related mortality rates are particularly high in Sub-Saharan Africa, which also has the highest rates of HIV infection in the world. Now, over 12 million HIV-infected (HIV[+]) women in Sub-Saharan Africa are living longer because of anti-retroviral therapy, thus increasing their likelihood of dying from ICC.[3] However, many of these women are already exposed to human papillomavirus (HPV), the viral cause of ICC, and will not benefit from or be targeted for prophylactic HPV vaccination. Thus, cervical-cancer screening will be needed for the foreseeable future.

However, setting up effective cytology for cervical-cancer screening is expensive and requires a complex clinical and lab infrastructure that generally does not exist in LMICs.[4;5] Moreover, it is now well understood that cytology has only a low- to moderate one-time sensitivity for precursor lesions and therefore must be done repeatedly over many years to reduce cancer risk. Alternative

strategies to address the ICC burden in LMICs, especially in SSA, must be developed and validated.

Persistent cervical infections by high-risk HPV (hrHPV) types cause virtually all ICC and its immediate precursor lesions, e.g. cervical intraepithelial neoplasia grade 3 (CIN3) and adenocarcinoma *in situ* (AIS) everywhere in the world.[6;7] hrHPV causes most anal and vaginal cancer and a significant proportion of vulvar, penile, and oropharyngeal cancers.[8] HPV16 is the most important causal type, responsible for ~60% of ICC.[9] HPV18 is the next most important, responsible for 10-15% of ICC, including 30-40% of adenocarcinoma of the cervix[9], which is on the rise in Western Countries.[10;11] Together, HPV16 and HPV18 account for ~70% of ICC, and the same 15 hrHPV types account for ~99% of ICC everywhere in the world.[9]

There is now overwhelming evidence to suggest that testing for hrHPV is more sensitive, albeit less specific, than high-quality cytology for identifying women with cervical precancer.[12-16] One-time hrHPV testing can reduce the risk of ICC incidence by approximately 40% in 6.5 years compared to cytology screening[16], and ICC mortality by approximately 40% (approximately 50% overall) in 8 years compared to cytology.[17] Importantly, a negative hrHPV test provides superior reassurance against CIN3+ [18] and against ICC[16;17], permitting safe extension of screening intervals.

The World Health Organization released cervical-cancer screening and treatment guidelines in 2013, recommending two evidence-based approaches to cervical-cancer screening[19]: (I) Use either hrHPV testing or visual inspection after acetic acid (VIA), which involves the inspection of the cervix with a speculum in place and following the application of dilute acetic acid to help identify potential CIN by its characteristic white coloring in the presence of acetic acid

(acetowhite), as alternative initial screening tests instead of Pap, and (II) immediately treat those who screen positive using the screening test, rather than require diagnostic verification through colposcopy and biopsy. This approach is commonly called screen-and-treat (S&T), and is increasingly thought to be more amenable to LMIC settings.

However, hrHPV testing is also a much more effective screen than VIA[17], which on a large-scale appears to only down-stage cancer rather than prevent it.[20] Thus, the recent American Society for Clinical Oncology (ASC) resource-stratified guidelines for secondary cervical-cancer prevention[21;22] emphasize that hrHPV testing is the preferred choice for screening, with VIA only being used until hrHPV testing becomes available, and that HIV-infected women, because of their higher risk, should be screened twice as frequently as the general (HIV-uninfected) population.

Recent data in HIV[+] women living in the U.S. suggest that hrHPV testing may have clinical utility similar to that in HIV-negative (HIV[-]) women. Several observational studies have shown that an extended screening interval is safe in HIV[+] women who test hrHPV and Pap negative as it is for HIV[-] women.[23;24] In a study of women enrolled in Women's Interagency Health Study (WIHS) in 2002, HIV[+] and HIV[-] women who tested hrHPV and Pap negative were at a similarly low risk of cervical intraepithelial neoplasia (CIN) grade 2 (CIN2) or more severe (CIN2+) histology over a 5-year follow-up.[23] In addition, no cases of histologically confirmed CIN2+ were diagnosed in the follow-up of hrHPV- and Pap-negative HIV[+] women aged 30-64 years who underwent routine three-year hrHPV and cytology cotesting at Kaiser Permanente Northern California.[24] Thus, both studies found very high negative predictive values (NPV) >99% in HIV[+] women who test hrHPV negative. However, how hrHPV testing can

best be used to screen HIV[+] women living in Sub-Saharan Africa to prevent ICC remains to be determined.

Recent studies[25-28] in HIV[+] women living in Sub-Saharan Africa have compared hrHPV, VIA, and/or Pap for the detection of cervical precancer/cancer. The results can be summarized as follows: 1) hrHPV detection was more sensitive but less specific than VIA; 2) surprisingly, cytology was equally or more sensitive but less specific than VIA; and 3) surprisingly, cytology was equally or more sensitive but less specific (vs. the converse) than hrHPV testing. Results and conclusions are varied, leaving unanswered the question of what screening strategy in HIV[+] women living in Sub-Saharan Africa has the greatest effectiveness and cost effectiveness.

Regardless of the screening method, most screen-positive women who go to colposcopy or are treated immediately without diagnostic verification do not have cervical precancer and cancer (positive predictive value [PPV] for screening tests are typically 10%-20%). In places like Sub-Saharan Africa that lack necessary infrastructure and personnel such as pathologists[29], excessive referral to colposcopy is problematic. Although WHO recommendations for S&T will hopefully overcome this bottleneck and increase the number of women living in LMICs who get screened, many countries may not adopt current S&T strategies because of concerns of low specificity and overtreatment, resulting in increased costs, unnecessary patient discomfort and concern, and wasting valuable healthcare resources that could otherwise be used to expand access to screening. Thus, methods to increase the accuracy of screening by reducing the numbers of women having colposcopy and biopsy or getting treated immediately in this context are highly desirable as they will likely increase the uptake of cervical-cancer screening.

In order to improve the specificity of screening tests, secondary tests (biomarkers) are used following a screen-positive result, with women who test positive for the triage undergoing further management (e.g., colposcopy or immediate treatment) and those who test negative typically being deferred to further evaluation in 6-18 months to allow hrHPV infections to clear. There are several very promising biomarkers that might be used to improve the specificity and positive predictive value (PPV) of the screening tests.[24] Given that HIV-infected women are more likely to test hrHPV positive[30-34], it is important to validate a triage strategy of using a secondary biomarker that sensitively and specifically rules-in women with cervical precancer and cancer among the HIV-positive, hrHPV-positive women.

We are therefore conducting a cervical-cancer screening study of >5,000 Rwandan women, ages 30-54 years, living with HIV infection. We are evaluating different screening tests (hrHPV DNA and VIA), those recommended by the WHO for cervical-cancer screening[19], and different triage tests and biomarkers for the management of screen-positive women (E6/E7 oncoprotein detection, p16INK4a immunocytochemistry, and hrHPV viral methylation). Screen-positive women undergo a rigorous colposcopic evaluation with multiple biopsies taken and the biopsies will undergo pathology review, to minimize the misclassification of endpoints. The primary objective of the study is to determine and compare clinical performance (Sensitivity (Se), Specificity (Sp), PPV, and NPV) and cost-effectiveness for identifying HIV[+] women with CIN3+ and CIN2+ of different cervical-cancer screening and management algorithms.

Methods and Analysis

Study design and population: We are recruiting those women receiving care for confirmed HIV infection at health centers (HC) and various hospitals operated by the Ministry of Health or

Rwanda Military Hospital during 2016-18 (**Table 1**). Sites were selected in collaboration with Rwanda Biomedical Center (RBC), which calculate estimated numbers of potentially eligible women using data from their HIV database (OpenMRS-Open Medical Records System; http://openmrs.org/). Sites were also selected from all provinces to ensure geographic representation. The total of 8,424 was the estimated number of women eligible for the study according to the inclusion/exclusion criteria (see below) at the beginning for the study in 2016. From each site, a convenience sample of women are being recruited to participate.

Inclusion criteria include 1) living and receiving HIV care in Rwanda, 2) ages 30-54 years, 3) confirmed HIV+ based on medical records, 4) no prior cervical-cancer screening, 5) no history of ICC, and 6) willing, able and competent to provide written, informed consent. We are extending age range beyond that of the age range (30-49 years) recommended by the WHO for cervical-cancer screening[19] because there is limited evidence for the optimal upper age for cervical-cancer screening of HIV-infected women. *Exclusion criteria*, in addition to not meeting the inclusion criteria, include 1) pregnant, 2) signs of abnormal, non-menstrual bleeding suggestive of ICC, 3) without a cervix due to hysterectomy, and 4) not sufficiently healthy to participate in a research study based on the judgment of the clinicians. Excluded women are being advised to seek routine cervical-cancer screening through government programs.

Prior to initiation of enrollment at a specific clinic, the local HIV-care provider team identifies potentially eligible women at their routine clinic visits and offers them enrollment. Women indicating interest in the study then are then registered by our research nurses using the eligibility criteria checklist. All eligible and willing women at that site enroll and receive screening before moving to another site. The study team of at least two research nurses schedules 12 to 15 women three to four days a week by calling them and confirming appointments over the telephone. Two

teams of nurses are in the field, meaning that two cervical-cancer screening clinics can be run simultaneously.

Enrollment Visit: The study participant flow is summarized in **Figure 1**. Enrollment visits, including pelvic exams with VIA and specimen collection, are being done entirely by a team of two study nurses. During their enrollment visit, women are being educated on cervical-cancer risk factors, mainly HPV infection, and why they are more at risk to develop ICC than HIV-uninfected women. They also are being allowed to ask questions before they commit to participating in the study. Women are then being asked to provide informed, written consent to participate in the study using a printed out consent form. Those who provide consent complete a short nurse-administered questionnaire (**Appendix I**) on cervical cancer risk factors and sociodemographic characteristics using a data capture screen in Microsoft Access. The questionnaire collects information on basic sociodemographics, factors associated with acquiring HPV (e.g., marital status and recent and lifetime number of sexual partners), factors associated with increased risk of progression of hrHPV infection to precancer and cancer (e.g., smoking and other tobacco use, parity, and oral and other contraceptive use), and other infections common in Rwanda such as malaria[35] and tuberculosis[36] that have been previously reported to be associated with precancer among hrHPV-infected women. The questionnaire was not pretested.

Enrolled women then undergo a pelvic exam, with VIA and a single cervical exfoliated ("Pap") specimen collected and placed into 20 ml PreservCyt (Hologic, Bedford, MA, USA) which is then being sent to the lab at RMH for hrHPV testing. Finally, a portable colposcope (http://www.mobileodt.com/; MobleODT, Tel Aviv, Israel) is being used for digital cervicography (comparable to VIA with magnification) and the image is being captured and saved for quality control, research, and to develop a digital library.

Colposcopy Visit: Screen-positive women (women who test hrHPV and/or VIA positive) are being called using a telephone as soon as the hrHPV result is available and being invited to return for colposcopy within one month. All screen-positive women receiving colposcopy will are having two additional specimens collected, one into PreservCyt for the evaluation of other molecular biomarkers (genotype-specific hrHPV viral methylation and load, and p16/Ki-67 immunocytochemistry CINtec® PLUS Cytology Kit [Roche, Tucson, AZ, USA]) and a second as a dry swab for HPV16, 18, 31, 33, 35, 45, 52, and 58 E6/E7 oncoprotein detection by the next generation lateral flow hrHPV oncoprotein test from Arbor Vita Corporation (Fremont, CA, USA) being included in this study as a triage for screen-positive women to identify those women who are at higher risk of having CIN3+. The residual PreservCyt specimens from both the screening and colposcopy visits are being stored at -20ºC, creating a biobank in Rwanda for future retrospective evaluations of promising new biomarkers and tests.

After specimen collection, a colposcopic evaluation of the cervix is being done with a modified version of the 4-quadrant microbiopsy procedure being performed.[37] Compared to the standard biopsy, the microbiopsy protocol improves disease ascertainment and reduces biases related to selecting the most visually obvious acetowhite lesions while removing less tissue (~13 mm^2 for 4 microbiopsies vs. ~28mm^2 for 1 standard biopsy). Modifications to the standard 4-quadrant microbiopsy procedure are: 1) endocervical curettage is being taken only for those women whose squamocolumnar junction is not entirely visible and the lesion extends into the endocervical canal; and 2) standard-size biopsies of very large lesions are being taken to increase the likelihood that the most severe area is being biopsied.

Pathology: Biopsies are being processed in a single cassette so that a single slide has a section from all biopsies taken. Biopsies are being read by a local pathologist at RMH and Dr. Hebert or

another pathologist at Montefiore Medical Center, Bronx, NY, USA. Women receiving a diagnosis of CIN2+ by the Rwandan pathologist (T.Z.) or, as a safety precaution, CIN3+ diagnosis by Montefiore pathologist (T.H.) are receiving treatment[38]: 1) CIN2, CIN3, or AIS are being referred to study doctors to undergo an excision procedure (e.g., loop electrosurgical excision procedure [LEEP] or cold-knife cone [CKC]) and 2) ICC are being referred to RMH Hospital for care. Women with <CIN2 are being advised to seek re-screening in a year.

A slide with biopsies also are undergoing p16 immunohistochemistry (IHC) using the CINtec® Histology Kit (Roche) for research purposes only.

Endpoints: The primary scientific endpoints of the study are histologically confirmed, consensus CIN2+ i.e., both pathologists diagnose CIN2+ (without adjudication) or CIN3+ by the study pathologist. The secondary, clinical endpoint is histologically confirmed CIN2+ diagnosed by the Rwandan pathologist. Additional endpoints using pathology review and p16 IHC are being used but not for evaluating the performance of p16 immunocytochemistry due to the possibility of p16-related autocorrelation.

Treatment: Women diagnosed with CIN2+ are being referred for treatment. Those precancerous lesions are being treated by ablation if they meet WHO criteria for cryotherapy.[39] Those who do not meet those criteria undergo an excision procedure (e.g., loop electrosurgical excision procedure [LEEP] or cold-knife cone [CKC]) or, in the case of an ICC diagnosis, referred for cancer management. Screen-positive women with <CIN2 are being advised to seek re-screening in a year through the existing healthcare system.

Data sources: Data are being collected from the following sources:

1. A nurse-administered questionnaire on sociodemographic characteristics and cervical cancer risk factors including age at first sexual intercourse, number of sexual partners, smoking, contraception, parity and socioeconomic status.

2. Pelvic exam, VIA, Mobile ODT and colposcopy data capture forms

3. Medical record data on HIV status (e.g., (http://www.who.int/hiv/pub/guidelines/HIVstaging150307.pdf), CD4 count, viral load, antiretroviral therapy (ART) regimen(s)), care, and dates.

Laboratory Testing: The following laboratory tests are being performed:

Xpert HPV Testing—cervical Pap specimens in PreservCyt are being sent to the RMH laboratory in Kigali, Rwanda for hrHPV DNA testing using the Xpert HPV test (Cepheid, Sunnyvale, CA, USA).[40-45] The Xpert HPV Assay is a new, qualitative, real-time PCR assay for the detection of hrHPV DNA. The Xpert HPV Assay includes simultaneous detection of 14 hrHPV types, hydroxymethylbilane synthase (HMBS), and an internal Probe Check Control (PCC). The 14 targeted hrHPV types are detected in 5 fluorescent channels: 1) HPV16, 2) HPV18 and hrHPV 45 (HPV18/45), 3) HPV31, 33, 35, 52, and 58, 4) HPV51 and HPV59, and 5) HPV39, 56, 66, and 68. HMBS (fluorescent channel 6) verifies specimen adequacy.

Specimens are being mixed and a 1-mL pre-aliquot is being removed using a disposable pipette and placed in the testing cartridge per the manufacturer's instructions. Unsatisfactory results due to insufficient cellular content are being re-tested. If the second test is also unsatisfactory, the final result are being recorded as unsatisfactory but women with unsatisfactory results are being referred to colposcopy for safety.

HPV Viral Methylation—We will conduct a retrospective analysis of HPV viral methylation and its association with CIN2+. To identify single hrHPV type infections, we will select single-channel positives from the Xpert HPV assay. For those that are hrHPV positive for a channel other than HPV16, which is detected singly, we will test them to identify the single type infections using a standard protocol for PCR amplification using MY09/11 L1 consensus primers and hrHPV genotype detection using dot-hybridization for 39 individual type-specific probes and a mixture of probes for 10 other uncommon hrHPV types as previously described.[46;47] To isolate the DNA, ThinPrep specimens (1.5 mL) will be pelleted, re-suspended in STM, digested with Proteinase K, precipitated overnight in ammonium acetate ethanol at -20°C, washed, and suspended and stored in TE buffer.

The isolated DNA then will undergo bisulfite conversion.[48] Following bisulfite conversion and DNA purification and de-sulphonation, bisulfite-treated DNA will be used as template for Next-Gen Sequencing (NGS) (HiSeq2000, Illumina, San Diego, CA) using barcoded-type specific primers. Sequences for pads and barcodes are not found in the targeted genomic region. Use of padding and barcodes will enable reads to be identified by amplicon (forward or reverse) or by sample during downstream bioinformatics analysis.[49]

All PCR products for NGS will be pooled (by assay) and a single DNA band containing multiple amplicons from different samples (with unique barcodes) will be isolated from a gel for NGS.[49] Briefly, equal concentrations of each barcoded PCR product (based on PCR band intensity) will be pooled and isolated. Upon confirmation of correct product size, all purified DNA pools will be combined and submitted for library preparation and paired-end 100 base pair Illumina HiSeq2000 sequencing at the Einstein Genomics Core Facility.

Methylation status are being determined in the lab of Dr. Robert Burk at Albert Einstein College of Medicine (USA). Prior to determination of methylation status, de-multiplexing based on the unique barcodes is being performed using in-house generated scripts to obtain paired-end NGS reads of each sample. Reads are being aligned with hrHPV reference genome sequences by bowtie v0.12.9.[50] Methylation status of each CpG site is then determined by bismark v0.7.7[51] using the default quality score parameter set to Q30, and the formula of the methylation ratio of the number of C read by the number of C+T read.

E6/E7 Oncoprotein Testing—Dry swab specimens, collected at the time of colposcopy, are being tested for individual E6/E7 oncoproteins as previously described[52;53], according to the manufacturer's instructions, at the RMH laboratory in Kigali, Rwanda. The E6/E7 oncoprotein test uses three lateral flow strips to detect 8 hrHPV types whereas the E6 oncoprotein test used a single lateral flow strip to detect 3 hrHPV types.

Analyses: We will evaluate combinations of the above mentioned screening strategies and tests to estimate the clinical performance (e.g., Se, Sp, PPV, and NPV) for the detection of consensus CIN3+ and community CIN2+. A log binomial model using generalized estimating equations will be used to take into account correlation between different tests from the same subject. Note while these models will be developed for the estimation and comparison of performance for two tests, the model can be extended to allow more than two tests by including more indicator variables for test type.

Some analyses of biomarkers, such as viral methylation will be restricted to hrHPV-positives. Comparisons of hrHPV viral methylation to other triage biomarkers will be restricted to the subset that gets tested for viral methylation as described.

Sample size calculations: We are basing our sample size on the ability to detect modest but minimally important differences in Se of 15%. We conservatively assume that the population prevalence of CIN3+ is 2% based on our past study in Rwanda.[33;35] We will enroll and have complete follow-up of at least 5,000 HIV[+] women. A sample size of 5,000 HIV[+] women with completed follow-up of the screen positives will yield 100 cases of CIN3+, which will have at least 80% power (α=5%) to crudely detect a 15% difference in Se between two screening strategies for a range of 10%-25% discordance. With this sample size of 5,000 women, 4,900 will not have CIN3+; we will also have at least 90% power (α=5%) to detect a difference in Sp of 3% for discordance up to 40%. Finally, we will have 80% power (α=5%) to crudely detect an 8%, 10%, or 11% difference in PPV if the reference PPV is 10%, 20%, or 30%, respectively.[54]

Cost Effectiveness: We will conduct assessments of the costs and cost-effectiveness of the different combinations of screening and triage tests, i.e., algorithms, as well as those of the entire community-based screening "system." Costs measurement will be conducted using a micro-costing (ingredients) approach in which resource use throughout each step in the screening process will be tracked and unit costs for each of the resources will be applied to generate an average screening cost per woman to be compared against what the estimated costs are for a possible program based on hrHPV screening and VIA triage or VIA screening. For estimating costs of the screening system and scale-up of screening to 100,000 women in a month, analyses will distinguish financial costs, which reflect actual expenditures of the program, from economic costs, including the value of donated and shared resources to more fully assess opportunity costs. Projections on budget impact and economic cost implications over time will be made under varying assumptions of screening uptake, follow-up compliance, and scenarios of changing disease burden.

Clinical outcomes will include true positive, true negative, false negative, and false positive test results, number of colposcopies, incident cancer, and cancer death. Cost-effectiveness will be measured as cost/CIN2+ detected, cost/CIN3+ detected, cost/invasive cancer prevented, cost/cancer death prevented, cost/life-year saved, and cost/quality-adjusted life year (QALY) saved; in addition, we will calculate harm/benefit ratios, using varying definitions of harms (colposcopies, false positive results) to benefits (cancers prevented, deaths prevented, life years and QALYs saved). Costs and effectiveness will be discounted at a 3% annual rate, with the rate varied from 0-5% in sensitivity analysis. For assessment of value-of-information (VOI), we will use net monetary benefits (NMB), defined as a function of the willingness-to-pay threshold (WTP) for different costs and outcomes as: $NMB=(WTP * Effectiveness) - Costs$.

Patient and Public Involvement

There was no patient engagement in the development or design of the study, recruitment, and the conduct of the study. Participants are receiving their results directly since it is related to their care. As this was not a randomized controlled trial, the burden of the intervention was not assessed by patients themselves. There were no patient advisors to acknowledge.

Ethics and Dissemination

Ethics: This study protocol was reviewed and approved by the Rwanda National Ethics Committee (RNEC) as well as the Institutional Review Board for human subjects research at Albert Einstein College of Medicine.

Confidentiality measures and protection against potential risks: The risks for those participating in our study include:

- Collection of Pap specimens/cervical swabs involves a modest risk of bleeding which is typically very limited when it occurs. Testing positive for any test may cause psychological distress (anxiety).
- Colposcopy and excisional treatments induce vaginal bleeding and may incur pain, infection, and short-term psychological distress (anxiety). A diagnosis of CIN2 or more severe may cause psychological distress (anxiety). A diagnosis of ICC may cause severe psychological distress.
- Questions in the questionnaire, regarding sexual behavior and other matters of a personal nature, may cause anxiety and embarrassment. Participants are advised that they are free not to answer specific questions.
- There is also the risk of psycho-social stress which could occur if there was inadvertent disclosure of confidential medical or other personal information.

Protection against the risk of inadvertent disclosure of confidential information is being addressed by the standard procedures at the Rwandan study site, including: (i) storing completed paper copies of questionnaires and other hard copy information (described above), identified by study number only, in a filing system separate from the name-address file of participants in the study; and (ii) only the designated local personnel have access to cross-reference the files; (iii) all paper files, including consent forms, are being maintained in locked cabinets in locked rooms, with access restricted to specific research personnel.

In addition, we will include the following security measures to protect the data:

- Controlled access to project data;

- A tracking system for data forms and activities;
- Date and time of stamping of all data records with electronic signatures;
- Audit trails to track all changes made to data records; and
- Data kept on password-protected computers and in locked rooms.

Potential Patient Benefits: There are potential direct benefits to study participants. HIV[+] women participating in the study are at very high risk of ICC. They are being rigorously screened and evaluated, more effectively than the standard of care anywhere in the world. As a result of the study, women with precancer who are at imminent risk of ICC are being diagnosed sooner and treated more effectively than women receiving routine care and thereby more likely averting the development of ICC. Women with cervical cancer are being diagnosed earlier thereby reducing the morbidity and the risk of mortality caused by ICC. Conversely, any pain, bleeding, or stress that might occur related to colposcopy or cervical swab are typically modest and well tolerated.

There are also substantial potential societal benefits related to the study due to its implications to improving cervical-cancer screening practices and guidelines in HIV[+] women – changes in practice which might also benefit the study participants themselves, if and when these changes are enacted. There is a great need to identify more effective and practical methods for cervical-cancer screening for HIV[+] women living in Africa, who are living longer than ever and are therefore at potentially greater risk of ICC.

Dissemination: We plan to publish a series of scientific reports in peer-reviewed scientific journals. As building research capacity in Rwanda is a major goal of this research project, all investigators of the research team are being asked and supported to lead at least one analysis and one manuscript preparation, based on interests and expertise.

In addition, an external advisory panel (EAP) composed of leaders from the Rwanda Ministry of Health, University of Rwanda, and Rwanda medical community has been formed. The responsibilities of the EAP include providing advice on the conduct of the project and interpretation for and dissemination of the study results to Rwandan stakeholders. The latter is important for the adoption of evidence-based best practices for cervical-cancer screening as warranted.

Limitations

There are several limitations to the study that bear mentioning. First, cervical cytology is not being included in the study. There is limited cervical cytology services available locally and of unknown quality and it is unlikely that cytology will be widely available in Rwanda, making its inclusion as a comparator test of limited value. Moreover, there are significant costs and logistical challenges in shipping PreservCyt specimens to and having cytology slides made and read in the U.S. Second, we are not conducting colposcopy and taking biopsies in screen-negative women, which would have allowed us to estimate absolute clinical performance. The burden of sending screen-negative women to colposcopy is deemed too great and it is impractical to send a sufficient numbers of screen-negative women to colposcopy to accurately estimate the false-negative disease (CIN3+) fraction. Thus, only relative clinical performance of the screening tests will be estimated from this study.

Contributor Statement:

KA, PEC, JCD, AA, and JDS conceived the original concept of the study and the interventions. GM, KA, JDS, and PEC drafted the protocol. PEC performed the sample size calculations, and PEC and GM will lead analysis of the results. GM, JDS, and Athanase Munyaneza are supporting patient recruitment. GM, TR, and Athanase Munyaneza are providing clinical care for patients. Anthere Murangwa and LM oversee laboratory testing, and TZ and TH are responsible for pathology. PM and LM oversee and administer the study activities at the clinical site in Rwanda. All authors (GM, JCD, TR, JDS, Athanase Munyaneza, Anthere Murangwa, TZ, TH, PM, AA, LM, KA, and PEC) contributed to the scientific design of the study and the protocol development, are involved in the implementation of the project, and have read and approved the final manuscript.

Figure Legends:

Figure 1. Study Design

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Table 1: Recruitment sites and estimated eligible population.

Province	Site	Type of site	Potential participants per site (approximate)
Kigali	Cor-unum HC	Public Health Center	1,405
Kigali	Kimironko HC	Public Health Center	1,227
Kigali	Rwampara HC	Public Referral Hospital	1,098
Kigali	Kicukiro HC	Public Health Center	1,054
Kigali	Kacyiru HC	Public Health Center	905
Kigali	Gikondo HC	Public Health Center	925
Kigali	Rwanda Military Hospital	Public Referral Hospital	300
Kigali	WEACTx for Hope	Private HIV Clinic	500
Kigali	Busanza HC	Public Health Center	100
Kigali	Nyarugunga HC	Public Health Center	100
West	Gisenyi DH	Public District Hospital	810
Total			8,424

Figure 1. Study diagram

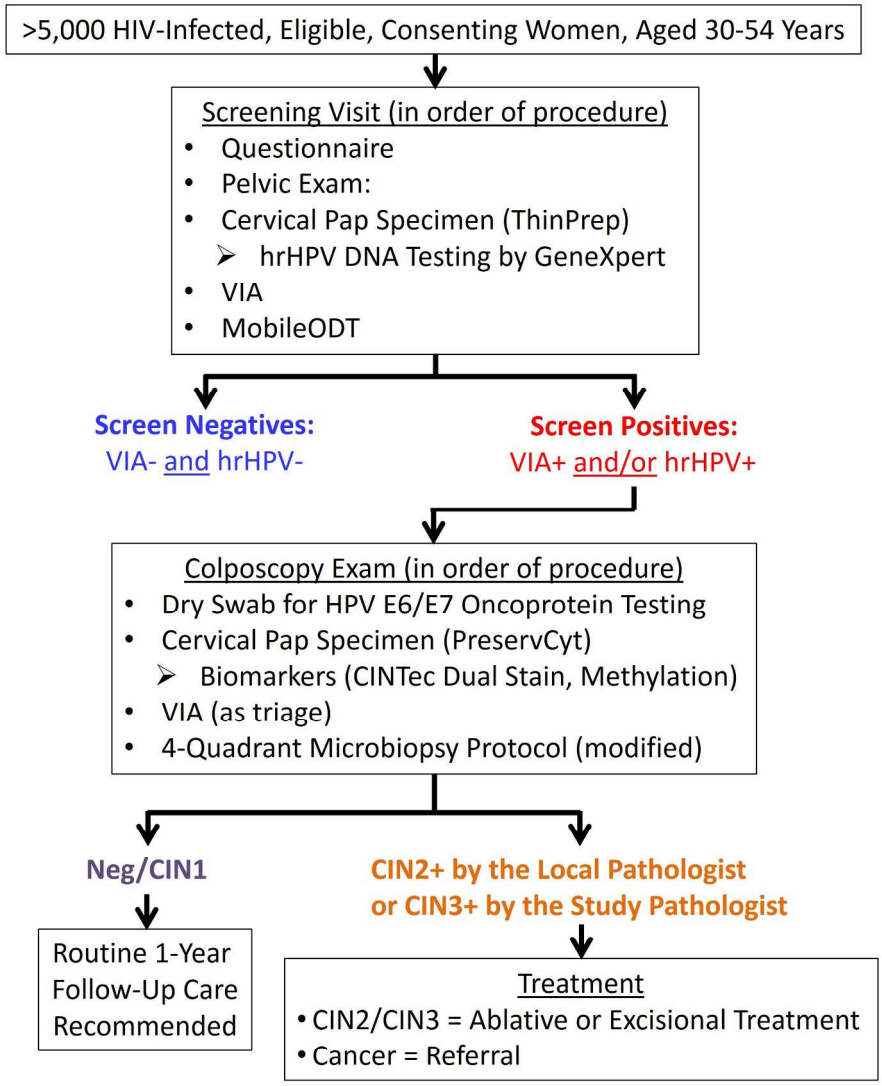

Figure 1. Study Design

190x254mm (300 x 300 DPI)

Appendix I. Study Questionnaire

A. Socio-demographics

1. **What is your date of birth? D D / M M / Y Y Y Y (enter all 0 if not remembered) (If date of birth or year of birth is given, skip to Question 3)**
2. **What is your age?** __ __ (Years)
3. **What is your marital status?**

☐ Married/Cohabiting
☐ Divorced
☐ Widowed
☐ Separated
☐ Single
☐ Choose not to answer

4. **Do you live in Kigali?**

☐ Yes
☐ No {Skip to Question A5}
☐ Choose not to answer {Skip to Question A5}

5. **In what area of Kigali City Province do you live?**

☐ Nyarugenge
☐ Kicukiro
☐ Gasabo urban (e.g., Kacyiru, Kimironko, Kinyinya, etc.)
☐ Gasabo rural (e.g., Nduba, Jabana, Gikomero, etc.)
☐ Do not know
☐ Choose not to answer

6. **What is your household income per month in FRW?**

☐ < 5,000
☐ 5,000 – 9,999
☐ 10,000 – 34,999
☐ 35,000 – 59,999

☐ 60,000 – 84,999
☐ 85,000 – 109,999
☐ 110,000 – 134,999
☐ 135,000 – 269,999
☐ 270,000 or more

7. **How many people live in your household?** __ __ {Enter 00 if refuse to answer}

8. **What is your occupation?**

☐ Employed by government, another institution, or company
☐ Self-employed (Small and medium enterprises)
☐ Self-employed (High income earnings)
☐ Farming (peasants)
☐ Unemployed/Does not work
☐ Other (specify) _____
☐ Choose not to answer {Skip to Question B1}

{The following questions are sensitive and personal in nature. Your answers will be kept confidential. You may choose not to answer certain questions. Answering any question is voluntary}

B. <u>Sexual Behaviors</u>

1. **How old were you when you first had sex?** __ __ (Years) {Enter 00 if refuse to answer}

2. **How many sexual partners have you had in your lifetime?**

☐ No Partners (Ineligible)
☐ 1 Partner
☐ 2-3 Partners
☐ 4-5 Partners
☐ 6-9 Partners
☐ 10 or more partners
☐ Choose not to answer

3. **How many sexual partners have you had in the last 6 months?**

☐ No Partners

☐ 1 Partner

☐ 2 or more Partners

☐ Choose not to answer

C. Parity

1. **What age did you have your first child? __ __** (Years) {Enter 00 if refuse to answer, Enter 99 if never pregnant} (if 00 or 99, skip to Question D1)

2. **How many live births have you had in your lifetime? __ __**

3. **Have you given birth in the last year?**

☐ Yes

☐ No

☐ Choose not to answer

D. Tobacco Use

1. **Have you ever smoked cigarettes?**

☐ Yes

☐ No (skip to Question D3)

☐ Choose not to answer (skip to Question D3)

2. **Do you currently smoke cigarettes?**

☐ Yes

☐ No

☐ Choose not to answer

3. **Have you ever chewed/used tobacco orally (Ubugoro)?**

☐ Yes

☐ No (skip to Question D5)

☐ Choose not to answer (skip to Question D5)

4. **Are you currently chewing/using tobacco orally (Ubugoro)?**

☐ Yes

☐ No

☐ Choose not to answer

5. Have you ever chewed/used tobacco orally (Tobacco leaves-Igikamba)?

☐ Yes
☐ No (skip to Question E1)
☐ Choose not to answer (skip to Question E1)

6. Are you currently chewing/using tobacco orally (Tobacco leaves-Igikamba)?

☐ Yes
☐ No
☐ Choose not to answer

E. Contraceptive Use

1. Have you ever used oral contraceptives?

☐ Yes
☐ No (Skip to Question E3)
☐ Choose not to answer (Skip to Question E3)

2. Do you currently use oral contraceptives?

☐ Yes
☐ No
☐ Choose not to answer

3. Have you ever used Depo Provera (contraceptive)?

☐ Yes
☐ No (Skip to Question E5)
☐ Choose not to answer (Skip to Question E5)

4. Do you currently use Depo Provera (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

5. Have you ever used Jadell (contraceptive)?

☐ Yes
☐ No (Skip to Question E7)

☐ Choose not to answer (Skip to Question E7)

6. Do you currently use Jadell (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

7. Have you ever used IUD (contraceptive)?

☐ Yes
☐ No (Skip to Question E9)
☐ Choose not to answer (Skip to Question E9)

8. Do you currently use IUD (contraceptive)?

☐ Yes
☐ No
☐ Choose not to answer

9. Do use condoms as a contraceptive?

☐ Yes
☐ No (skip to Question F1)
☐ Choose not to answer (skip to Question F1)

10. Have you used condoms as a contraceptive in the last 6 months?

☐ Yes
☐ No
☐ Choose not to answer

F. Infections

1. Have you ever had Malaria

☐ Yes
☐ No (skip to Question F8)
☐ Choose not to answer (skip to Question F8)

2. How many times have you had Malaria? __ __

3. **What was the year that you last had Malaria? Y Y Y Y**

4. **Have you had your Malaria treated using drugs?**

☐ Yes
☐ No
☐ Choose not to answer

5. **If Yes, how was it treated?**

☐ Treated only with traditional medicine
☐ Treated only with drugs (e.g. Coartem)
☐ Treated with both (traditional and modern)
☐ Not treated
☐ Choose not to answer

6. **Was your last episode of Malaria treated using drugs?**

☐ Yes
☐ No
☐ Choose not to answer

7. **If Yes, how was it treated?**

☐ Treated only with traditional medicine
☐ Treated only with drugs (e.g. Coartem)
☐ Treated with both (traditional and modern)
☐ Not treated
☐ Choose not to answer

8. **Have you ever had Tuberculosis (TB)?**

☐ Yes
☐ No (skip to end)
☐ Choose not to answer (skip to end)

9. **If Yes, how many times have you had TB in your lifetime? __ __**

10. **What year did you last have Tuberculosis (TB)? Y Y Y Y**

END OF QUESTIONNAIRE