UTILITY OF P16^{INK4A}/KI-67 IMMUNOSTAIN AS AN ADJUNCT TO CONVENTIONAL PAP SMEAR FOR THE DIAGNOSIS OF CERVICAL LESIONS AMONG WOMEN LIVING WITH HIV AT COMPREHENSIVE CARE CLINIC INKNH, KENYA

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DECLARATION FORM

I hereby declare that this dissertation is my original work under the guidance of the supervisors and has not been submitted to the University of Nairobi or any other higher learning institution.

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DEDICATION

I dedicate this work first and foremost, to my Lord Jesus Christ for this rare opportunity in my life. To my wife Mabel Mwila Ngoma, my wonderful children George Dalitso (MANE-G) and Febian Junior, for being there for me. I further dedicate this work to the women in Kenya, who have been always ready to fight cancer of the cervix.

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ABBREVIATIONS

AIDS:	Acquired Immune Deficiency Syndrome
ASCUS:	Atypical Squamous Cells of Undetermined Significance
ASC-H:	Atypical Squamous Cells-cannot exclude HSIL
CCC:	Comprehensive Care Clinic
CDKN:	Cyclin Dependent Kinase Inhibitor
CDK:	Cyclin Dependent Kinase
DNA:	Deoxyribonucleic Acid
DPX:	Diestrene Plasticizer Xylene
EA:	Eosin Azure
ER:	Early Region
H&E:	Hematoxylin and Eosin
HSIL:	High Grade Squamous Intraepithelial lesion
HPV:	Human Papilloma Virus
ICC:	Immunocytochemistry
IR:	Incidence Rate
KNH:	Kenyatta National Hospital
KMLTTB:	Kenya Medical Laboratory Technician and Technologist Board
LSIL:	Low Grade Squamous Intraepithelial lesion
LCR:	Late Control Region
LR:	Late Region
MOH:	Ministry of Health
OG:	Orange G
ORF:	Open Reading Frames
PAP:	Papanicolaou
PI:	Principal Investigator
PPV:	Positive Predictive Value
pRB:	Phosphorylate Retinoblastoma Gene
QA:	Quality Assurance
SOP:	Standard Operating Procedure

UoN:	University of Nairobi
URR:	Upstream Regulatory Region
VIA:	Visual Inspection with Acetic Acid
VILI:	Visual Inspection with Lugol’s Iodine

ABSTRACT

Background: The current cervical cancer screening methods pose limitations. Since use of biomarkers has shown efficiency, there is need for a disease specific biomarker such as the $p16^{INK4a}$ /ki-67 Immunocytochemical (ICC) stain. Thus, the study focus was on evaluating the diagnostic utility of $p16^{INK4a}$ /Ki-67 ICC stain as an adjunct to conventional smear in cervical diagnosis.

Objective: The aim of the study was to determine the diagnostic utility of $p16^{INK4a}$/ki-67 ICC stain as an adjunct to conventional Pap smear in the diagnosis of cervical lesions among women living with HIV at the Comprehensive Care Clinic (CCC) in KNH, Kenya.

Methodology: This was a descriptive cross sectional study which was undertaken at CCC at KNH for the duration of three months. A total of 61 women living with HIV who met the inclusion criteria were screened for cervical lesions. Only 35 (57.4% - response rate) of them were included in the comparative analysis. Twenty six (26) of the study participants were not included in the final analysis since most material was lost during cell block preparation. Ethical clearance was obtained from KNH/UON ethics and research committee. Consecutive cervical cytobrush liquid based cervical samples were stained for Pap method and a cell block was prepared from the residues for $p16^{INK4a}$/Ki-67 ICC stain. Analysis was done using SPSS software for Windows v.22, at a significance level of $P \leq 0.05$.

Results: The mean age of the 35 participant's was 41.1 years (SD ± 10.9). The cytomorphology results by Pap smear were 26 (74.3%) were NILM, 4(11.4%) HSIL, 2(5.7%) LSIL, 1 (2.9%) each of the ASCUS, ASC-H and SCC ($P = 0.001$). The $p16^{INK4a}$ and Ki-67 combination were 9/35 (25.7%) women tested positive ($P = 0.004$). Using Cohen's kappa statistics, there was moderate and significant agreement of the combined $p16^{INK4a}$ and Ki-67 with cytology (Kappa = 0.481; $P = 0.001$) and By Spearman test ($r = 0.95$ and $P = 0.001$).

Conclusions: The combined $p16^{INK4a}$/Ki-67 ICC staining on cell block showed moderate agreement and strongly correlated with cytological results, indicating the usefulness of the biomarker for diagnosis of cervical cancer, especially in cases of ASCUS and ASC-H while improving the diagnostic accuracy of LSIL and HSIL.

Recommendation: The use of $p16^{INK4a}$/ Ki-67 ICC can be used as a means of analyzing cervical cancer when used in conjunction with conventional cytology, therefore $p16^{INK4a}$/Ki-67 biomarkers should be incorporated into the cervical cancer screening algorithms. However, a larger sample size should be conducted to validate these findings.

1.0 INTRODUCTION

Cases of cervical cancer are on the rise globally. This is the second most common cancer among women worldwide with the highest incidence rate reported in the developing countries despite the availability of screening methods in place. [(1)] Cervical cancer comprises approximately 12% of all cases in women, where 99.7% of the cases are due to the persistent infection with the high risk oncogenic HPV type. [(1)] There is a high association between women living with HIV and the development of premalignant lesions of the cervix; hence cancer of the cervix has been classified as an AIDS defining disease. [(1, 2)] Although cancer of the cervix is preventable, sub-Saharan region has reported the highest burden of the disease, Kenya accounts for 8% to 20% of the cases with a peak age group of 35 to 45 years. This coincides with the age group mostly affected with HIV/AIDS. [(2)]

Cervical cancer screening methods currently in use include, conventional Pap smear, visual inspection with acetic acid (VIA), visual inspection with lugol‘s Iodine (VILI) and molecular HPV DNA methods. [(2)] However, the methods mentioned above have shortfalls. These include limited reproducibility with low sensitivity to detect precancerous lesions in comparison to HPV DNA test which has a high sensitivity but low specificity leading to inaccurate diagnosis. [(3)] The major challenge, however, with molecular HPV tests is that they lack specificity, hence use of HPV tests is unable to differentiate low risk from high risk oncogenic HPV. [(3)] It has been shown that diagnostic tests based on biomarkers have high specificity and sensitivity as compared to molecular HPV tests. Biomarkers such as $p16^{INK4a}$/ki-67, $p16^{INK4a}$ and the Ki-67(MIB-1) have the potential to minimize the errors in cytomorphology interpretation which highly contributes to inter-observer variability. The use of $p16^{INK4a}$/ki-67 dual stain is much more superior to a single biomarker as it increases sensitivity and specificity. Further, the use of cell block specimen to demonstrate $p16^{INK4a}$/ki-67 biomarkers has been cited to improve on cytodiagnosis. [(4, 5)]

Despite the advantages mentioned on the diagnostic utility of the $p16^{INK4a}$/ki-67 biomarker, it appears to be underutilized in the health facilities in Kenya. [(3, 4, 5)] Therefore, the dissertation intended to evaluate the diagnostic utility of $p16^{INK4a}$/ Ki-67 dual stain as a suitable adjunct to conventional cytology in the diagnosis of uterine cervical pre-neoplastic lesion on a cell block.

2.0 LITERATURE REVIEW

2.1 Epidemiology of cervical cancer

2.1.1 Global

Cervical cancer is an important public health problem, which ranks second among women worldwide. In the developing world, it is the second most common cancer among women especially those infected with HIV/AIDS. It is estimated that 471, 000 new cases were reported with an approximately 233,000 deaths in the year 2000. [1, 5] Over 85% of the global burden of cervical cancer occurs in Third World countries, these include Latin America, Caribbean and the sub-Saharan region included. In the western world, such as North America and Western Europe, the incidence rates (IR) are as low as 6.6 to 7.3 per 100,000 populations, while New Zealand and Western Asia recording the lowest IR at 5.5 per 100,000 populations and 4.4 per 100,000 populations respectively. [5] It has been shown that Screening programmes with high quality methods resulted into a significant reduction of cervical lesions. [5]

In a study done in the United States of America, the incidence rates were as low as 2% to 3%, reducing the incidence from 14.8 per 100,000 in 1975 to 6.6 per 100,000 populations in 2006. [2, 5] This however, is not the situation in Sub-Saharan region where the incidence rates of cervical cancer still range from 20% to 36%. [5] The Western World reportedly experienced such high incidence rate in the 1960's and 1970's before they introduced organized screening activities as those currently seen in the sub-Saharan countries. Africa has shown limited progress in cervical cancer screening despite the use of new diagnostic methods. [1, 2, 5]

2.1.2. Africa

According to White et al, (2013), averages of 24% to 34% women die of cervical cancer in the sub-Saharan region, partly due to the high prevalence of HIV cases among the community. [5] Other studies reported similar findings in Zambia, Zimbabwe and South Africa. [4, 5] Currently, cervical cancer screening services offered in the Sub-Saharan region include VIA/VILLI, HPV, Colposcopy, Pap smear and histological method. [6]

2.1.3 Kenya

The Incidence of cervical cancer in Kenya is high. [3, 5] The Ministry of health, Kenya, report indicated that by the year 2008, an estimated of 529,409 new cases occurred and that approximately 274,883 women died. Kenya accounted for 44% of the breast and cervical cancer cases with about 86% of the new cancers cases that mainly occur in Sub-Saharan Africa resulting into 80% to 90% of the deaths. [2, 6] Kenya accounts for 8% to 20% of the cancer of the cervix in its population with a peak age of 35-45 years. This is the most productive age group as well as the most affected women with HIV/AIDS. [7] Therefore, there is need to screen women living with HIV with accurate diagnostic screening tools. Currently in Kenya, methods for screening for cervical cancer include VIA, VILI, Pap smears and HPV tests. However, there is limited information in regard to the use of $p16^{INK4a}$/ Ki-67 dual stain on cell block in screening for cervical uterine lesions. [9] In this study, $p16^{INK4a}$/ Ki-67 was used as an adjunct to conventional Pap smear in an effort to increase the diagnostic accuracy in the detection of cervical lesions.

2.2 Human papillomaviruses (HPV) and transmission

Cervical cancer is caused by high risk oncogenic HPV DNA virus, which is transmitted through unprotected sexual contact. Women who encounter coitus at the tender age are highly vulnerable. The HPV infection is common in healthy women. The immune system can easily encounter and eliminate HPV, however, persistent HR-HPV presence in the cervix in conjunction with other factors can bring about progression to pre-neoplastic lesions. [5, 10] Factors that may influence cervical cancer include: long use of oral contraceptives, smoking, high parity, and HIV/AIDS infection. [10]

The HPV belongs to the family of papillomaviridae, which are over 100 types, these are formed by the 189 papillomavirus types isolated from human beings and differentiated by DNA sequences. [11] The structure of HPV has an enclosed circular double stranded DNA with a genome of almost 8,000 base pairs encoded up to 9 open reading frames. [10, 11] The HPV is a major cause of cervical cancer and intraepithelial neoplasm worldwide. [11] The human papilloma virus is divided into low risk and high risk. The high risk HPV types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82. While the low risk HPV types include 6, 11, 40, 42, 44, 54, 61, 70, 72 and 8. The high risks HPV are associated with pre-cancerous lesions and cancer,

whereas the low risk is mainly found in genital warts and other benign lesions. The HPV have access into human beings through any open area of the body or minute abrasions and therefore infect keratinocytes in the skin or the mucous membrane. [11, 12]

2.3 Human papilloma virus (HPV) major transcription structure

The HPVconsists of 7200 to 8000 base pairs and is organized into three functional sites. The Functional sites have different activities. The late control region (LCR) is divided into the upstream regulatory region (URR), the early region (ER) and the late region (LR). The early region which represents 50% of the entire region comprise of E1, E2, E3, E4, E5, E6 and E7, 40% of the genome consist of the late region (LR) and 10% is represented by the Genomic regulatory region (GRR). [10, 11, 12] The DNA fragments are encoded into the single DNA described as ORF which are classified as E1, E2, E3, E4, E6 and E7. [11, 12]

The E1 proteins are involved in the viral DNA replication, whereas E2 proteins are responsible for the initiation of transcription. [11, 12] The structural framework of keratin is disturbed by the E4 proteins thus associated with the formation of koilocytic cells. The HPV koilocytic changes manifest in mature squamous epithelium. During the process of carcinogenesis, the E4 binds to the keratin cytoskeleton and virus assemble and are released in the process. [11, 12]

The E5 protein which is involved in DNA replication develops mechanism in the infected cell to escape recognition from the human immune system. [11, 12] The E6 and E7 proteins play a major role in HPV based carcinogenesis. They impair the cycle regulation and cell maturation. The oncogenic E6 from high risk HPV binds to the p53 protein causing proteolytic degradation. [12] It promotes the proliferation (E7) of infected cells leading to their resistance to apoptosis. However, the E6 protein from low risk HPV do not bind to p53, therefore have no effect on p53. [13] Further, the E6 from the high risk group can stimulate the catalytic subunit of the telomerase which adds hexamer repeats to the telomeric ends of the chromosome. Such a situation may lead to predisposal to long term infection hence the latency observed from time of exposure in association to outcome of visible changes, this may take as long as 10 to 20 years before HPV can manifest into cervical cancer in women. [12, 13]

Below is a diagram showing the structure of the Human papilloma virus.

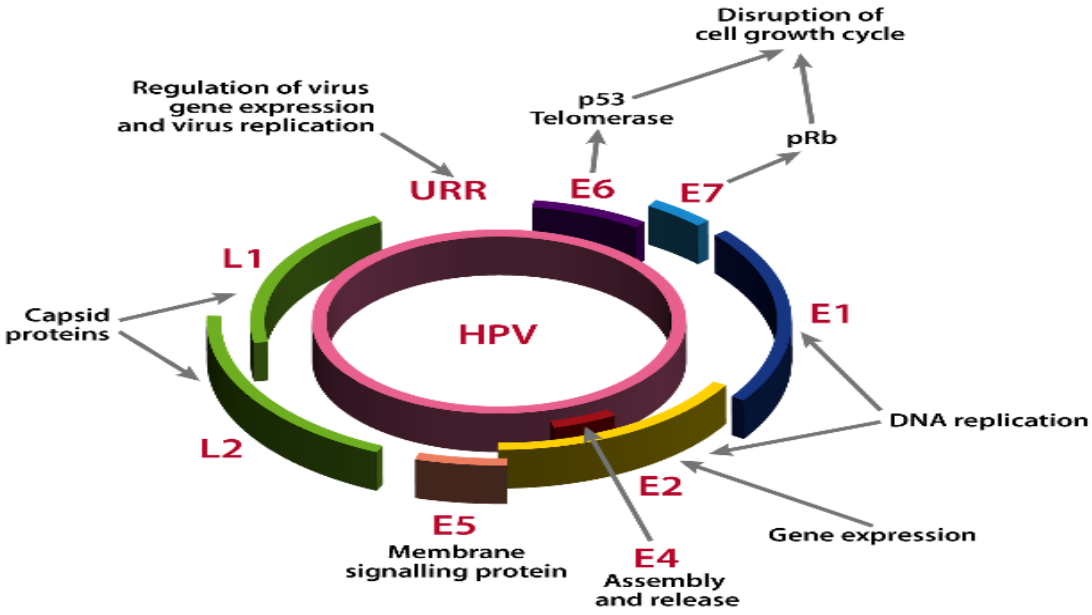


Figure 1: HPV pathogenic proteins sites. (*Diagram adopted from John B et al,* ***Virology principles and applications****, 2007: Saunders, London*).

The E7 protein mainly maintains the differentiation in the S-phase of the cell cycle, where it binds to the retinoblastoma (pRB) and a suppressor protein thereby disrupting the association between pRB growth factors. [12, 13] In the current study, diagnosis of cervical cancer was based on demonstration of HPV infected cells expressing E6 and E7 oncogenic HPV virus using the $p16^{INK4a}$ and Ki-67 biomarkers.

2.4 Human papilloma virus (HPV) and cervical pre-neoplasia

The HPV is epitheliophilic, therefore, when it gains access to the surface lining tissue, it replicates solely in keratinocytes of the stratified squamous epithelium of the skin and the mucosal areas. The Human papilloma virus is mainly a sexually transmitted infection, therefore individuals with multiple sexual partners are at increased risk of acquiring the virus. [13].

The cycle is initiated by exposure of the stratified epithelium, mainly of the basal cell line to the viral particles. The human papilloma virus particles will bind to the host cell and penetrate through minute abrasion usually formed during coitus. The entry of the HPV viral genome establishes itself within the basal epithelium. [12, 13] While a stable pool of infected cells is maintained in this region, the infected cells may lose cohesion and migrate into the suprabasal layer. In the suprabasal layer, cells exit the cell cycle and begin to differentiate. [13]

In HPV infected keratinocytes, the ability to restrain the cell-cycle progression is lost by stimulating the G-1 to the S-phase through expression of E6 and E7 genes. The HPV takes 10 to 20 years before it progresses into the pre-neoplastic lesion. The manifestation may be clinically asymptomatic. [13]

2.5 Human immunodeficiency (HIV) and cervical pre-neoplasia

The HIV leads to chronic immunosuppression that propagates the HPV, the etiologic agent for cervical cancer. There is a high prevalence of HPV infection among HIV infected women, ranging between 30% - 56%. [12, 13] The high risk HPV types 16 and 18 account for 56.6% and 18% respectively of the invasive cervical cancers worldwide. Meta-analysis carried out on the correlation between HIV, HPV and cervical pre-neoplasia have shown that HIV infected women have a higher prevalence of HPV infection and cervical intraepithelial neoplasia (CIN) that tend to increase in proportion with the degree of immunodeficiency. [13]

2.6 Screening for cervical cancer

It has been established that women living with HIV have an increased risk of developing cervical cancer, therefore require frequent screening. [13] A consensus on the method performance for primary screening recommends that Sensitivity (SE) and specificity (SP) of a screening method should exceed 90% and that the test must detect ASCUS, ASC-H, LSIL and HSIL. [14] The screening methods currently in Kenya such as VIA/VILI, Pap test and HPV DNA tests have exhibited limiting factors. Since HPV DNA molecular tests are generally expensive and technically challenging than immunocytochemical stains, ICC have the potential to be used as screening tool. [14]

2.7 Prevention of cervical cancer

Cervical cancer is preventable if measures are put in place way back before one acquires the causative agent. The World Health Organization (WHO) recommends that girls aged between 9 and 13 years receive the HPV vaccination before they encounter the virus, as at this age, most girls would not have begun sexual activity. [5, 14] Since vaccination is effective if administered before a person is infected, primary preventive measures have a major impact on the reduction of incidences of cervical cancer. [7, 14] Prevention programs include HPV vaccine, male circumcision, condoms use, abstinence and faithfulness in partners. Currently in Kenya, the HPV vaccination is available, but it is limited to girls and only to those who are below the age of 12 years. Further, secondary preventive measures include screening, diagnosis and treatment at tertiary level of cervical cancer management. [5, 14]

2.8 Classification of cervical neoplastic lesions

Solomon,*et al,* described the detailed classification of the cervical non-neoplastic lesions and neoplastic lesions, in the Bethesda 2001 report. This was developed in order to provide a standard system of reporting cervical Pap smear. [15] The 2001 Bethesda reporting system was categorized into three basic groups which included the description of the specimen adequacy, general categorization and the interpretation of results. The table below shows the general categorization of cervical lesions in the Bethesda system of reporting. [15]

Table 1: Categorization of Cervical cancer cytology by the Bethesda system 2001.

	Lesion	**Cells Affected**	**Cytological Features**
1.	ASCUS (Atypical Squamous cells of undetermined Significance)	Mature superficial and intermediate squamous cells	Minimal pleomorphism, Nuclear enlargement, Slight increase in Nuclear/ Cytoplasm ratio, Pseudokoilocyte, binucleation, Occasional parakeratosis.
2.	LSIL (Low Grade squamous intraepithelial Lesion)	Mature superficial and intermediate squamous cells	Pleomorphism, >3 nuclear enlargement, increase in N/C ratio, coarse granular hyperchromasia, marked nuclear irregularity, koilocytic cytoplasm, Binucleation, no nucleoli.
3.	ASC-H	Immature Parabasal sized cells	Mild pleomorphism, high N/C ratio, marked hyperplasia, these may mimic atypical repair, HSIL.
4.	HSIL	Parabasal and metaplastic cells	Marked pleomorphism, high N/C ration and Nuclear enlargement, marked hyperchromasia and nuclear irregularity,Presence of HCG. No nucleoli.
	SCC (Squamous cell carcinoma)	Immature and mature cells	Marked pleomorphism, nuclear enlargement, hyperchromasia and irregularity. Dyskeratocyte, prominent nucleoli.
5.	Glandular cell abnormalities	Endocervical, Endomen	Adenocarcinoma

2.9 Screening and Diagnosis

The standard practice worldwide in cervical cancer screening is that there is a variation in the approach of screening depending on commitment and policies of governments. A majority of developed countries such as USA and Canada have organized screening programmes, unlike in the sub-Saharan region. [16] A report in the IARC, et al, 2013 on the global screening programmes, highlighted diverse approaches intertwined with a number of successes and challenges. [5, 16] It recommends the use of liquid based cytology, molecular HPV testing and use of Biomarkers in screening algorithms. It is reported that 89% of the Canadian women aged 20 to 69 years have had more than one Pap test in their life time, which is not the situation in the Sub-Saharan region. [4, 16]

The Sub-SaharanAfrica region lacks organized screening programmes. [16] Unfortunately this region has the highest burden of cervical cancer. [5, 17] A study done in Zimbabwe notes that while Africa requires a cheaper and easy method of screening, there is little or no government policy to drive the agenda. [18] Claeys et al, 2003 notes that, while cervical cancer screening services have well been integrated into family planning service in Kenya, the venture is a huge challenge in that only few women, approximately 43.5% can access these services with the screening services currently offered in Kenya. [3, 19]

A study done by Arbyn et al, 2008, that compared VIA (79.2%), VILI (91.2%), Cytology (57%) and HPV (62%) diagnostic tests, highlighted the limitations in the screening tools currently in use. [18, 19] It was noted that VILI had the highest sensitivity, but low specificity, while HPV had the highest specificity of 94% as compared to VIA (87.7%), VILI (84.5%) and cytology 93%. [19] A study done to demonstrate sensitivity and specificity on convention cytology, indicated that Pap smear had a sensitivity of 11-99% with a specificity of 14-97%, which had a very wide variation leading to high possibilitiesof inaccurate misdiagnosis of cervical cancer. [19] A study done by Benevolent et al, 2008, on the role of $p16^{INK4a}$ biomarkers compared to HPV and histological diagnosis, indicates that biomarkers are far much superior to the usual screening methods currently in use. The Authors, however, point out gray areas in the use of biomarkers, such as sporadic immunostaining and lack of standardization. [20, 21] This study was aimed at

evaluating the diagnostic utility of $p16^{INK4a}$/Ki-67 dual stain as a method of choice to enhance accuracy in screening for cervical cancer in women living with HIV at CCC, KNH.

2.10. Clinical Significance of Biomarkers in HPV Diagnosis

The use of immunnobiomarker to screen for cervical cancer could enhance the detection of ASCUS, ASC-H, LSIL and cases of HSIL. Biomarkers have been in use for some time now with new methods coming onto the market. [22] These include $p16^{INK4a}$, [20, 25, 26] Ki-67 (MIB-1), ProEx C, L1, p63/p73, phosphatidylinositol 3-kinase gene, PIK3CA, and telomerase biomarkers. These biomarkers can be demonstrated on Liquid based cytology, conventional smear or cell block preparation. [27, 29, 30] The majority of HPV infection is transient and testing using molecular HPV DNA tests may not discriminate between a transient and a transforming infection, this challenge can be intercepted by use of biomarkers. [23, 24]

2.10.1 Protein $p16^{INK4a}$ biomarker

The $p16^{INK4a}$ protein is derived from the host $p16^{INK4a}$ /CDKN2A tumor suppressor gene, found at chromosome 9. [6, 31] The cytoplasmic antigen $p16^{INK4a}$ may have diverse nomenclature, such as $p16^{INK4a}$, Cyclin-dependent kinase inhibitor 2A (CDKN-A), CDKN2, CDK 4 inhibitor and Multiple suppressor 1 (MTSI). In humans it has been identified as a biomarker for transforming HPV infection and therefore can be used as a surrogate marker of HR-HPV infection. [14, 31] In the absence of HPV, p16 ^{INK4a} blocks the activityof the cyclin-dependent kinases CDK4/6, resulting in greater binding of pRB to the transcription factor E2F, thus down-regulating progression through the G1-S-phase transition checkpoint of the cell cycle. The unbound E2 acts in a negative feedback loop with pRB. [14, 32] When HR-HPV infects the host cell, the viral oncoprotein E7 binds and inactivates pRB to release E2F. This promotes cell cycle progression, a molecular switch that is usually activated by CDK4/6. [32] The p16 ^{INK4a} induced feedback loop is thus lost and $p16^{INK4}$ is over-expressed in cells. This results into accumulation of the protein in the nucleus and cytoplasm of affected cells which can be detected by Immunocytochemical stains. [14, 33] In cytological samples, $p16^{INK4a}$ positive cells will stain brown in the cytoplasm while the nucleus remains unstained. [31, 33]

Majority of women with pre-neoplastic lesion express $p16^{INK4a}$ and Ki-67 biomarkers. The presence of these biomarkers can be demonstrated with immunocytochemical stains to demonstrate specific disease characteristics. [34] The demonstration of $p16^{INK4}$ in a cell strongly suggest presence of HSIL by 97.5% or otherwise CIN2 and CIN3 categories, but less sensitive for LSIL (CIN 1) by 74%. [22, 34] The $p16^{INK4}$ biomarker is therefore highly useful in differentiating pre-neoplastic lesion from benign mimics such as immature squamous metaplastic cell, atrophy and reparative changes. [35]

2.10.2 The Ki-67 protein

The Ki-67 (MIB-1) is a nuclear antigen proliferative biomarker which is highly expressed in HPV infected mature squamous cells. These usually manifest in a proliferating cell, hence they are closely linked to tumors of the cervix. [14, 33] The detection of Ki-67 (MIB-1) nuclear biomarker is useful in the demonstration of LSIL. Biomarkers can help to predict the prognosis of which cases of ASCUS and LSIL will progress to HSIL and Invasive cases. [35]

2.11 Use of a combination of $p16^{INK4a}$ and Ki-67 Biomarkers

The use of a combination of $p16^{INK4a}$ and Ki-67 Biomarkers, such as the CINtec plus Cytology dual kit, is of an advantage. The immunocytochemical assay provides simultaneous qualitative detection of the $P16^{INK4a}$ and Ki-67 proteinon the cervical cytology preparations. [36] Several studies done on the use of dual biomarkers reported that a specimen prepared from a cell block would be ideal for immunocytochemical stains unlike the conventional Pap smear. Cell block specimens are easy to store and can be used on other ancillary studies. [37-40] Biomarkers can be used to triage patients with less aggressive or other high risk HPV genotypes and identify women with transforming lesions which are positive for $p16^{INK4a}$ and Ki-67. [24, 41] The advantage in the use of biomarkers in the laboratory is that they are manual methods of staining which can be applied in most basic laboratory set ups and easy to interpret on conventional smear. This can help in the reduction of unnecessary biopsies and ensure patients are treated appropriately. [42, 43]

2.12 Study justification

Despite increased advances in the screening methods for cervical cancer, diagnosisof pre-neoplastic lesions among women living with HIV still poses some challenges.The current screening methods such as VIA/VILI are highly sensitive but with low specificity, whereas Conventional Pap smear have low sensitivity but with high specificity resulting into under diagnosis. While the molecular methods such as HPV DNA testing offer high sensitivity, its specificity for cervical diagnosis is limited. The use of biomarkers such as $p16^{INK4a}$, MIB-1 and BD-ProExhas proven to be very efficient and cheap as opposed to the use of a single biomarker such as $p16^{INK4a}$ which exhibits limitations, such as demonstration of HPV activities only in the cytoplasm. There is a dire need for incorporating a disease specific biomarker such as the dual immunostain $p16^{INK4a}$/ki-67 to differentiate low risk from high risk oncogenic HPV since use of biomarkers on cell block can increase accuracy and interpretation. Immunocytochemical stains are manual methods of staining that most basic laboratories across Kenya can make use of, but this is not happening even in most of the high level health facilities in Kenya. This study focused on evaluating the utility of $p16^{INK4a}$/ki-67 Immunocytochemical dual stain as a suitable adjunct to conventional cytology in women living with HIV.

2.13 Research Question

Is $p16^{INK4a}$/ Ki-67 Immunocytochemical stain useful as an adjunct to conventional cytology for screening of uterine cervical dysplasia among women living with HIV?

2.14 Objectives

2.14.1 Broad Objectives

To establish the diagnostic utility of $p16^{INK4a}$/ki-67 ICC stain as an adjunct to Conventional Pap smear in the diagnosis of cervical pre-neoplastic lesion among women living with HIV attending comprehensive care clinic at KNH.

2.14.2 Specific objectives

i. To describe the cytomorphological features of conventional cervical smear.

ii. To detect dysplastic cells expressing biomarkers $p16^{INK4a}$andki-67 ICC on the cervical scrape cell block specimens.

iii. To correlate cytomorphological findings on the Conventional Pap and the $p16^{INK4a}$/ Ki-67 ICC stain expression on the cell block

3.0 METHODOLOGY

3.1 Study design

This was a descriptive cross-sectional study.

3.2 Study site

The study was done at the comprehensive care clinic (CCC) at KNH for the period of three months. The clinic operates from Monday to Friday and opens from 08:00hours to 16:00hours. It serves an average of 60 to 80 female patients per day. The patients are attended by consultant gynecologists and registrars, assisted by nurses who are experienced in collection of cervical samples. While the CCC has the basic facilities for collection of cervical specimens, I provided some essential cervical collecting tools such as cytobrush, universal container and fixatives.

3.3 Study population

This included women living with HIV aged between 18 to 65 years attending CCC in KNH.

3.4 Sampling method

Consecutive sampling method was used until the required sample size was achieved.

3.5 Selection criteria

3.5.1 Inclusion criteria

i. This included women living with HIV aged18 to 65 years attending CCC in KNH.
ii. Women who gave consent.
iii. Women with the history of cancer of the cervix

3.5.2 Exclusion criteria

i. Women who were on menses.
ii. Women who were uncomfortable with the collection procedure.

3.6 Sample size determination

The sample size of the study was calculated using Fischer's formulae, [44] with a prevalence of 20%. [3]

$$n = \frac{Z^2 P (1-p)}{d^2}$$

n= required sample size

Z= Statistic for level of confidence on normal distribution Critical value set at 1.96

This corresponds to 95% confidence interval (CI).

P= Expected prevalence of proportions expected at a particular characteristic=20% [3]

d= degree of precision was set at plus or minus 10%

$$n = \frac{1.96^2 – 0.2 (1- 0.2)}{0.1^2}$$

n = 61 (Minimum sample size)

3.7 Sampling method

3.7.1 Recruitment

Before recruitment and consenting, group counseling was offered by a nurse (counselor). The nurse explained the procedure and the possible expectations. The study risks and benefits where clearly explained to legible participants in the informed consent explanation form (appendix I).

3.7.2 Administration of the questionnaire and Consenting

Those who accepted to participate in the study where attended to in a secluded room for specimen collection. The nurse administered a pre-designed questionnaire and proceeded to consent the client (appendix III). The questionnaire had only identification numbers, while the register or log book was used to capture the name and address of the patient for the purpose of communicating results (appendix II).

3.7.3 Specimen collection, transportation and storage

A cervical sample scraping was collected using a cytobrush sampling device by an experienced nurse, gynecologist or the resident registrars available on that particular time. Before collection of a cervical sample, one frosted slide and a universal sterile container with a balanced salt solution was labeled with the clients study identification number. For conventional cytology, one slide was smeared with cervical material and immediately fixed in 95% alcohol within 15 seconds and left to fix for not less than 15 minutes. After fixation the slides were stored in a slide box file and taken to the laboratory. The rest of the cytobrush tip was snapped off into a sterile universal plastic container containing 14mls of a balanced salt solution (appendix VI). The residue sample was stored at room temperature and transported in a sealed biohazard box to Cytology laboratory, KNH.

3.7.4 Specimen preparation

The slides which were fixed in 95% ethanol were stained with Papanicolaou stain. A cell block was prepared from the residue in the balanced salt solution with cytobroom tip. These were further processed in a tissue processor (appendix VI and VII), where two sections was cut at 3 to 5 micrometers. The sectioning and staining was done by the PI with the help of research assistants, who were qualified Medical laboratory technologist. The two sectioned slides were stained with $p16^{INK4a}$/ki-6ICC stain and Hematoxylin and Eosin stain (appendixVIII and IX).

3.8 Format of reporting and analysis

3.8.1 Conventional Pap smear and cell block

The Conventional Pap smears and H & E cell block were screened by the Principal investigator and reviewed independently by a cytologist and pathologist using the Bethesda system for reporting cervical cytology. Discordant cases were reviewed by a third pathologist as a tie breaker. (Appendix X)

3.8.2 Immunocytochemistry

The $p16^{INK4a}$/ki-67 ICC stained slides were screened by the Principal investigator and reviewed independently by a Cytologist and pathologists for confirmation of $p16^{INK4a}$/ki-67 stained cells. Discrepant findings were evaluated by a third pathologist (appendix XI).

3.8.3 Results Interpretation

The slides were evaluated based on the binary rating score system composed of the ratings "Positive" and "Negative" All the reviewers were blinded to the conventional Pap result to avoid bias (appendix XI).

3.8.4 Positive result for $p16^{INK4a}$/ Ki-67

The $p16^{INK4a}$ immunostain stained the cytoplasmic contents brown and the Ki-67 stained the nucleic and cytoplasmic structures brown. This showed focal, sporadic or a diffuse staining pattern. [36, 37]

3.8.5 Negativeresult for $p16^{INK4a}$/ Ki-67

The epithelial cells without any visibly stained cytoplasmic content or nucleic content were considered negative for $p16^{INK4a}$/Ki-67. [32, 37, 37]

3.9 Variables

3.9.1 Independent variables

These included age, sex.

3.9.2 Dependent variables

These included outcomes from Conventional Pap smear, H &E and $p16^{INK4a}$/ Ki-67 immunostain.

3.10 Biosafety measures

During specimen collection and processing, standard operating procedure were followed. Personal protective equipment such as laboratory coats and gloves were used during sample collection, processing and analysis. Standard biosafety measures were observed and all

procedures done according to the KNH laboratory biosafety and biohazard waste disposal guidelines.

3.11 DATA MANAGEMENT

3.11.1 Data storage

Data was collected and stored in a hard cover register and entered into SPSS version 22 software which was used for data analysis. Information stored in soft copies was pass-word protected against access from any unauthorized persons. The register was kept in lockable cabinets where only the researcher and the supervisors had access in order to maintain confidentiality. The questionnaire was identified by a study identification number. Data collected will be kept for a minimum period of 5 years.

3.11.2 Data presentation

The data was presented in tables, charts, graphs and percentages. Photomicrographs representing these patterns were clearly displayed.

3.11.3 Data analysis

Data was entered in the statistical software SPSS version 22. Descriptive statistics frequency (%), mean, standard deviation and medium (interquartile ranges at 25% and 75%) were used to present the quantitative data. Spearman correlation coefficient was used to correlate $P16^{INK4a}$, Ki-67 and combined $P16^{INK4a}$/Ki67 immunostaining score with cytology. Data analysis was done using SPSS software for Windows v.22, while $P \leq 0.05$ was considered significant analysis using frequencies and proportions were computed. Socio-demographic factors associated with cervical cancer were analysed using chi-square tests. A P-value of $P<0.05$ was considered significant. Descriptive statistics were presented as proportions and in form of tables, charts and graphs, whereas, microphotographs were used to present cytomorphology and $p16^{INK4a}$/ Ki-67 biomarker stains in cytodiagnosis.

3.12 QUALITY ASSURANCE

3.12.1 Pre-analytical

The PI and the research assistants made sure that all questionnaires and necessary forms were filled appropriately by the client and identified each client with a study number, which was used

on all proceeding procedures. Standard prescribed collection tools were used to obtain samples in order to avoid contamination of the specimen. Clients were attended to, one at a time where a qualified nurse, gynecologist or resident registrars collected the cervical specimens using a cytobrush and the PI proceeded with fixation of sample.

3.12.2 Analytical stage

Quality control was done on all the stains before use. The stains were kept covered and daily filtering was done before use. The reagents were stored in a refrigerator at the recommended temperature by the manufacturer. Contamination of slides was avoided by using standard staining rack, while standard operating procedures were used at every stage. The recommended concentration of fixatives was used in every procedure. Internal positive and negative controls were used in combination with external controls. To avoid background staining, cell block and correct antibody dilutions were used as recommended by the manufacturers. All the smears were screened by the principal investigator, and independently confirmed by a cytologist and pathologists.

3.12.3 Post- analytical

To ensure precise interpretation of results all slides were verified before release of the report. A third pathologist was used as a tie breaker in cases where a cytologist disagreed with the pathologist. A 10% of Positive and negative smears, randomly selected, were re-examined by an independent pathologist. Results were communicated to the clinicians for further action based on the Bethesda 2001 recommendations.

3.13 Ethical consideration

Before commencement of the study, ethical clearance was obtained from KNH/UON, Ethics and research Committee. Permission to conduct research in the unit was granted from the Deputy Director Clinical Services, in-charge of CCC and the Cytology laboratory in Kenyatta National Hospital. Permission to use patient's samples for the current and future studies was sought from the participants and ethics committee. To ensure participant's privacy and confidentiality, the collected data was kept in a lockable cabinet where only the researcher and the supervisors had access. Consent form had detailed information on the benefits, rights and possible outcome in

the research. Conventional Pap smear results were communicated to the study participants within seven working days for appropriate management.

3.14 Data dissemination

The data was presented to the Kenyatta National Hospital, Department of Human Pathology and Comprehensive Care Clinic. It will also be published in peer reviewed journals and presented in conferences and seminars.

4.0. RESULTS

4.1 Social Demographic Characteristics of Study Population

A total of 61 women living with HIV who met the inclusion criteria were screened for cervical lesions. Only 35 (57.4% - response rate) of them were included in the comparative analysis. Twenty six (26) of the study participants were not included in the final analysis since most material was lost during cell block preparation. From the 35 participants studied, 25.7% had abnormal lesion detected by Pap smear, whereas 74.3% had normal Pap result. Of the 35 participants a combination of $p16^{INK4a}$ and Ki-67 ICC stain was performed. The mean age of the participants was 41.1 years (SD ± 10.9), median (IQR) 42 (32 to 48) ranging from 19 to 69 years. Majority (51.4%) were married verses 11.4 who were divorced. Most of the participants (62.9%) had been pregnant 1 to 3 times, while 28.6% having had ≥ 4 pregnancies. About 37.1% were currently using barrier (condoms) for their family planning methods while 34.3% were using hormonal family planning methods and intrauterine devices. There were near distribution between the study population having had a previous Pap smear test done (40%) verses (60%) of those who had not undergone the test. The majority (85.7%) of the study population had had between 1 to 2 sexual partners, while 14.3% having had ≥ 3 different sexual partners. Most women (65.7%) in the study population had their age of first pregnancy ≤ 20 years while 25.7% had the age of first pregnancy at 20 years.

Table 3: Summary of participants' demographic profile

Variable	Sample size (N = 35) No	%	chi-square	df	P
Age (Years)					
Mean (± SD)	41.1	(± 10.9)			
Median (IQR)	42	(32 - 48)			
Range	50	(19 - 69)			
15-20	1	2.9			
21-30	4	11.4			
31-40	12	34.3	13.714	4	0.008
41-50	12	34.3			
≥ 51	6	17.1			
Marrital status					
Single	6	17.1			
Married	18	51.4	13.571	3	0.003
Divorced	4	11.4			
Widowed	7	20.0			
Parity					
Mean (± SD)	2.9	(± 1.9)			
Median (IQR)	3	(2 - 4)			
Range	11	(0 - 11)			
Nulliparous	3	8.6			
1 to 3	22	62.9	15.829	2	0.001
≥ 4	10	28.6			
Family planning					
Barrier (Condoms)	13	37.1			
Hormonal (Pills/IUD/Injectable)	12	34.3	0.4	2	0.87
None	10	28.6			
Previous PAP					
Yes	14	40.0	1.4	1	0.311
No	21	60.0			
Sexual partner					
Mean (± SD)	1.9	(± 0.8)			
Median (IQR)	2	(1 - 2)			
Range	3	(1 - 4)			
1-2	30	85.7	17.857	1	0.001
≥ 3	5	14.3			
Age at first pregnancy (Years)					
Mean (± SD)	18.8	(± 7.5)			
Median (IQR)	19	(17 - 210)			
Range	38	(0 - 38)			
≤ 20	3	8.6	18.057	2	0.001
≥ 21	23	65.7			
None	9	25.7			

No - Number; % - Percentage; SD - Standard deviation; IQR - Interquartile range; PAP -
df - Degree of freedom; P - Level of significance; P ≤ 0.05 indicates the relationship is significant

4.2 Clinical history of the study population

The majority (68.6%) of the study population had normal cervical appearance as compared to 31.4% who had eroded cervices, whereas 77.1% of the study population had been using ART for more than four (4), while only 14.3% used ART for 1 to 3 years.

Table 4: Clinical characteristics of the study population

Variable	Sample size (N = 35)		chi-square	df	P
	No	%			
Appearance of the cervix					
Normal	24	68.6	4.829	1	0.028
Erroded	11	31.4			
Years of ART use (Years)					
1 to 3	5	14.3			
≥ 4	27	77.1	30.4	2	0.001
None	3	8.6			

No - Number; % - Percentage; df - Degree of freedom; P - Level of significance;
$P \leq 0.05$ indicates the relationship is significant

4.3 Cytomorphological features of conventional cervical smears

Out of a total of 35 Pap smears, 26 (74.3%) were negative for intraepithelial lesion (NILM), while 9/35 (25.7%) had abnormal lesions. These included, high grade squamous intraepithelial lesion (HSIL) which were 4(11.4%), Low grade squamous intraepithelial lesion (LSIL) 2(5.7%)), Atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells-cannot exclude high grade lesion (ASC-H), and Squamous cell carcinoma (SCC) were 2.9% respectively as shown in the figure below.

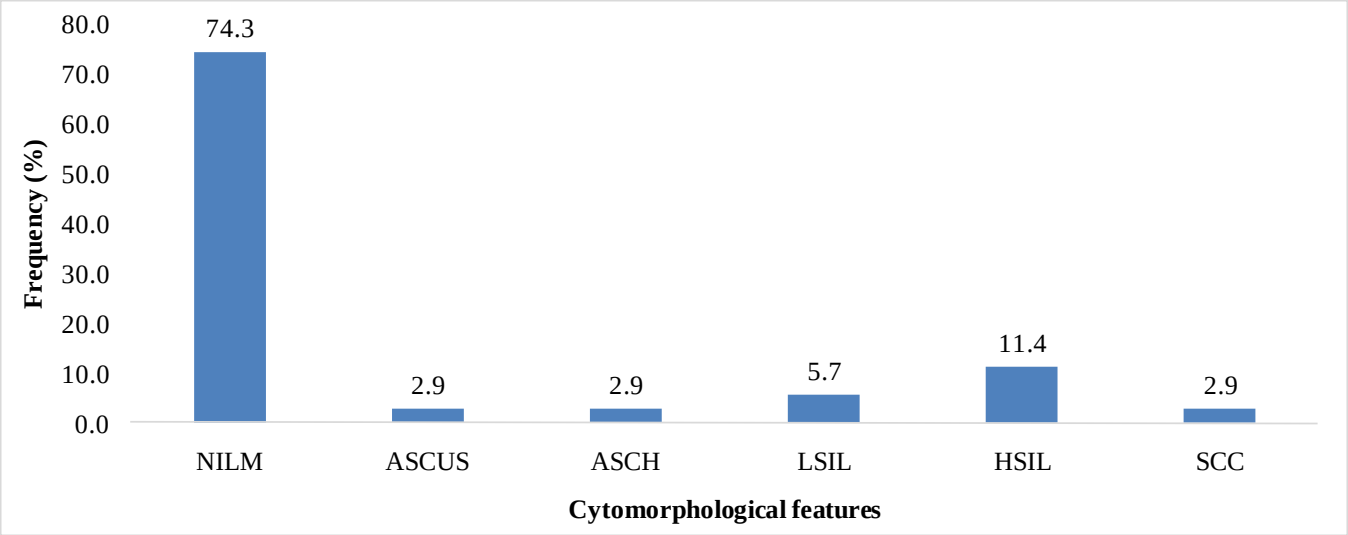


Figure 2: Cytomorphological features of Pap smears

4.4 Immunocytochemical (P16INK4a and Ki-67) staining results

The immunocytostains using $P16^{INK4a}$ showed 25/35 (71.4%) as negative (no staining), while 7/35 (20%) were sporadic positive, and 3/35 (8.6%) focal positive. The Ki-67 proliferation index staining showed 17/35 (48.6%) negative (no staining), 13/35 (37.1%) sporadic positive and 5/35 (14.3%) focal positive. Out of the 35 specimens, 9/35 (25.7%) were positive for the combined $P16^{INK4a}$/ Ki-67 immunostaining, while 26/35 (74.3%) were negative, as shown in table 5.

Table 5: Cytomorphological features of Pap smears and immunostaining results

Test outcome	Sample size		chi-square	df	P
	No	%			
Cytology					
ASCUS	1	2.9			
ASCH	1	2.9			
LSIL	2	5.7	84.829	5	0.001
HSIL	4	11.4			
SCC	1	2.9			
NILM	26	74.3			
P16					
Sporadic positive	6	17			
Focal positive	4	11.4	23.029	2	0.001
Negative	25	71.4			
Ki 67					
Sporadic positive	13	37.1			
Focal positive	5	14.3	6.4	2	0.041
Negative	17	48.6			
Dual P16/Ki 67					
Positive	9	26	8.257	2	0.004
Negative	26	73			

No - Number; % - Percentage; df - Degree of freedom; P - Level of significance;
$P \leq 0.05$ indicates the relationship is significant

4.5 Correlation of combined p16INK4a/Ki-67 immunostaining with conventional pap smears

The $p16^{INK4a}$ expression was concordant with cervical cytology in 34 of 35 cases (97.1% concordance). Only one NILM was focal positive by $p16^{INK4a}$. Using Cohen's kappa statistics to measure the agreement between $p16^{INK4a}$ and conventional pap smears, a fair and significant agreement (Kappa = 0.381; P = 0.001) was obtained. By Spearman test (which measure the strength and direction of association that exists between two variables measured on at least an ordinal scale), there was a strong and significant correlation between $p16^{INK4a}$ and conventional Pap smear (r = 0.781 and P = 0.001).

4.5.1 Ki-67 Immunostaining Results

The Ki-67 Immunostaining results were concordant with conventional Pap smear cytology in 26 of 35 cases (74.3% concordance). There were 9 NILM by cytology that was positive by Ki-67. Using Cohen's kappa statistics, there was slight and significant agreement (Kappa = 0.268; P = 0.001). By Spearman test, there was a slightly strong and significant correlation between P16 and cytology (r = 0.516 and P = 0.001)

4.5.2 P16INK4a and Ki-67 combined immunostaining results

The combined $p16^{INK4a}$/Ki-67 immunostaining result was concordant with Conventional Pap cytology in 35 of 35 cases (100% concordance) (Figure 6). Using Cohen's kappa statistics, there was moderate and significant agreement with cytology (Kappa = 0.481; P = 0.001). By Spearman test, there was a much higher and significant correlation between $p16^{INK4a}$ and cytology (r = 0.95 and P = 0.001) (Table 5)

Table 6: Correlation of $P16^{INK4a}$, Ki 67, $P16^{INK4a}$/ Ki-67 with Pap cytology interpretation

Cytology	*N*	P16	*N*	Ki-67	*N*	P16/Ki-67	*N*
NILM	26	Focal Positive Negative	1 25	Sporadic Positive Focal Positive Negative	**7** **2** 17	Negative	26
ASCUS	1	Focal Positive	1	Sporadic Positive	1	Positive	1
ASC - H	1	Focal Positive	1	Sporadic Positive	1	Positive	1
LSIL	2	Sporadic Positive Focal Positive	1 1	Sporadic Positive	2	Positive	2
HSIL	4	Sporadic Positive Focal Positive	3 1	Sporadic Positive Focal Positive	3 1	Positive	4
SCC	1	Sporadic Positive	1	Focal Positive	1	Positive	1
		Agreement using Cohen's kappa statistics					
kappa		0.381		0.268		0.481	
p		0.001		0.001		0.001	
		Correlation with cytology					
r		0.781		0.516		0.95	
p		0.001		0.001		0.001	

N - Sample size; r - Spearman's correlation coefficient; p - significant at the 0.05 level (2-tailed)
kappa - measure of agreement between two tests

Flow chart of the Schematic correlation of $p16^{INK4a}$, Ki 67, $p16^{INK4a}$/ Ki-67 with Pap cytology interpretation.

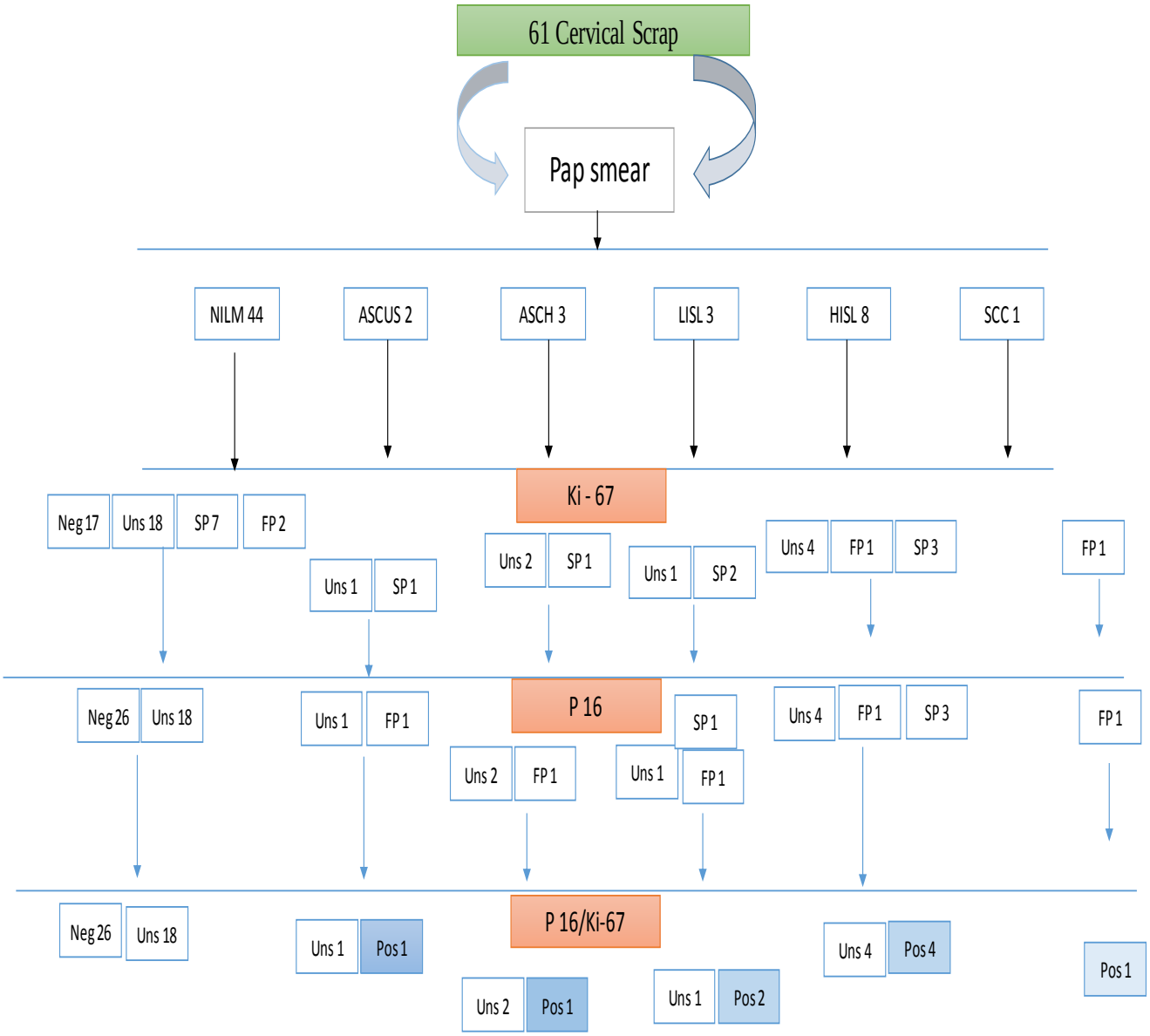


Figure 6: Cervical cancer screening results by four type specific tests. Categories of Conventional Pap cytology were tested by $P16^{INK4a}$,Ki -67 and for dual $P16^{INK4a}$/ Ki-67immunostain categorized as negative (Neg); Sporadic positive (SP); focal positive ("FP) or unsatisfactory (Uns).

4.6 Photomicrographs of Conventional cervical Pap smears and ICC stains

The photomicrographs below are showings cytomorphological features of various cervical lesions on Pap smears and Immunocytochemical stains. The ICC stains were only done on cell blocks.

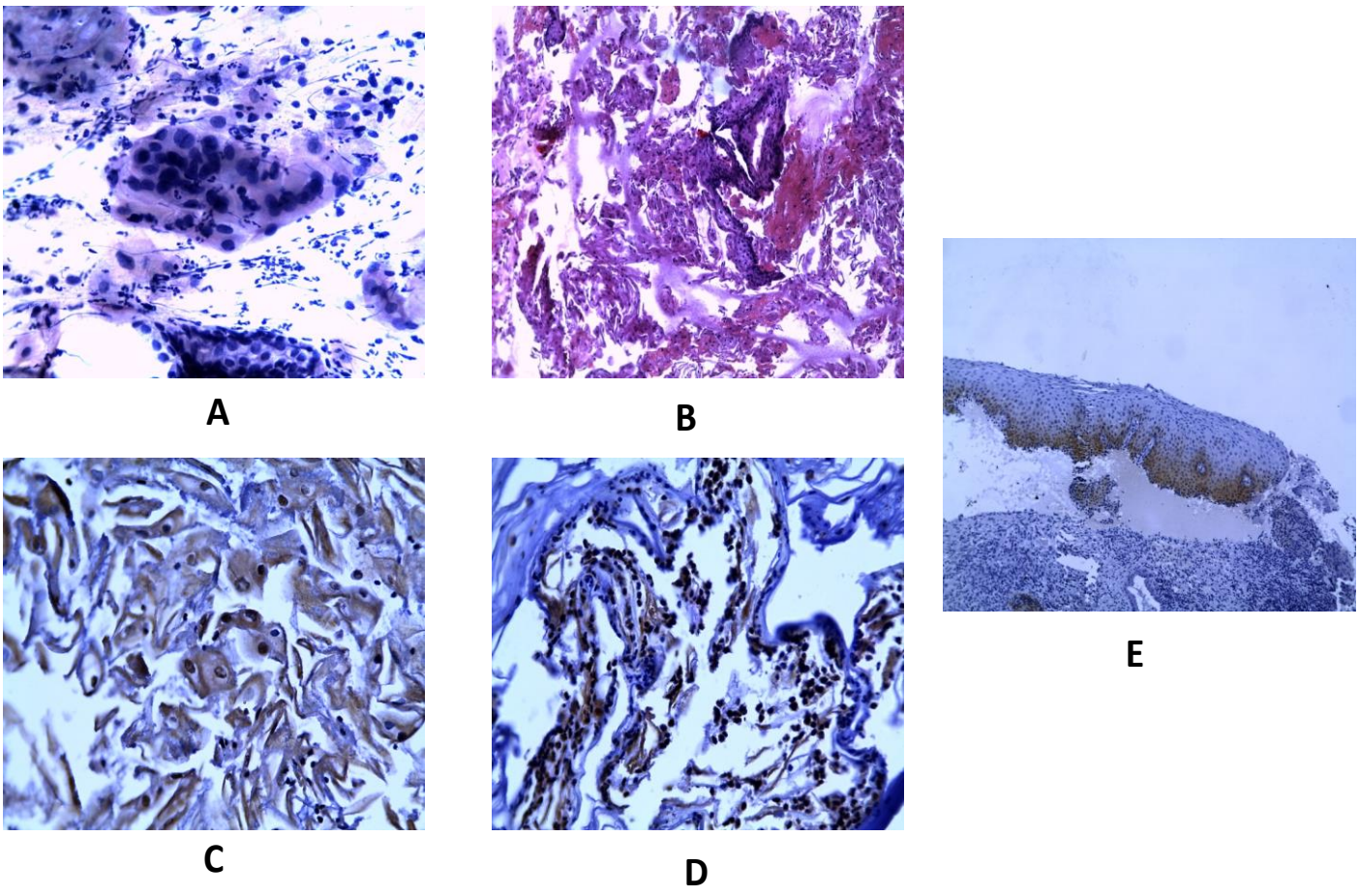
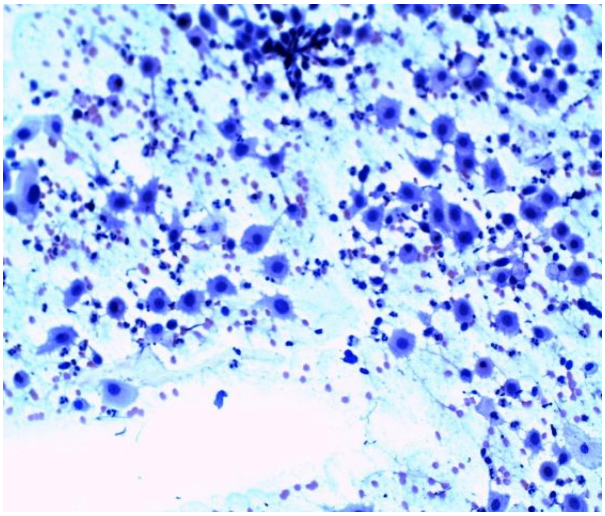
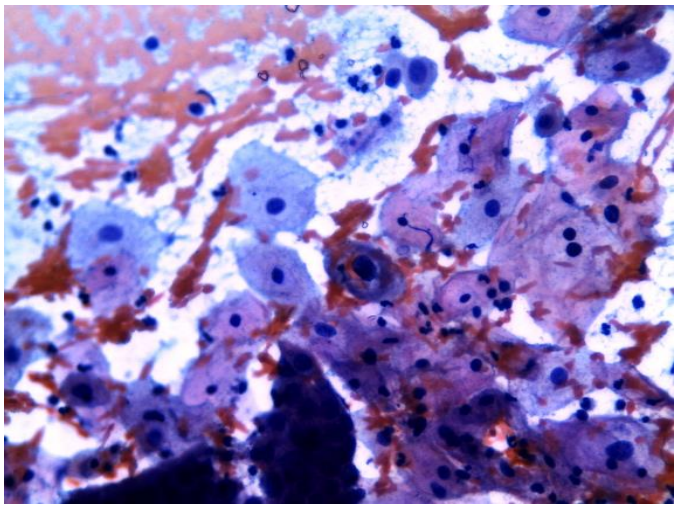
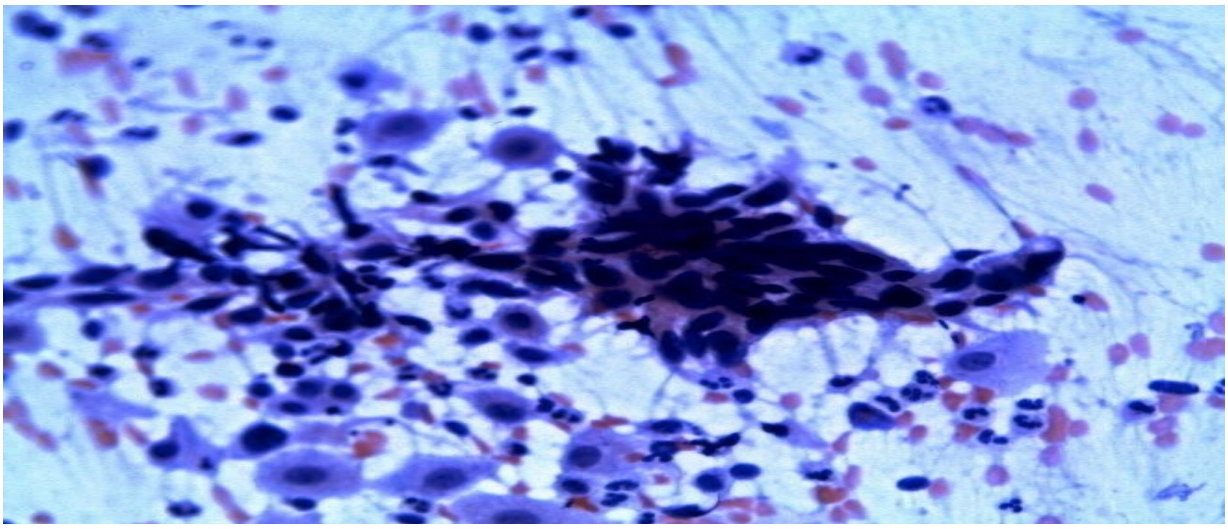


Figure 4: Photomicrographs: Representation of cytological and immunostaining. Where, A. is HSIL by Pap staining, B. Is cell block in H&E, C. Ki-67 Positive, D is Ki-67 Negative and E Positive control $p16^{INK4a}$





(A) Atrophic smear (B).ASCUS



B(C) HSIL in atrophy

Figure 5: Photomicrographs: Representation of cytomorphology include: (A) Atrophic smear, (B) ASCUS and (C) HSIL in atrophy.

5.0 Discussion

This study set out to establish the diagnostic utility of $p16^{INK4a}$/Ki-67 Immunocytochemical stain as an adjunct to conventional smear in the diagnosis of cervical cancer in women living with HIV at comprehensive care clinic at Kenyatta National Hospital. A total of 61 participants underwent conventional Pap tests and were eligible for the study. Of this 26/61 (42.6%) specimens could not be enrolled for immunostain, thus the total number of participants done for both Pap smear and immunostain was 35. The median age of the 35 participants was 41.1 years with a range of 19 to 69 years. Of the 35 participants, 25.7% had an abnormal Pap smear result, where HSIL recorded the highest (11.4%), followed by LSIL 5.7%, while ASCUS, ASC-H and SCC had 2.9% each. These abnormal findings were comparable to those reported by Memiah *et al* (26.7%) in Kenya, Ndambi *et al*, 2016 (unpublished data) and Joyce *et al*, 201 (unpublished data). The high prevalence of cervical cancer could be attributed to the fact that the women who are on HAART have a longer life span hence the manifestation of cervical cancer. In the study by Memiah *et al*, the sample size was large enough hence gave a wider population representation unlike in this current study which was confined to CCC at KNH. Contrary to our findings, a study done in Johannesburg, South Africa 2013, reported a prevalence of 57% in women living with HIV. This was also reported in Nigeria, while other studies done in South Africa reported a lower percentage of prevalence of less than 50%. This difference may be due to the different sample size, extent of sickness or the different risk factors encountered by the women. The HIV percentage in South Africa is higher than that of Kenya. . In this study we expected to detect more lesions, however, the percentage was similar to that of other researchers. This could have been due to a high loss of the respondent rate (42.6%).

In this study, $p16^{INK4a}$ and Ki-67 was done on all the 35 participants, this included the 25.7% abnormal and the normal Pap smear. The $p16^{INK4a}$ detected 1/4 (25%) focal and 3/4 (75%) sporadically stained, which were reported as HSIL, followed by 1/2 (50%) of the LSIL sporadic and focal, the rest were either focal or sporadically stained. Contrary to the findings of Keating *et al*., 2001 and Agoff *et al*, 2003, in their studies of Ki-67, cyclin E, and $p16^{INK4a}$ done on cervical biopsies, which found 50% to 70% of HSIL lesions, where they showed diffuse strong expression of $p16^{INK4a}$ and 20% to 23% had focal strong staining. This difference was mainly due to the type of specimen used. In this study we used a cell block preparation unlike in their

studies. [53, 54] Additionally, Keating *et al.* 2001, show in their study that low-risk HPV is associated with less $p16^{INK4a}$ expression, and they suggest that different stages of high-risk HPV-induced cervical neoplasia may have different levels of $p16^{INK4a}$ expression. [54]

Further to note in this study is that about 4% of the NILM cytology was sporadically stained with $P16^{INK4a}$. Contrary reports have been shown by Murphy *et al*, 2003. [57] However, many more studies have detected $p16^{INK4A}$ immunostaining in normal-looking cervical cells similar to this study. [46, 55, 56] Many more Studies have shown a close correlation between the numbers of $p16^{INK4a}$-stained cells and the HPV type, being most intense in HPV16 type and lowest in low-risk HPV lesions. They also reported that this correlation is also a significant diagnostic sign, with higher mean count of $p16^{INK4a}$positive reactions detectable in HSIL than in other diagnostic categories. [36, 46]

In the current study a combination of $p16^{INK4a}$ and Ki-67, 9/35 (25.7%) of the cases were positive. In this study we subjected both abnormal and the normal Pap smears to immunostain. These findings are different from those of Filho *et al*, 2005, which showed that HPV-positive group, 59 of 101 cases (58.4%) were positive for both $p16^{INK4a}$ and Ki-67 immunostaining, whereas only 17 of 101 (16.8%) were negative for both. [46] This explains why there was 25.7% positive rate in contrast to 58.4%, as their target group was HPV positive women by molecular tests. Studies done by Sahebali *et al*, showed that there is a strong correlation between $p16^{INK4a}$ and HPV positive women, while other studies have shown that $p16^{INK4a}$ and Ki-67 ICC staining can be used to clarify the grey areas such as cases of ASCUS and ASC-H cytology. [36, 46] In consonant with this study, Immunostaining for $p16^{INK4a}$ and Ki-67 on cell block preparations can help to improve the diagnostic accuracy of HSIL and SCC, as reported by Yu *et al*, 2010. In the study done by Donàet *et al*, 2012, they showed that $p16^{INK4a}$ and Ki-67 immunostaining might have a relevant clinical role, since the dual staining was significantly associated with HR-HPV infection.[58, 59]

5.1 LIMITATIONS: There was a high loss of cervical cells during cell block preparation, this was partly due to the method used in concentrating the sample.

CONCLUSIONS: The combined $p16^{INK4a}$/Ki-67 ICC staining on cell block showed moderate agreement and strongly correlated with cytological results, indicating the usefulness of combined $p16^{INK4a}$/Ki-67 biomarker for diagnosis of cervical cancer, especially in cases of ASCUS and ACS-H while improving the diagnostic accuracy of LSIL and HSIL.

RECOMMENDATION: The use of $p16^{INK4a}$/ Ki-67 ICC can be used as a means of analyzing cervical cancer when used in conjunction with conventional cytology, therefore $p16^{INK4a}$/Ki-67 biomarkers should be considered in the cervical cancer screening algorithms. However, a larger sample size should be conducted to validate these findings.

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APPENDICES

APPENDIX I: INFORMED CONSENT EXPLANATION

TITLE: UTILITY OF p16^{INK4A}/KI-67 IMMUNOCYTOCHEMICAL DUAL STAIN AS AN ADJUNCT TO CONVENTIONAL PAP SMEAR FOR THE DIAGNOSIS OF CERVICAL LESION AMONG WOMEN LIVING WITH HIV ATTENDING CCC AT KNH, KENYA.

Self introduction

My name is Ngoma Febian, a postgraduate student at the University of Nairobi pursuing a Master of Science in Clinical Cytology at the Department of Human Pathology. I kindly request you to participate in the above study.

Purpose of the study:

The purpose of this study was to highlight the importance of p16^{INK4A}/KI-67 immunostain as an adjunct to Pap smear in diagnosing cervical cancer in women living with HIV.

Benefits and risks of the study to you:

i. **Benefits**

The potential benefit to the patient in participating in this study was that an additional test which was more accurate was offered for free. This gave an assurance of a true negative or positive test result. Currently this was not provided in the routine investigation for cervical cancer.

ii. **Risks**

Some slight discomfort was felt, but the procedure was associated with any major complications. The instruments that used were sterile and disposable. In case of serious discomfort with the process, the client was assured to freely withdraw from the study without any consequences.

Specimen collection

After agreeing to participate in the study, the patient was required to lie on an examination couch where the nurse or a doctor inserted a speculuminto the vaginal canal to visualize the cervix for sample collection. No further specimens were collected after this procedure. The specimens

weresent to the laboratory for examination. The informationprovided helped in complementing demographical datain the study. The patient was requested to feel free to ask any questions pertaining to the study.The patient was at liberty to be included in the study or not without any coercion. The client was entitled to have a copy of this consent for her records.

Confidentiality

Names were not required in the study. The patients were identified by study numbers. Questionnaires will be kept for one year before destroying. Any information given to us remained confidential and only for the patient's own benefit. The patients results were sent to the patients file where the clinician took the necessary steps during the patients next visit.

Withdrawal from the study

Participation in this study was voluntary and this was part of the patient's routine evaluation. Declining to participate in the study had noeffectin the services the patient was seeking. The patient was free to withdraw any time without losing the benefits to which she was entitled to in this institution.

Contact information

Any important questions regarding the participation in the study was directed to the following people:

Principal investigator: NgomaFebian (Telephone number +254732414347),
Supervisor: Dr. Waweru (Telephone number +254 722759523),
Supervisor: Dr. E. O. Walong (Telephone +2547338590623)
Supervisor: Mrs. Josephine. N. Rioki (Telephone +25477531874)
ERC Chairperson: Prof M. L. Chindia 02-2726300 -KNH/ UON- ERC

APPENDIX II: CONSENT FORM

The above details about the study and basis of participation were explained to me and I solely agreed to give permission for use of my specimen for the proposed study and future studies. I.....do hereby give informed consent to participate in the study fully aware of the benefits and risks. I am aware that I can withdraw from the study without loss of any benefit or quality of management to which I am entitled. I am fully aware that the results of the study will be used for scientific purposes and may be published.

Participant's Signature/Thumb print …….....Date…….....

Doctor/Nurse…….....……Date…….....

Principal investigator…….....……Date…….....

FOMU YA IDHINI: CONSENT FORM

TITLE: UTILITY OF P16^{INK4A}/KI-67 IMMUNOCYTOCHEMICAL DUAL STAIN AS AN ADJUNCT TO CONVENTIONAL PAP SMEAR FOR THE DIAGNOSIS OF CERVICAL LESION AMONG WOMEN LIVING WITH HIV ATTENDING CCC AT KNH, KENYA.

Utangulizi-Introduction

JinalanguniNgomaFebian (+254732414347s) kutokaIdaraya Human Pathology katika Chuo Kikuu cha Nairobi.Lengo la ujumbehuunikuombaruhusayakokatikautafitinitakao anya. Nitakuelezaumuhimuwautafitihuukwakobinafsiiliuwezekuamuakamautajihusisha au la. Kama muhusikaunauhuruwakuulizaswalililoloteutakalokuhusuumuhimuwautafitihuukwako, hatarinafaidautakazopatanahakizakokwawakatiwautafitihuu.Unaweza pia kutojihusishawakatiwowoteunapohisihukotayarikuendeleatena.

MadhumuniyaUtafiti

Lengo la utafitihuunikutathminiumuhimuwap16^{ink4a}/ki-67 kamanjiayakuchunguzasarataniyakizazikwawamamawalionavirusivya HIV, kandonamatumiziya pap smear katika Comprehensive Care Clinic yahospitalikuuya Kenyatta.

Faida

Ni muhimukuhusikakwenyeutafitihuukwanimatumiziyap16^{ink4a}/ki-67 biomarkers yataletamatokeo bora nakupunguzagharamayaupimajiwasarataniyakizazi. Faidahizizitafanyaushughulikiwekwanjia bora zaidi.

Madhara

Uchunguzihuuhautakusababishiamadharayakukuhatarishakwaniutaratibuutakaotumiwautazingati ahaliyajuuyausafinakinga. Una ruhusayakutohusikaukihisiumohatarini.

UchukuajiwaSampuli

Baadayakujazafomuyakukubalikuhusika,

,sampuliitachukuliwakutokasehemuyakoyauzazikwakutumia speculum

kwanjiasafiisiyonamaumivu.

Hakunasampulinyingineitachukuliwa.Sampulihiyoitapelekwakwenyemahabarakufanyiwauchunguzi. Una hakiyakuulizamaswaliyoyotenakupewa copy yafomukwamatumiziyako. Kumbukakwambaunahusikakwahiari.

Siri katikautafiti

Jinalakohalitatumikakatikautafitihuu.Utakabidhiwanambaribadalayajina.Utapokeamatokeoyakokwenyefailiyakowakatiwaklinikiijayo. Muuguziatachukuahatuamadhubutikulingananamatokeo.

Kutohusika

Iwapoutahisikuwahutakikuendeleanautafitihuu,ukohurukujiondoawakatiwowote. Hospitaliitaeendeleakukuhudumiavilivyo.

Mawasiliano

Iwapounajambololoteungependakufahamishwa, unawezakuwasilianawahusikawafuatao:

Principal investigator: NgomaFebian (Telephone number +254732414347),

Supervisor: Dr. Waweru (Telephone number +254 722759523),

Supervisor: Dr. E. O. Walong (Telephone +2547338590623)

Supervisor: Mrs. Josephine. N. Rioki (Telephone +25477531874)

ERC Chairperson: Prof M. L. Chindia 02-2726300 -KNH/ UON- ERC

IDHINI KUTOKA KWA MSHIRIKI

Mimi ----------nimesomanakushauriwabilakushurutishwanamtu au kwaainayeyotekujitoleakatikautafitihuu. Nimeelewahatari, umuhimunafaidazotezinazohusika.Natoaidhiniyangukwambanimeitikiakujihusishanautafitihuu. Naelewa pia kwambakutohusikahakutaadhirihudumaninayopokeakwahospitalihiinaninawezakujiondoawakati wowote. Pia, naelewautafitihuuutachangiakuongezamaarifakatikautafitiwakisayansi.

Sahihiya mshiriki.....Tarehe.....

Sahihiya muuguzi/shahidi.....Tarehe.....

SahihiyaMchunguziMkuu……..…Tarehe…….....

APPENDIX III: QUESTIONNAIRE

PROJECT TITLE: UTILITY OF PINK4A/KI-67 DUAL IMMUNOCYTOCHEMICAL STAIN AS AN ADJUNT TO CONVENTIONAL PAP SMEAR FOR THE DIAGNOSIS OF CERVICAL LESION AMONG WOMEN LIVING WITH HIV AT CCC, KNH

Consenting participants were required to fill the questionnaire before specimen collection.

Kindly tick one of the choices given.

Study Identification Number .....**Date** ( ) ( ) ( )

DD/ MM/ YR

Section A Social demographic information:

i. a). Age: ( ) ( ) ( )

DD/ MM /YR

ii. Marital status: Tick were applicable:

Single ( ) Married ( ) Divorced ( ) Widowed ( )

iii. Parity (Number of pregnancies, both living and deceased): ☐

iv. Last date of menses: ( ) ( ) ( )

DD/ MM/ YR

v. Have you ever had a Pap smear: Yes ( ) or No ( )

vi. Date of previous Pap smear done: ( ) ( ) ( )

DD/ MM/ YR

vii. Family planning method in use:

Natural ( ) Condom ( ) Pill ( ) IUCD ( ) None ( )

viii. Number of sexual partners: ( ). Age at first Pregnancy ( ) ( ) ( )

DD/ MM / YR

Section B: Clinical history

Appearance of the cervix with speculum:

i. Normal ( ) Eroded ( ) Inflamed ( ) Suspicious

ii. History of Antiretroviral treatment:

a). Less than 6 months ( ) 6-12 months ( ) 1-5 years ( ) > 5 years ( )

iii. History of Hysterectomy: Yes ( ) or No ( )

Section C:

Results for the Study:

i. Conventional Pap smear report:

a) Specimen adequacy: Satisfactory ( ) or Unsatisfactory ( )

b) Squamous epithelial cell abnormalities.....
.....

c) Glandular cell abnormalities.....
.....

d) Organisms present.....
.....

e) Other non-neoplastic factors.....
.....

f) Recommendation.....
.....

ii. Immunocytochemistry: p^{INK4a}/Ki-67 Immunostain results

A). p^{INK4a}/Ki-67 immunostain positive with simultaneous brown stained cytoplasm and red stained nucleus

B). p^{INK4a}/Ki-67immunostain negative refer to no visible stained cytoplasm or nucleus.

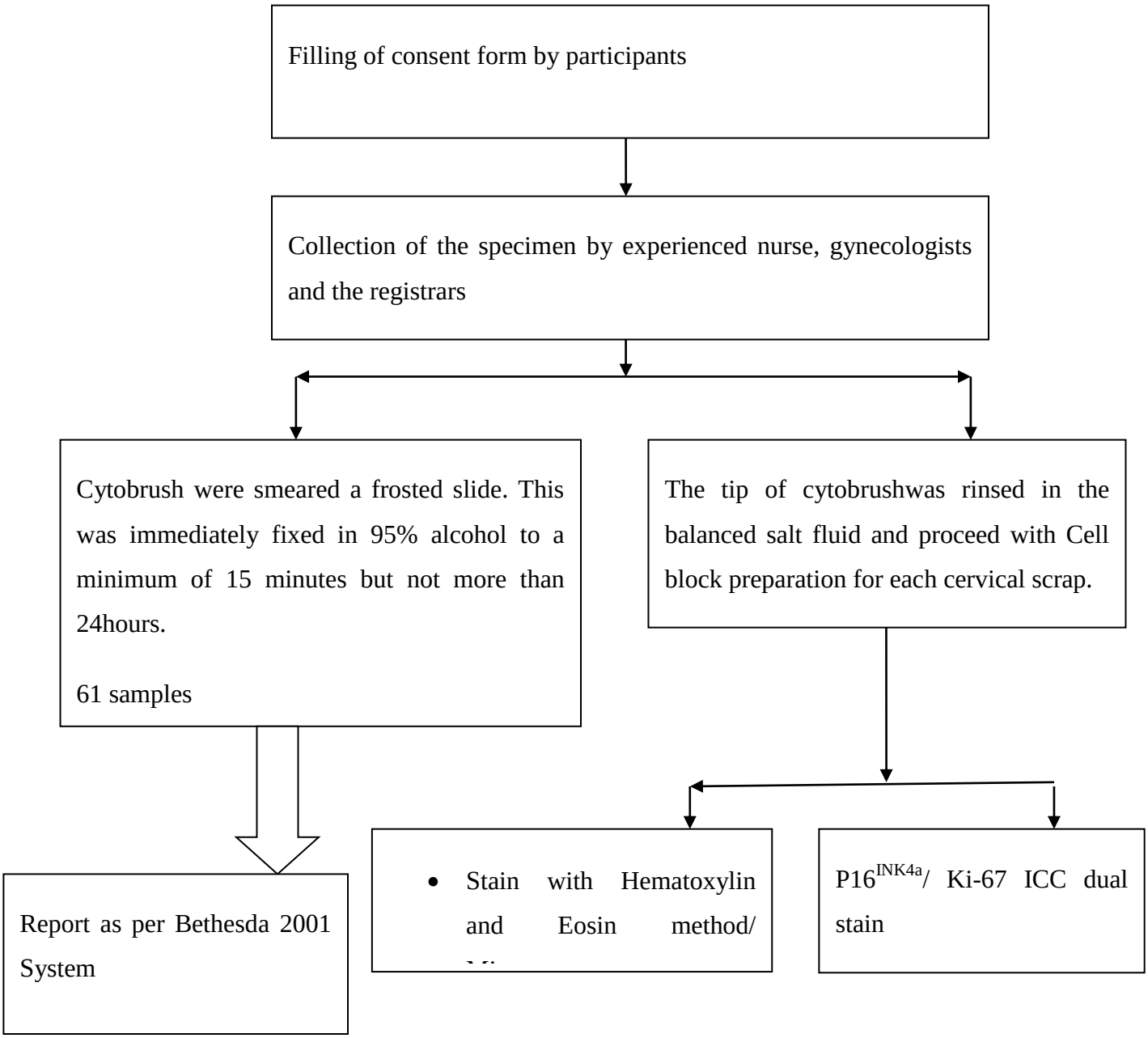
Signatures

Principal investigator.....Date.....

Cytologist.....Date.....

Pathologist.....Date.....

APPENDIX IV: SAMPLE COLLECTION PROCEDURE –FLOW CHART



APPENDIX V: PAPANICOLAOU STAINING PROCEDURE

Principle of the stain

Hematoxylin stains the nuclei blue by dye-like formation. The eosin azure solution being acidic stains the cytoplasm which is basic so that the eosin has affinity for the mature cells while light green has affinity for the young cells. Orange G also being an acidic dye has an affinity for the cytoplasm and stain keratin.[12, 14]

Staining procedure

1. The smears were fixed in 95% alcohol for a minimum of 15 minutes
2. Then hydrated by passing them through degrading levels of ethanol of 80%, 70% and 50%, ten dips in each.
3. Rinsed in distilled water 10 dips
4. Proceeded to stain in Harris Hematoxylin for 4 minutes
5. Rinsed in tap water
6. Differentiated in 0.05% acid water, 10 dips
7. Rinsed in tap water and blueing in Scott's tap water, 10 dips
8. Rinsed in 95% ethanol, 10 dips.
9. Proceeded to stain in Orange G 6 for 1.5 minutes
10. Rinsed in 95% ethanol, 10 dips.
11. Proceeded to stain in E.A 50 for 4 minutes
12. Rinsed in 95% ethanol, 10 dips
13. Dehydrated in changes of absolute ethanol, 10 dips each.
14. Cleared in 3 changes of xylene 10 dips each.
15. Finally mounted in D.P.X[(6, 14)]

APPENDIX VI: CELL BLOCK PREPARATION

Procedure

1. The cervical scrap were collected ina 14mls container of a balanced salt solution, the cytobroom tip was rinsed thoroughly to ensure all the cell contents remained in the fluid.
2. A constitution of balance salt solution: Mixed the contents.
    - 95% Ethanol -34 miles
    - 10% Formalin -4mile
    - Glacial acetic acid -2miles
3. The suspension was centrifuged for 10 minutes at 2000 rpm.
4. Then cell button re-suspended in 10% formal saline for fixation and centrifuged for 10 minutes at 3000 rpm. This was washed with normal saline prior to mixing with plasma.
5. The button was thentreated with thromboplastin agent to form a clot and colored with 1% Eosin.
6. This was then fixed in 10% formalin, processed and wrapped into a lens paper and transferred into a tissue cassette for processing into an automatic tissue processor.
7. The samples were embedded using paraffin wax and then sectioned using microtome.
8. Staining sections were achieved by using $P16^{INK}$4a/ Ki-67 dual ICC stain and Hematoxylin and Eosin. [6, 14]

APPENDICES VII: AUTOMATIC TISSUE PROCESSING

Procedure:

The processing of cell block using an automatic tissue processor was set in the fluids as follows.

1. 70% ethanol	1 hour
2. 96% ethanol	1 hour
3. 96% ethanol	2 hours
4. Absolute ethanol	1 hour
5. Absolute ethanol	2 hours
6. Absolute ethanol	2 hours
7. Absolute ethanol	1 hours
8. Xylene	$1^{1/}_{2}$ hours
9. Xylene	$2^{1/}_{2}$ hours
10. Xylene	3 hours
11. Molten paraffin wax bath	2 hours
12. Molten paraffin wax bath	3 hours
Total hours	**22 hours**

APPENDIX VIII: IMMUNOCYTOCHEMISTRY- SOP

STAINING PROCEDURE

1. The sectioned slides were left overnight at 37^{o} c to allow removal of excess wax.
2. Cool on a cooling bench
3. Dewax in xylene 3 changes for 2 minutes in each.
4. Hydrate in alcohol 3 changes for 2 minutes in each
5. Let slides air dry and use Nova pen to outline cell block section
6. Put slides in a coupling filled with citric buffer PH 6.0 or EDTA buffer PH 8.0
7. Put in the microwave or steamer for 20 minutes checking at 5 minutes intervals for the level of the buffer.
8. Cool the slides for 10minutes then rinse with distilled water
9. Apply peroxidase block for 5 minutes (To prevent Endogenous peroxidase reaction) then rinse with Tris/ PBS buffer.
10. Apply protein block for 10 minutes to ensure protein do not react. Then wash thoroughly with Tri/ PBS buffer.
11. Apply Primary Antibody for 30 minutes then wash thoroughly with Tri/ PBS buffer.
12. Apply post primary conjugate for 30 minutes (to enhance staining) then wash thoroughly with Tri/PBS buffer.
13. Apply polymer for 30 minutes (to enhance binding of DAB) then wash thoroughly with Tris /PBS buffer
14. Apply DAB chromogen diluted 1:2 with substrate buffer for 2 to 5 minutes then wash with Tri/PBS buffer
15. Counterstain in Hematoxylin for 1-2 minutes then wash with distilled water.
16. Differentiate in 1% Acid alcohol then wash with distilled water.
17. Blue in Scotts tap water then wash with distilled water.
18. Dehydrate with alcohol 3 changes for 2 minutes in each
19. Clear with xylene 3 changes for 2 minutes in each
20. Mount with DPX and examine microscopically.

APPENDICES IX: HEMATOXYLIN AND EOSIN STAINING (H & E)

Principle of the stain

Hematoxylin stains the nuclei blue by dye-like formation. The eosin azure solution being acidic stains the cytoplasm which is basic so that the eosin has affinity for the mature cells while light green has affinity for the young cells.

Staining procedure

1. The Cell block sections were dewaxed in three changes of xylene for 5 minutes
2. The smears were hydrated by passing them through ethanol grades of 80%, 70% and 50%, ten dips in each.
3. Rinsed in distilled water 10 dips.
4. Proceeded to stain in Harris Hematoxylin for 4 minutes
5. Rinsed in tap water
6. Differentiated in 0.05% acid water, 10 dips
7. Rinsed in tap water and blueing in Scott's tap water, 10 dips
8. Rinsed in 95% ethanol, 10 dips.
9. Rinsed in 95% ethanol, 10 dips.
10. Proceeded to stain in E.A 50 for 4 minutes
11. Rinsed in 95% ethanol, 10 dips.
12. Dehydrated in changes of absolute ethanol, 10 dips each.
13. Cleared in 3 changes of xylene 10 dips each.
14. Finally mounted in D.P.X. [14]

APPENDIX XI: INTERPRETATION OF P16^{INK4A}/ KI-67 RESULTS

Table Evaluation of Nuclear score.[44, 45]

SCORE	Staining intensity	Extent of stained cell (%)	INTERPRETEATION
Score 0	No staining	< 0-1% Positive Cells	Negative for p16/ ki-67
Score 1	Weak staining	1-5% Positive Epithelial cells	Sporadically positive Isolated cells were positive 1-10% cells
Score 2	Moderate staining	5- 25% Positive	Focally positive Small cell clusters, but less than 25% of the cell were positive 11-50% cell content
Score 3	Strong staining	>25% Positive epithelial cells	Diffusely positive >25% of cells positive 50-80%- cell content
Score 4	Prominent staining	>25%	81-100 % cell content

Note

1. The simultaneous red nuclear staining and the brown cytoplasm in at least 1 (one) or more than 30 epithelial cervical cells will be considered as positive criteria.
2. This was interpreted as positive p^{INK4a}/Ki-67.

APPENDIX XII: DUMMY TABLES

TABLE 1: Sociodemographic Characteristics

Age Range (years)	Frequency	%
25 -29		
30-34		
35-39		
40-44		
45-49		
50-54		
55-60		

A bar chart was used to show age distribution

Table 2: Conventional Pap Smears Results

Lesions	Frequency	Percentage
NILM		
ASCUS		
LSIL		
ASC-H		
HSIL		
SCC		
AGC		
AIS		
Adenocarcinoma		

Table 3: Results for p16^{INK4a}/ Ki-67 immunostaining in relation to Conventional cytology results in women with cervical lesions

LESIONS	P16^{INK4a}/ Ki-67TESTING		TOTAL
	POSITIVE	NEGATIVE	
NILM			
ASCUS			
LSIL			
ASCH			
HSIL			
SCC			
AGC			
AIS			
ADENOCARCINOMA			
TOTAL			

A bar chart was used to highlight the major findings here.

TABLE 4: Comparison of Pap smear results and p16^{INK4a}/ Ki-67 test results

		P16INK4a TESTING		TOTAL
		POSITIVE	NEGATIVE	
PAP SMEAR	Positive			
	Negative			
TOTAL				

Table5: Comparison of Pap smear results and $p16^{INK4a}$/ Ki-67 test results

		$P16^{INK4a}$/Ki-67 staining results		TOTAL
		Positive	Negative	
PAP SMEAR	Positive	a	b	a+b
	Negative	c	d	c+d
TOTAL		a+c	b +d	a +b+c+d

Overall agreement= $\frac{(a + b)}{(a + b + c + d)}$

Table 6. Comparison for Conventional Pap and Cell block H & E findings

Neoplasm's	MalignantHistology	Benign histology	Total
Malignant on Cytology			
Benign of Cytology			

Table 7. Comparison for Cytology findings and Histology findings

Infections	Positive on Cell block	Negative on Cell block	Total
Positive on cytology			
Negative on cytology			
Total			

APPENDIX XIII: ROLE OF STUDY PERSONNEL

Summary of responsibilities

Study personnel	Responsibility
a.) Principal investigator (NgomaFebian)	1. Making Cell block from residual samples
	2.Fix Pap smears in 95% ethanol
	Stain Pap smears Stain Cell Block in H& E Immunostain
	4.Screening all Pap smears, H&E and Immunostain Reports
	5.Data record ,analysis and management
	6.Results and recommendations
(b.) Supervisors (Pathologists: Dr.Waweru Dr E:Walong Ms. Josephine	Supervision of proposal development,conduct of the entire study, report /dissertation /writing /publication. Proof reading of the Immunostain, Pap and H&E reports
(C.)Research assistant : At CCC	Collection of Cervical scraps