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The utility of p16^{Ink4a}/Ki-67 dual immunostain to triage women with Hr-HPV positive and negative Pap smear results (NILM) in Harare, Zimbabwe

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Abstract
Background: Women who test Hr-HPV+/NILM have a 3-7% risk harboring of undetected underlying precancerous cervical lesions. The biomarkers: p16 ^{ink4a} and Ki67 are surrogate markers of cell-cycle dysregulation mediated by transforming HPV infections. Therefore, the p16 ^{ink4a} /Ki67 dual immunostain can be used to identify women with transforming HPV infections; these women have a higher risk of harboring undetected cervical precancerous lesions.
Objectives: (1) To determine the proportion of women with prior Hr-HPV+/NILM screening results who are positive for p16 ^{ink4a}/Ki67 dual immunostain. (2) To compare Hr-HPV persistence rates between women with positive and negative immunostain results after 12 months.
Design: Cross sectional descriptive study.
Setting: Cimas Medical Laboratories and Parirenyatwa Central Hospital Laboratory.
Subjects: Residual LBC samples from women with prior HR-HPV+/NILM results.
Materials and Methods: Cell blocks were prepared from the residual LBC samples using the plasma-thrombin method. A paraffin serial section was cut from the cell blocks and stained manually with a mix of two monoclonal antibodies against p16^{Ink4a} and Ki-67 proteins using the CINtec PLUS kit**.** The slides were evaluated using the binary rating score system: positive or negative. Follow-up Hr-HPV persistence rates were compared using an independent t-test.
Results: Of the 82 residual LBC samples evaluated: 17.1% [14/82] were positive and 82.9% [68/82] were negative for the p16 ^{ink4a}/Ki67 dual immunostain. The 12 month follow up attrition rate was 75.6% [62/82]. The follow up Hr-HPV persistence rates in p16 ^{ink4a}/Ki67 dual positive and negative women were 72.0% [8/11] and 47.1% [24/51] respectively. These two Hr-HPV persistence rates were distinct (p=0.01).
Conclusions: Approximately 20% of patients with Hr-HPV+/ NILM screening results are positive for the p16^{Ink4a}/Ki67 dual immunostain. Hr-HPV infections in p16^{Ink4a}/Ki67 dual immunostain positive women are more likely to persist compared to women with negative dual immunostain results.
Recommendation: p16^{Ink4a}/Ki67 dual immunostain should be done on all patients with Hr-HPV+/ NILM results to stratify them according to the risk of harboring cervical precancerous lesions.

Keywords: Human papillomavirus, liquid based cytology, p16^{Ink4a}/Ki67 dual immunostain, cervical cancer, negative for intraepithelial lesion or malignancy

1. Introduction
The Pap smear has led to an enormous decrease in cervical cancer incidence over the past seven decades [1]. The establishment of a cause-effect relationship between high risk HPV (Hr-HPV) and cervical cancer led to the introduction of Hr-HPV testing as a primary screening method [2]. Hr-HPV DNA testing has a higher sensitivity and negative predictive value (NPV) than cytology [2]. A negative Hr-HPV result is reassuring as less than 1% of women develop cervical cancer without an Hr-HPV infection [3].
However, both Pap smear cytology and Hr-HPV DNA screening methods have their limitations and shortfall [4]. The shortfalls include: low sensitivity, sampling errors and inter-observer subjectivity in Pap smears [5]. The major demerit of Hr-HPV DNA is low specificity and low positive predictive value (PPV) resulting in a poor correlation between a positive Hr-HPV DNA test result and clinical disease [5]. The poor correlation is because most Hr-HPV infections are transient and are cleared by the immune system within 24 months [6]. Therefore, women who test Hr-HPV positive/NILM need additional triaging to minimize unnecessary referrals for colposcopy [7].

Several studies have evaluated the clinical utility of p16/Ki-67 dual immunostaining for triaging Hr-HPV positive/NILM and recommended its use. Petry *et al.* reported that p16/Ki-67 dual immunostaining had a sensitivity of 96.4% and a specificity of 82.1% for the detection of CIN2+ [8]. The sensitivity of the dual immunostain in that study is better than the pooled sensitivity of Hr-HPV DNA testing (sensitivity: 62% and specificity: 95%) and Pap smear (sensitivity: 57% and specificity: 93%) reported in a study done in 11 African countries and in India [4].

P16 ^{Ink4a} (p16) is a cell-cycle regulatory protein that induces cell-cycle arrest under normal physiological conditions, and Ki-67 is a marker expressed during cell proliferation [7]. The simultaneous detection of p16 and Ki-67 within the same cervical epithelial cell will not occur under physiological conditions and may be used as a surrogate marker of cell-cycle deregulation mediated by transforming high-risk HPV infections [7]. Therefore, p16^{ink4a}/ki-67 biomarkers are more superior to HPV DNA testing as the later cannot distinguish between a transient and a transforming Hr-HPV infection [7].

Dual staining with p16^{Ink4a}/Ki-67 immunostain is better than using a single as it results in higher specificity and sensitivity [9]. The p16^{Ink4a} protein is derived from the host p16^{Ink4a} /CDKN2A tumor suppressor gene, found at chromosome 9 [8]. In humans, it has been identified as a biomarker for transforming HPV infection [8]. In the absence of HPV; p16 ^{Ink4a} blocks the activity of 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 [8]. The unbound E2F acts in a negative feedback loop with pRB.10 When HR-HPV infects the host cell, the viral oncoprotein E7 binds and inactivates pRB to release E2F [8]. This promotes cell cycle progression, a molecular switch that is usually activated by CDK4/6 [8]. The p16 ^{Ink4a} induced feedback loop is thus lost and p16^{Ink4a} is over-expressed in cells [8]. This results into accumulation of the protein in the nucleus and cytoplasm of affected cells which can be detected by immuno cytochemical stains [9]. In cytological samples, p16^{Ink4a} positive cells will stain brown in the cytoplasm while the nucleus remains unstained [9].

Most patients with precancerous lesions express p16^{Ink4a} and Ki-67 molecular biomarkers which are demonstrable using immuno cytochemical methods [8] p16^{Ink4a} positive cells strongly suggest a HSIL (CIN II-III) diagnosis but is less sensitive for LSIL (CIN 1) by 74% [10]. Therefore, the p16^{Ink4a} biomarker is a powerful tool in differentiating precancerous lesions from other benign mimics such as reactive changes, reparative changes, atrophy and immature squamous metaplasia [7].

The Ki-67 is a nuclear antigen proliferative biomarker which is highly expressed in HPV infected mature squamous cells [9]. These usually manifest in a proliferating cell, hence they are closely linked to tumors of the cervix [9]. The detection of Ki-67 nuclear biomarker is useful in the demonstration of LSIL [9]. Biomarkers can help to predict the prognosis of ASCUS and LSIL cases [9].

Dual immunostaining for p16^{Ink4a} and Ki-67 biomarkers is of an advantage as it helps to detect different grades of precancerous lesions [8]. The ideal specimen for immunostaining is a cell block prepared from residual liquid based cytology samples [9] p16^{Ink4a} and Ki-67 biomarkers can be useful in triaging patients other high risk HPV genotypes apart from HPV 16 and HPV 18 and screen for women with transforming lesions which are positive for these HPV subtypes [9].

The additional advantage of these biomarkers is that they are manual processes which can be done in most routine histological laboratories [7]. If incorporated into cervical cancer screening programs, the biomarkers can greatly reduce the need of confirmatory biopsies and the number of referrals for colposcopy; this will aid to a cheaper health delivery system [10].

Despite the above stated advantages of p16^{Ink4a}/Ki-67 biomarkers, these are rarely done in Zimbabwe as routine screening tests. This study seeks to utilize the p16^{Ink4a}/ki-67 biomarkers to triage women with HR-HPV+/NILM screening results and to determine the proportion of women harboring precancerous lesions who need colposcopy referral.

2. Material and Methods

2.1 Study design: This study was a cross-sectional descriptive study conducted from March 2019 to March 2021.

2.2 Sampling method: Consecutive sampling method.

2.3 Subjects: Residual LBC samples from women with prior Hr-HPV+/NILM results.

2.3.1 Inclusion criteria: Women who have never been diagnosed of cervical cancer.

2.3.2 Exclusion criteria: Samples with insufficient cellularity for p16^{Ink4a}/Ki67 dual immunostaining interpretation.

2.4 Study sites: Cimas Medical Laboratories and Parirenyatwa Central Hospital Laboratory.

2.5 Sample size: A total of 82 LBC samples with prior Hr-HPV+/NILM results were evaluated for the presence p16^{Ink4a}/Ki67 dual immunostain positivity.

2.6 Study objectives

1. To determine the proportion of women with prior Hr-HPV+/NILM screening results who are positive for the p16 ^{ink4a}/Ki67 dual immunostain.
2. To compare Hr-HPV persistence rates in those with positive and negative p16 ^{ink4a}/Ki67 dual immunostain results after 12 months of follow up.

2.7 Laboratory processing of samples for p16^{Ink4a}/Ki-67 immunocytochemistry

Cell blocks were prepared from the residual LBC samples using the plasma-thrombin method. The cell blocks were further processed in a Shandon Citadel 2000 tissue processor. A paraffin serial section was cut from the cell blocks and stained manually with a mix of two monoclonal antibodies against p16^{Ink4a} and Ki-67, respectively using the CINtec PLUS kit**.**

2.8 p16^{Ink4a}/Ki-67 Immunocytochemistry reporting and interpretation

The slides were evaluated based on the binary rating score system composed of the ratings ‘Positive’ and ‘Negative’. A sample was deemed positive if one or more cells were stained by the red nuclear stain for Ki-67 and a brown

cytoplasmic stain for p16. A sample was deemed negative when no staining or only single staining of either p16 or Ki-67 was observed in a single cell. A sample was deemed inadequate if there was insufficient specimen cellularity (≥5000 cells per slide). The slides were interpreted by two Pathologists. Discrepant findings were evaluated by a third Pathologist. All the three reviewers were blinded of the prior Hr-HPV and Pap smear results.

2.9 Data management
Patients with positive Hr-HPV DNA and NILM baseline results had their samples evaluated by the p16^{Ink4a}/ki-67 dual immunostain. The immunostain results were recorded as positive or negative. The patients were re-tested for Hr-HPV DNA after 12 months and the results were captured. Follow-up Hr-HPV persistence rates were compared using an independent t-test. The results were presented in tables and charts.

2.10 Ethical approval
Ethical approval was obtained from the Joint Research Ethical Committee of University of Zimbabwe and Parirenyatwa Hospital (JREC), certificate number: JREC 124/2019. Permission was also granted by Cimas Medical Laboratories. During the study, strict patient confidentiality was observed by use of unique study numbers.

3. Results and Discussion
A total of 86 residual LBC samples were used to process cell blocks. Four (4.7%) were excluded because the sections did not have enough cellularity (5000 well preserved and well visualized cells) for immunostaining.

3.1 Age characteristics of the women from whom the samples were collected
The age characteristics of the women from whom the samples were collected are summarized in table 1 below:

Table 1: The age characteristics of the women from whom the samples were collected are summarized

Age characteristic	Years
Age mean	39.9
Age SD	8.4
Age range	30-81
Peak age group	30-40

3.2 p16^{Ink4a}/Ki67 immunostain results
Of the 82 residual LBC samples evaluated: 17.1% [14/82] were positive and 82.9% [68/82] were negative for the p16 ^{ink4a}/Ki67 dual immunostain.

3.3 Follow up Hr-HPV DNA testing results
The 12 month follow up attrition rate was 75.6% [62/82]. The follow up Hr-HPV persistence rates in p16 ^{ink4a}/Ki67 dual positive and negative women were 72.0% [8/11] and 47.1% [24/51] respectively. These two Hr-HPV persistence rates were distinct (p=0.01). The other results are summarized in Figure 1 below

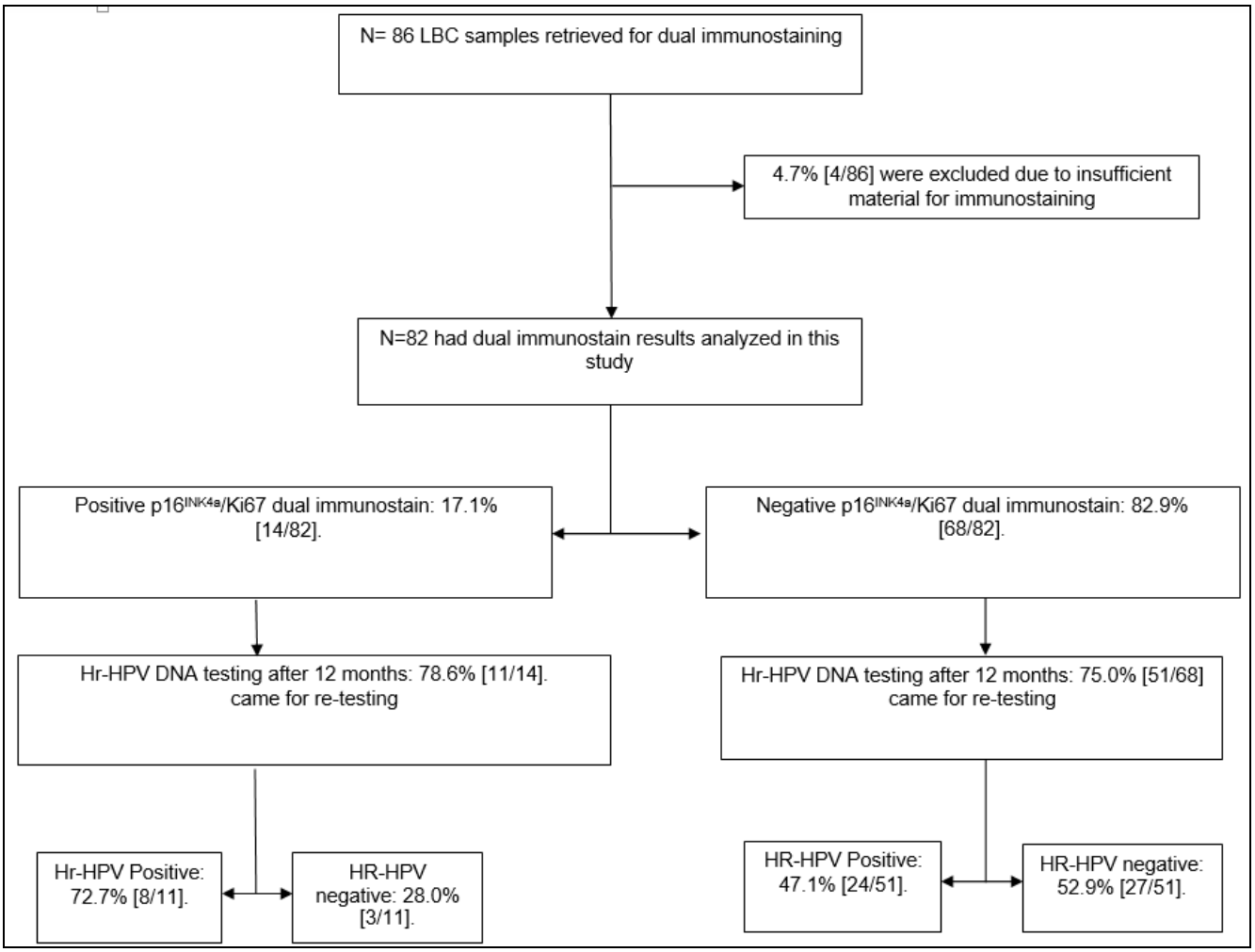

Fig 1: p16^{Ink4a}/Ki67 immunostain and follow up HR-HPV results

4. Discussion

Studies done previously confirmed that p16/Ki-67 dual immunostain can be used to triage women with Hr-HPV+/NILM results. This approach helps to detect the underlying cervical precancerous lesions that would have been missed by the two screening methods. Petry *et al.* reported that as much as 70% of underlying cervical precancerous lesions can be detected in HR-HPV+/NILM women by triaging with p16/Ki-67 dual immunostain [8]. Petry *et al.* also recommended its use for identifying a subgroup of women who would benefit from immediate referral to colposcopy [8]. Carozzi *et al.* evaluated the use of p16/Ki-67 dual immunostain and reported that incorporation of p16/Ki-67 dual immunostain in cervical cancer screening services improves the specificity and sensitivity of both screening methods: Hr-HPV DNA testing and Pap smear [11].

In this study, 17.1% and 82.9% of the Hr-HPV+/NILM women were positive and negative for p16/Ki-67 dual immunostain respectively. The p16/Ki-67 dual immunostain positivity in this study was significantly lower than the 31% reported by Wentzensen *et al.* in USA [12]. The positivity rate in this study was also slightly lower than the 25% reported by Petri *et al.* in Germany and 22% that was reported by Hu *et al.* in China [13-14].

Several reasons could have caused the lower p16/Ki-67 dual immunostain positivity rate in this study. Firstly, we used a manual method of staining which may be prone to several human errors unlike the other two methods that used automated methods of p16/Ki-67 dual immunostaining. Secondly, the dual immunostain detect $p16^{Ink4a}$ and Ki67 proteins in smears. We therefore, hypothesize that the longer storage period of our samples (over 12 months) may have contributed to the denaturation of these proteins which became undetectable. It is reported by Ngugi *et al.* that optimum results may be obtained within six weeks of sample collection [15].

In another study done in Thika district, Kenya; Ngugi et reported an even lower p16/Ki-67 dual immunostain positivity of 8.2% [15]. A noteworthy finding in this study is that they utilized the same manual kit we used [15]. We had therefore, anticipated to obtain more or less similar findings to that study. The different findings can possibly be explained by higher mutations in our population that predisposes the women to more likelihood of transforming infection. However, this cannot be stated with certainty unless another large study confirms this.

Ngugi *et al.* also enrolled women of a wide age range including some who were less than 21 years [15]. In that study Ngugi *et al.* reported that p16/Ki-67 dual immunostain positivity increased with age and they did not report any positive immunostain in women below the age of 21 years [15]. This meant that the test did not detect any transient or non-transforming Hr-HPV infections. This is consistent with the reasoning of authors of this study who only enrolled women above the age of 30 to increase the chance of detecting transforming infections only.

After the assessment of the baseline samples for p16/Ki-67 dual immunostain, the patients with Hr-HPV positive/NILM were followed up for 12 months to determine the Hr-HPV persistence rates in the two groups: p16/Ki-67 dual immunostain positive and p16/Ki-67 dual immunostain negative. The Hr-HPV persistence rates were 72% and 47% in p16/Ki-67 dual immunostain positive and negative respectively. These persistence rates were statistically different (p=0.01). This can be explained by the fact that most Hr-HPV infections detected in p16/Ki-67 dual immunostain positive were transforming infections that were more likely to persist than clear. On the other hand, only a few Hr-HPV infections persisted in the p16/Ki-67 dual immunostain negative group because they were transient and non-transforming infections. However, unfortunately, to the best of our knowledge; no other study that determined Hr-HPV in the two groups after follow up has been done. Therefore it was difficult to compare our findings with other previous study. A study by Rodriguez *et al.* reported an overall Hr-HPV persistence of 38%. However, that study did not stratify the women into either p16/Ki-67 dual immunostain positive or negative [16].

Moriarty *et al.* reported that knowing the Hr-HPV results before the interpretation of the Pap smears or the p16/Ki-67 dual immunostain introduces a potential bias to the interpretation which falsely increases the sensitivity of the immunostains.[17] We therefore, blinded our interpreters to the previous Hr-HPV and Pap smear findings. In addition, to make sure we had the best and correct opinions; discrepant findings were reviewed by a third and independent pathologist.

This study had a few limitations. The major one being the lack of a complete disease ascertainment on all women with Hr-HPV+/NILM screening test results using biopsy histological findings, which may lead to an under- or overestimation of the exact performance of dual-stained cytology testing in the triaging of such women. Secondly, we excluded 4.7% of our samples because they did not have the required minimum cellularity. In as much as it appears like a small proportion, we would have appreciated to have them in the study. However, our sample inadequate rate was far much superior to the 46% recorded by Ebisch *et al.* [18].

To summarize, the results of this study have shown that p16/Ki-67 dual immunostaining is a powerful tool in triaging women with Hr-HPV positive/NILM in order to identify those with transforming Hr-HPV infections for colposcopy referral.

5. Conclusions

Approximately 20% of patients with Hr-HPV+/ NILM screening results are positive for the $p16^{Ink4a}$/Ki67 dual immunostain. Hr-HPV infections in $p16^{Ink4a}$/Ki67 dual immunostain positive women are more likely to persist compared to women with negative dual immunostain results.

6. Recommendation

$p16^{Ink4a}$/Ki67 dual immunostain should be done on all patients with Hr-HPV+/ NILM results to stratify them according to the risk of harboring cervical precancerous lesions.

7. Acknowledgements

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