

David Tvildiani Medical University

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**Characteristics of expression p16<sup>INK4a</sup> /Ki-67 dual  
biomarkers in  
cervical cytology material and their role in detection of  
cervical precancerous lesion**

**Thesis**

**of Dissertation for the Academic Degree of PhD in Medicine**

**Tbilisi 2024**

The PhD research was performed at David Tvildiani Medical University,  
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The dissertation could be obtained from the Daphne Hare Medical Library,  
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The Thesis will be distributed on \_\_\_\_\_ 2024

Scientific Secretary

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# General description of the study

## Relevance of the problem

Early detection of precancerous lesions can reduce cancer morbidity and mortality. In terms of objective detection of precancerous lesions in cytological diagnostic material during cervical cancer screening, there are no clear recommendations for the widespread use of cellular biomarkers, based of existing literature. It is mostly due to the paucity of prospective studies, heterogeneity of results, and the small size of the study group on the conventional cytology and immunocytochemical results based on cytological material taken from the cervix of women with pathological screening results. Therefore, the findings do not provide an opportunity to improve the current cyto-histopathological classification and management algorithm for women involved in cervical cancer screening. In countries where screening coverage is low, like in Georgia country, and the possibility of referral for screening materials is limited, due to the low sensitivity of cytomorphology (Colgan et.al. 2001) and the low specificity of HPV testing (Schiffman et.al. 2007), it is not uncommon, that women of young reproductive age with repeated pathological screening (LSIL/ASC-US) results have conducted unnecessary instrumental intervention on the cervix (Habbema et.al. 2017), on the other hand, due to improper assessment of underling precancerous lesions, the risk of developing invasive cancer is high. Despite the achieved results, a enduring cervical cancer screening guideline still needs

to be refined. Therefore, we decided to study the features of the expression of the cell cycle regulatory proteins -p16INK4a (hereafter p16) and Ki-67 biomarkers (Petry et.al. 2011) in the cervical cytological diagnostic material of women with repeated cytopathological results during cervical cancer screening and their role in early detection of cervical precancerous lesions.

### **Aim of the study**

The aim of our study is to determine the possibility of timely detection of precancerous lesion of the cervix with a biomarker-based, non-invasive, objective methodology and by preventing possible cytomorphological errors in cytological screening and improving the current guidelines for cervical cancer screening & management, to decrease morbidity and mortality caused by cervical cancer as well as unnecessary interventions on the cervix in women of reproductive age.

### **Objectives**

- Stain the cervical cytological diagnostic material of women involved in cervical cancer screening with p16/Ki-67 double immunocytochemical method, studying the expression of

biomarkers in cervical epithelial cells and determining immunocytochemical staining categories.

- Study expression of p16 and Ki-67 markers in cytological diagnostic material of the cervix together with the cytomorphological characteristics of the cells.
- Analyse expression of p16 and Ki-67 markers in cervical cytological diagnostic material along with Pap test and histomorphological results.
- Observe the long-term results of the cervical cancer screening.

### **Scientific Novelty of the Research**

For the first time, during our research was conducted p16/Ki-67 dual immunocytostaining of cervical smear in parallel to the pap test, cervical cytologic material collected during opportunistic cervical cancer screening, from different diagnostic center, of women with abnormal previous pap test results.

Despite small size of study group, our results show that expression of p16/Ki-67 dual biomarkers in cervical cytology is statistically significant irrespective to the morphology of stained cell, and sensitivity

to detect high grade cervical intraepithelial neoplasia is much high that it was described by other authors.

For the first time during our research was conducted categorization and analysis of results based on immunostaining and morphology criteria of the stained cells as well according presence of inflammatory infiltrates in cytology diagnostic material.

For the first time during our research was conducted description of screening follow-up results of woman with negative p16/Ki-67 DS results during opportunistic cervical cancer screening

### **Theoretical and Practical Value of Expected Results**

The results of our research on the expression of cell cycle control proteins in the cervical cytological diagnostic material along with the morphological features of the cells during high grade cervical dysplasia, will contribute to the deepening of fundamental knowledge on the mechanisms of precancerous lesions. The results of our study may help in the filling of the gap existing between cell biology and cytopathology and will improve the diagnostic value of cytology in cervical screening. The results of our study may have practical value for early detection of cervical precancerous lesion based on objective criteria and for effective triage and management

of the screening population. The results will be the basis for the development of sustainable guidelines for cervical cancer screening and management, in order to reduce cervical cancer morbidity and mortality and unnecessary interventions on the cervix in women of reproductive age.

### **Approbation of the Research Work**

Dissertation approval took place at David Tvildiani Medical University on December 7, 2023.

Dissertation data and results were presented step by step:

1. Symposium of the Association of Pathologists of Georgia. Tbilisi, Georgia. 2011წ.
2. Regional Congress of Central Europe, “Actual Topics on Woman Health”. Batumi, Georgia. 2012
3. First International conference of “women’s rehabilitation association” (WRA), Healthy family, healthy pregnant woman, healthy child.” Tbilisi, Georgia. 2012
4. Students and Young Scientists Association of Davit Tvildiani Medical University, First Scientific Conference. Tbilisi, Georgia. 2013

5. Students and Young Scientists Association of Davit Tvildiani Medical University, Second Scientific Conference. Tbilisi, Georgia.2014
6. Symposium of Georgian Medical Women's Association. Tbilisi, Georgia.2015
7. Students and Young Scientists Association of Davit Tvildiani Medical University, Eleventh Scientific Conference "Nobel Day" Tbilisi, Georgia. 2022.
8. GAU International Scientific Conference 'Build a Better Future Today with Science'. Tbilisi, Georgia. 2022.
9. [2nd International Conference on Global Advanced Nursing and Healthcare](#)  
August 11-12, 2023 | London, UK. (virtual)
10. TMA International Scientific Conference -Medicine: Innovations and Challenges 2023, (Conference has been accredited by EACCME ® with 15 European CME credits). Tbilisi, Georgia. 30.11.2023

The following Activities were Conducted During Working on the Research:

1. p16/Ki-67 Dual Immunocytochemistry training course, Certified Specialist, 2011 mtm Laboratories AG, Heidelberg, Germany.



2. Implementing Dual immunocytochemistry methodology in first Georgia during 2011-2013.
3. Introduction of teaching practical skills of morphological, immunocyto/histochemical, including double immunocytochemistry diagnosis based on tissue and cytological diagnostic material, Georgian-American University School of Medicine, Pathology Laboratory; 2022 წ.
4. Participation in the discussion of recommendations of “Enduring Consensus Cervical Cancer Screening and Management Guidelines with use of *p16/ki-67 Dual Stain*” for use of p16/Ki-67 Dual stain, developed by the working group of American Society for Colposcopy and Cervical Pathology (ASCCP). June 1,2023.
5. <https://www.asccp.org/PublicComments2023>

Five articles and one abstract have been published around the dissertation.

### **Structure and volume of Dissertation**

The dissertation consists of 7 parts: introduction, literature review, research material and methods, statistical analysis of the

results, discussion, conclusions, practical recommendations. The work is accompanied by a list of used literature.

The work includes 100 printed pages, illustrated with 18 micrographs, 12 diagrams and 15 tables. The work is accompanied by 9 appendices. The bibliography includes 97 sources.

## **Materials and methods of the study**

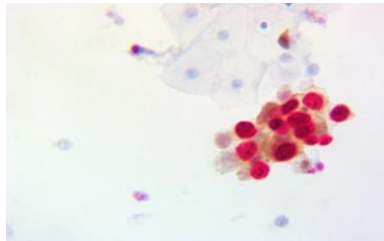
Our study is a cross-sectional design. We studied the cervical material of women between 21 and 65 years of age who, due to the pathological results of the Pap test, were referred for repeated screening. We studied the cytological diagnostic material of the cervix with p16/Ki-67 double immunocytochemistry (for the first time in Georgia), analyzed the obtained results as well as the results of routine cervical screening pathomorphological studies. The reference standard was the results of cervical biopsy H&E histopathology. Within the scope of the study, we studied the results of the Pap test of 162 cases (women), histopathology of 29 cases and HPV-PCR test of 33 cases. Assessment of biomarker expression was performed at three medical centers by independent pathologists. All appropriate smear collection methods for immunocytochemical study were taken into account when collecting cervical material. Immunocytochemical examination of cervical cytological material for dual p16/Ki-67 biomarkers was performed using the CINtec PLUS Kit (REF 9531, mtm laboratories, Heidelberg, Germany) according to the manufacturer's instructions. The Shandon Coverplate TM system was used for staining the slides. p16 immunohistochemical examination of cervical biopsy specimens was performed using the CINtec histology kit (REF 9511, mtm laboratories, Heidelberg, Germany) according to the manufacturer's instructions. The criteria for inclusion in the study were taken into account during the collection of cervical material. Follow-up of screening results after the study was carried out

during the years 2014-2023. The study was carried out based on the conclusion of the 2011 bioethical commission of Davit Tvildian Medical University. The collection of pathomorphological diagnoses of the cervix of the patients, as well as other clinical data, was carried out in the clinics on the basis of medical histories, taking into account the decision of the bioethical commissions of the respective clinics.

## **The results of our study**

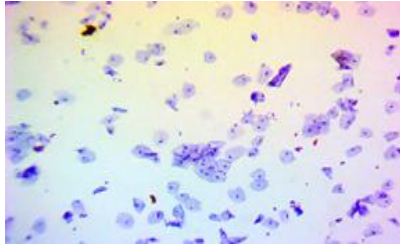
### **Immunostaining results and categorization of results**

According to the manufacturer's instructions, when we interpreted the slides stained by double p16/Ki-67 immunocytochemistry by light microscopy, the presence of more than one epithelial cell with brown cytoplasmic and red nuclear staining in a cell cluster was rated as double p16/Ki-67 positive immunostaining (p16/Ki-67 DS), Figure N1; Cases without expression of p16/Ki-67 DS were rated as negative for staining, Figure N2.



**Figure N1.**

Positive p16/Ki-67DS, with atypia x 400 magnification



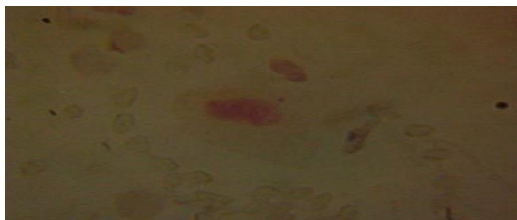
**Figure N2.**

Negative p16/Ki-67DS, without atypia x 100 magnification

Along with the results of immunocytostaining, taking into account the criteria developed by Wentzensen and colleagues (Wentzensen et al 2005) scoring according to the nucleus (Increased nuclear/cytoplasmic ratio, Chromatin granule, Irregular nuclear shape, and Anisonucleosis), we create additional categories, where according to the characteristic of the nucleus. The presence of one or more of the above nuclear features corresponded to the atypical cell category characteristic of dysplasia: 1) p16/Ki-67 DS positive category, stained cells with or without atypia. 2) p16/Ki-67 DS negative:

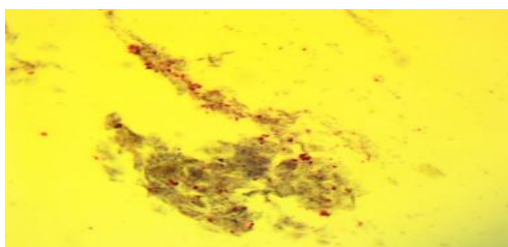
- a) p16 staining category, stained cells with or without atypia.
- b) Ki-67 staining category, stained cells with or without atypia.
- c) separate p16 and Ki-67 staining category ( without double staining), with or without atypia.
- d) cells without staining category, with or without atypia.

In each category we studied presence of the inflammatory infiltrates. Different staining categories are presented in the Figures N3-7 and Diagram N1.



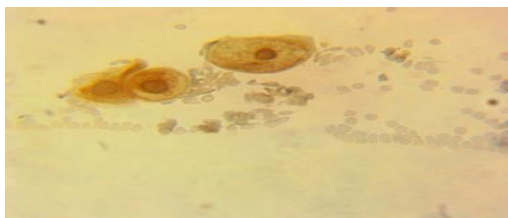
**Figure N3.**

Positive p16/Ki-67 DS, without atypia; x 400 magnification



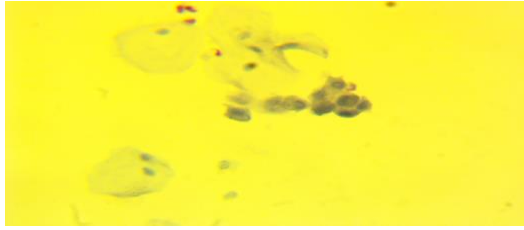
**Figure N4.**

Negative p16/Ki-67 DS, separate p16 and Ki-67 staining. x 100 magnification



**Figure N6.**

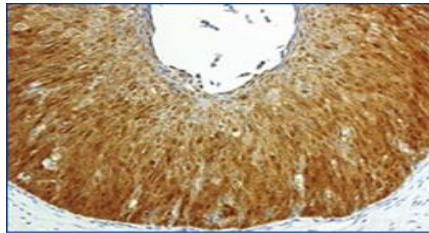
Negative p16/Ki-67 DS, p16 staining with atypia. x 400 magnification



**Figure N7.**

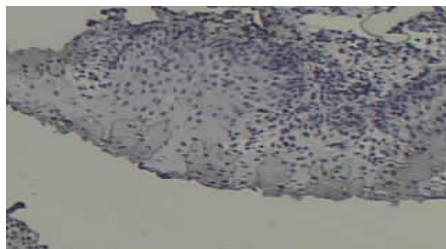
Negative p16/Ki-67 DS, without atypia. x 400 magnification

We interpreted the p16 IHC staining results of the histological material according to the results of the LAST study (Darragh et.al. 2012), where the p16 IHC positive category corresponded to HSIL, and the p16 IHC negative category corresponded to <CIN1, (Figures N8-9).



**Figure N8.**

Positive p16 IHC x 100 magnification.



**Figure N9.**

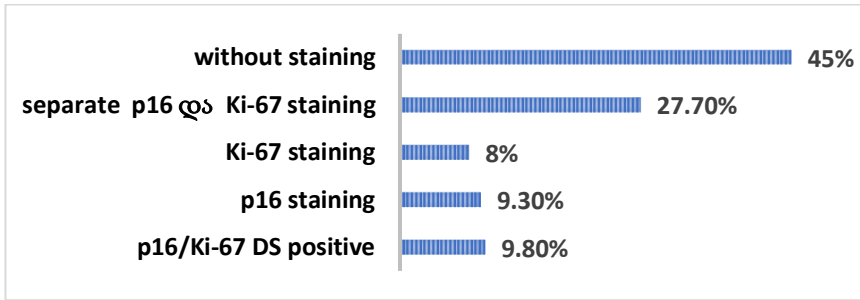
Negative p16 IHC x 100 magnification.

## **Statistical analysis of the results**

Taking into account the criteria for inclusion in the study, the obtained data were entered into the database and statistical analysis was performed. Data were analyzed with SPSS software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). For comparison between categorical variables,  $\chi^2$  test, Fisher's exact test, Cohen's kappa ( $\kappa$ ) statistic were used, considering the fit to the data. The accuracy of detection of high-grade dysplasia (CIN2+) in the study of cervical cytological diagnostic material by immunocytochemical and cytomorphological methodologies was evaluated by measuring the sensitivity, specificity, positive (PPV) and negative (NPV) predictive values and accuracy of the diagnostic tests. The gold standard of diagnosis was histomorphological result.

In our study, the categories of p16/Ki-67 DS immunocytochemical staining of slides of the cervical cytological material are given in chart N1. The consensus of the diagnosis was 100% in the assessment of p16/Ki-67 double immunocytochemical staining of cytology slides among three independent pathologists.

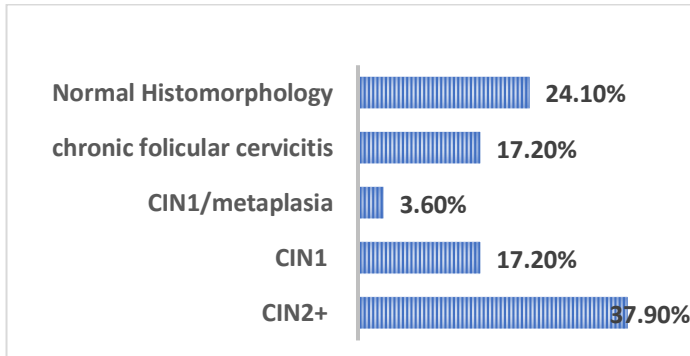




**Diagram N1.**

Categories of slides of p16/Ki-67 DS immunocytochemical staining.

H&E histopathological results of cervical biopsy material are given in the Diagram N2;



**Diagram N2.**

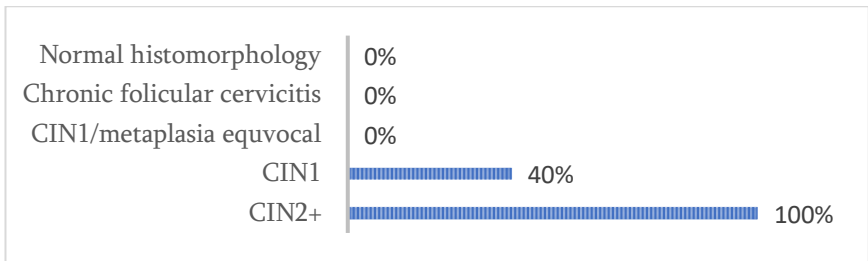
Distribution of histopathology results .

The results of positive p16/Ki-67 DS immunocytochemistry of cervical smear and H&E histopathology of corresponding cervical biopsy material are shown in Table N1. Diagram N3.

H&E Histopathology	Histopathology, n	p16/Ki-67DS immunocytochemistry, irrespective to the morphology of stained cells	Percentage of positive dual staining
CIN2+	11	11	100%
CIN1	5	2	40%
CIN1/metaplasia equivocal	1	0	0%
Chronic follicular cervicitis	5	0	0%
Normal histopathology	7	0	0%
Total	29	13	

**Table N1.**

Results of p16/Ki-67 DS immunocytochemistry and H&E histopathology



**Diagram N3.**

Distribution of H&E histopathological findings in cervical smear with positive p16/Ki-67 DS

Statistical analysis based our data revealed, that correlation between p16/Ki-67 dual biomarker expression, irrespective of morphologic features of stained cells, in cervical cytological diagnostic material and histopathological CIN2+ lesions is statistical significant,  $p=2,5 \times 10^{-6}$ , Table N2.

	<i>p</i> value, correlation between p16/Ki-67DS and H&E CIN2+
p16/Ki-67DS immunocytochemistry, irrespective to the morphology of stained cells	$p=2,5 \times 10^{-6} \ll 0,001$
p16/Ki-67DS immunocytochemistry with cellular atypia	$p=5,75 \times 10^{-6} \ll 0,001$

**Table N2.**

Correlation of high-grade uterine dysplasia and p16/Ki-67DS positive results

The results of positive p16/Ki-67 DS immunocytochemistry with cellular atypia and H&E histopathology of the corresponding biopsy material are given in Table N3.

Histopathology n	p16/Ki-67 DS immunocytochemistry positive , with cellular atypia
CIN1 n=5	1
CIN1/metaplasia n=1	0
CIN2+ n=11	10
Cronic follicular cervicitis n=5	0

**Table N3.**

Distribution of H&E histopathology and p16/Ki-67 DS immunocytochemistry results with atypia

Statistical analysis of our data revealed, that considering cellular atypia of p16/Ki-67 DS positive results of cervical cytological diagnostic material and histopathological CIN2+ lesions is statistically significant,

$p=5,75 \times 10^{-6} \ll 0,01$ , Table N2.

In our study, the sensitivity, specificity, PPV, NPV and accuracy of detection of high-grade dysplasia (CIN2+) by p16/Ki-67 DS immunocytochemistry in uterine cytological material, considering histopathology as the gold standard of diagnosis, are given in Table N4.

Test to detect CIN2+ in cervical smear	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV %	NPV %	Accuracy%
p16/Ki-67 DS positive, irrespective to the morphology of stained cells	100	89	85	100	93
p16/Ki-67 DS positive with cellular atypia	91	94	91	94	93
Pap test	9	100	85	64	66

**Table N4.**

Sensitivity, Specificity, PPV, NPV and Accuracy of CIN2+ Detection

Considering the atypia of cells stained by immunocytochemistry in the cytological diagnostic material, the specificity, PPV and NPV of detection of high-grade dysplasia of the cervix was improved, although the sensitivity decreased, while the accuracy remained unchanged. Taking into account the morphological characteristics, Type 1 error was detected. It is worth noting, that there was no variability in interpretation between cytotechnologists and pathologists when evaluating the results of p16/Ki-67 DS immunocytostaining, although variability was detected

when adding a component of morphological evaluation along with marker expression. All but one case of positive p16/Ki-67 DS cervical cytology, where the H&E histopathology result of the corresponding biopsy material was CIN2+, showed cellular atypia of the double-stained cells in the cytological material. At the same time, in two cases of a positive p16/Ki-67 DS cytology slide, in which the result of H&E histopathology of the corresponding tissue material was CIN1, in one case, cellular atypia was detected during immunocytochemistry staining, and in the other case, double staining without atypia, Table N5.

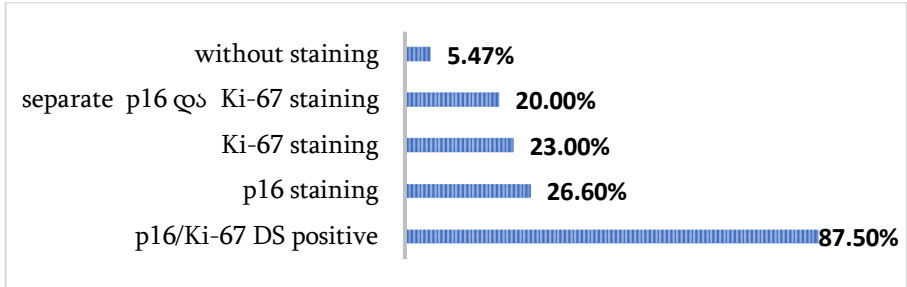
Histopathology	p16/Ki-67 DS positive, without cellular atypia
CIN1 n=5	1
CIN1/metaplasia n=1	0
CIN2+ n=11	1
Chronic follicular cervicitis n=5	0

**Table N5.**

Distribution of H&E histopathology and p16/Ki-67 DS immunocytochemistry results without atypia

Based age grouping of the woman, a positive result of p16/Ki67 DS was detected in the cytological smear of the cervix in the age range of 28-50 years. No influence of female age on the expression variable of double biomarkers p16/Ki67 in cervical diagnostic material was detected ( $p>0.05$ ). However, p16/Ki67 DS positive results were more prevalent in women over 40 years of age.

Categories of cytological slides with immunostaining of epithelial cells and morphological features are given in diagram N4. The highest rate of atypia was found in the category of positive double staining.



**Diagram N4.**

Frequency of atypical cells in different categories during p16/Ki-67 DS immunocytochemistry

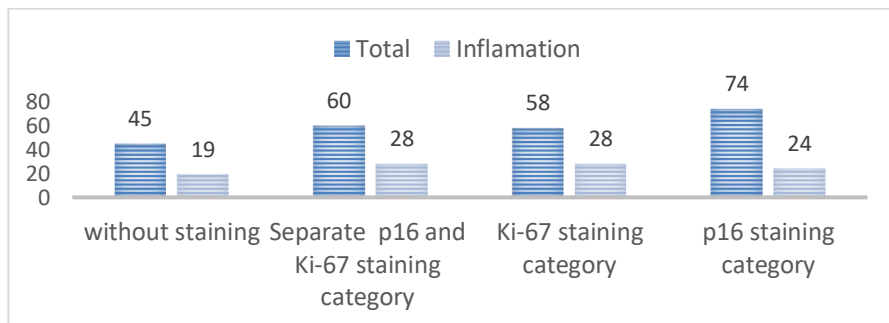
When comparing different categories according to immunostaining and cellular atypia, in p16/Ki-67 DS negative category, no significant correlation was revealed,  $p > 0.57$ ,  $p > 0.73$ ,  $p > 0.81$ , Table N6.

	Cellular atypia of stained cells	Inflammatory infiltrates
P 16 staining category	$p > 0.57$	$p > 0,29$
Ki-67 staining category	$p > 0.73$	$p > 0,18$
Separate staining category	$p > 0.81$	$p > 0,89$

**Table N6.**

Correlation of cellular atypia and inflammatory infiltrates in different immunostaining categories

The categories of cytological slides according to different immunostaining of epithelial cells and the presence of inflammatory infiltrates are given in diagram N5.



**Diagram N5**

Frequency of inflammatory infiltrates in different staining categories with p16/Ki-67 DS negative slides

Statistical analysis between inflammatory infiltrates and different staining groups (p16 positive, Ki-67 positive, separate p16 positive and Ki-67 positive) of p16/Ki-67 DS negative category, doesn't reveal statistically significant correlation,  $p > 0.29$ ,  $p > 0.18$ ,  $p > 0.89$ , Table N6.

Out of the 29 histopathology results of cervical biopsy, two cases was CIN2/CIN3, and one case was CIN1/metaplasia. On p16 IHC staining of the equivocal histological material, the cytology slides corresponding to the p16 IHC positive cases were immunocytochemically p16/Ki-67 DS positive. The results are given in Table N 7.

Equivocal H&E histopathology	p16/Ki-67 DS positive	p16 IHC positive	p16 IHC negative
CIN1/metaplasia, n=1	0	0	1
CIN2/3, n=2	2	2	0

**Table N7.**

Distribution of H&E histopathological and immunostaining results in cervical diagnostic material

The Pap cytology results of 10 women with DS-negative cervical cytology, who had a mean age of 41,5 years at enrollment, were assessed after a median follow-up time of 6,5 years (ranging from 3 to 10 years). The results are provided in Table N8.

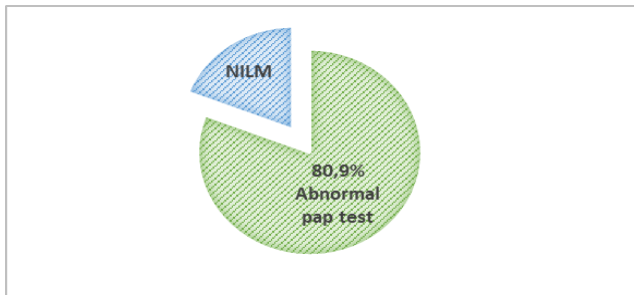


Time of Initial screening	Age at Initial screening	Results of p16/Ki-67 DS at initial screening	Results of Pap test at initial screening	Time of follow up screening	Results of follow up Pap test screening
2011	30	Negative	LSIL	2014	NILM
2011	34	Negative	LSIL	2015	NILM
2012	40	Negative	NILM	2016	NILM
2011	60	Negative	NILM	2017	NILM
2011	39	Negative	LSIL	2018	NILM
2012	40	Negative	LSIL	2018	NILM
2011	41	Negative	LSIL	2019	NILM
2012	42	Negative	LSIL	2020	NILM
2011	50	Negative	LSIL	2021	NILM
2011	63	Negative	LSIL	2021	NILM

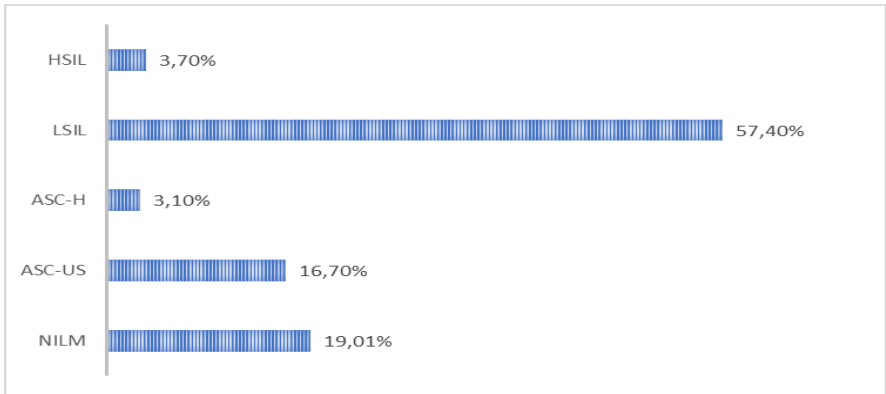
**Table N8.**

Median 6,5 years follow-up cytology results in p16/Ki-67 DS negative women.

Out of all Pap cytology results, various pathologies (SIL, ASC) were detected in 80.9% cases, based on the medical histories of the women of our study (Diagram N6.a). The most frequent and the rarest Pap cytology results were LSIL and ASC- H, respectively (Diagram N6.b).



a.



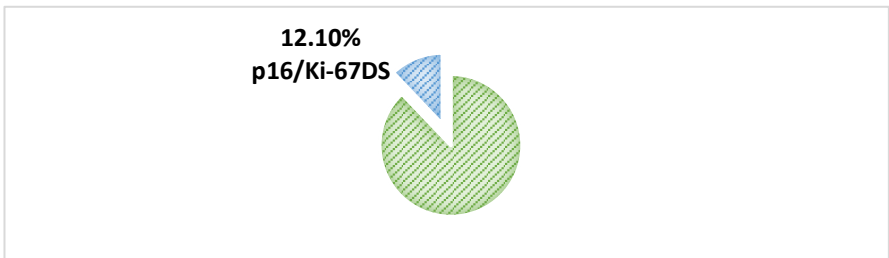
b.

**Diagram N6.**

A. The overall incidence of an abnormal Pap test.

b. Distribution of Pap test results.

The percentage distribution of abnormal Pap test results based Bethesda categories was as follows: ASC-US 27 (20.6%); ASC-H 5 (3.8%); LSIL 93 (70.9%); HSIL 6 (4.6 %). p16/Ki-67 DS positive result was in 12,2 % out of all woman with abnormal Pap test results, Diagram N7.



**Diagram N7.**

Incidence of dual p16/Ki-67 staining in women with abnormal Pap test results.

p16/Ki-67 DS immunocytochemistry staining of cervical cytology material of women with the same pathological results by Pap test, revealed different categories based on biomarker expression, Table N9.

	Pap test NILM (31)	Pap test ASC-US (27)	Pap test ASC-H (5)	Pap test LSIL (93)	Pap test HSIL (6)	Total
p16/Ki-67 DS positive	0	1	2	11	2	16
p16 staining	4	3	0	6	2	15
Ki-67 staining	3	0	0	10	0	13
Separate p16 and Ki-67 staining	7	13	1	23	1	45
Without staining	17	10	2	43	1	73
Total	31	27	5	93	6	162

**Table N9.**

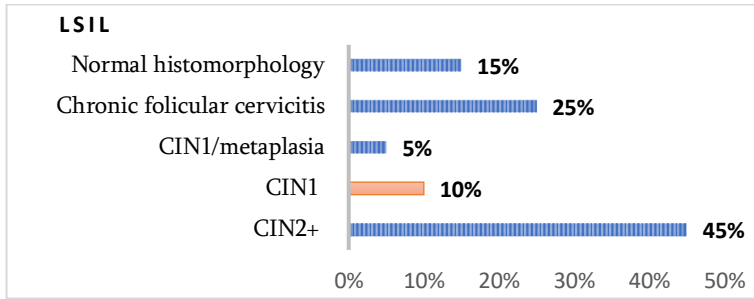
Distribution of p16/Ki-67 DS immunocytochemical staining and Pap test results of cervical cytology diagnostic material.

The distribution of histopathology and Pap test results of the women included in our study is shown in Table N10, Diagrams N8 and N9.

	NILM	ASC-US	ASC-H	LSIL	HSIL	Total
CIN2+		1		9	1	11(37,9%)
CIN1			3	2		5(17,2%)
CIN1/metaplasia				1		1(3,6%)
chronoc follicular cervicitis				5		5(17,2%)
Normal histomorphology		4		3		7(24,1%)
Total	0	5(17,2%)	3(10%)	20(69%)	1(3,4%)	29

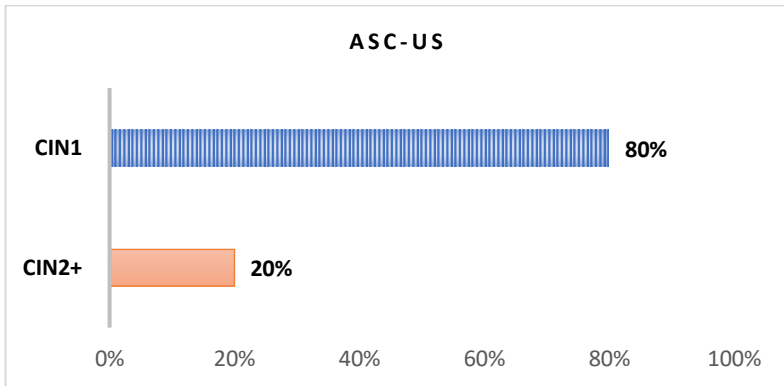
**Table N10.**

Distribution of Pap test and H&E histopathology results.



**Diagram N8.**

Distribution of H&E histopathological results  
in LSIL group with Pap test



**Diagram N9.**

Distribution of H&E histopathological results  
in ASC-US group with Pap test

According to the data of our study, the sensitivity, specificity, PPV, NPV and accuracy for Pap test in detection of CIN2+ are given in Table N4.

In order to detect high-grade dysplasia of the cervix, when comparing the results obtained from the examination of cervical cytological material by p16/Ki-67 DS immunocytochemical and Pap cytological methods, a low rate of concordance was revealed, according to Cohen's kappa ( $\kappa$ ) statistical analysis of the data (Table N11).

p16/Ki-67/Pap test	Total 162
Negative/negative	144(88%0
Positive/positive	2(1,23%)
Positive /negative	12(7,4%)
Negative/positive	4(2,46%)
Agreement, %	% of agreement:90,12
Cohen's kappa ( $\kappa$ )	0,15 Slight agreement

**Table N11.**

Cohen's kappa ( $\kappa$ ) analysis of p16/Ki-67 DS and Pap test results

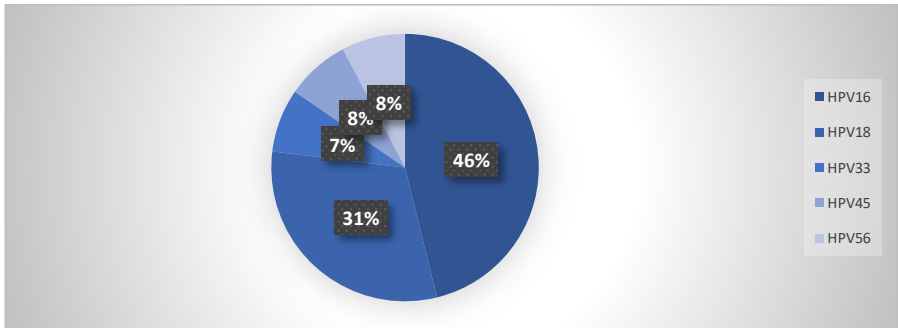
HPV-PCR test results were available in 33 cases out of 162 women in our study. Of these, 7 cases had LR-HPV and 8 HR-HPV positive PCR results, while 18 cases were negative for HPV infection. From the mentioned 33 cases, the relevant histopathology results were available in 7 cases. The distribution of p16/Ki-67 DS, HPV PCR, Pap test, and histopathological results is given in Table N12.

HPV PCR	H&E CIN2+	Chronic follicular cervicitis	H&E N	Pap test LSIL	Pap test ASC- US	P16/Ki67
HPV negative		3		18		
LR-HPV		1	1		4	
HR-HPV	1			4	2	1

**Table N12.**

Distribution of p16/Ki-67 DS, HPV PCR, Pap test and histopathological results.

The prevalence of HR-HPV infection in our study was 24%. The prevalence of HR-HPV 16,18,33,45 and 56 genotypes is given in the Diagram N10.



**Diagram N10.**

Prevalence of HR-HPV genotypes.

## Discussion

Pap cytology based screening was introduced half a century later in Georgia compared to USA and the leading countries of Europe.

Therefore, taking into account the possibility of observing long-term outcomes in these countries, our aim was to identify an alternative, improved possibility of early detection of cervical precancerous lesions. At the beginning of our study, in the background of three years of experience of pap cytology-based screening in Georgia, taking into account the country's small population and low screening coverage at country level, selecting an unbiased study sample was a main task for us. First of all, it was important for us to have the opportunity to evaluate the expression of biomarkers in the cytological material of the uterus by independent experts, on the other hand, the results of the expression of biomarkers should be compared with the results of the morphological (cyto/pathological) examination of the cervix evaluated by independent pathologists. Of particular interest was the presence of different Pap cytology results by different laboratories of the same woman. In order to solve the mentioned task, knowledge and skills about immunocytochemical diagnostics were shared with cytotechnologists, pathologists, gynecologists of different medical centers. Finally, it was possible that collected cervical material from nine medical institutions was processed and studied by independent gynecologists, cytotechnologists and pathologists, respectively, and a statistical analysis of the results was carried out.

Our study revealed a statistically significant correlation between p16/Ki-67 DS positive results in cervical cytological diagnostic material and the diagnosis of precancerous lesion (CIN2+) in cervical tissue material by H&E histopathology. According to the results of our study, the expression of dual p16/Ki-67 biomarkers in cervical diagnostic material has high sensitivity and diagnostic role in screening for CIN2+(HSIL) cervical precancerous lesions, regardless of the morphological characteristics of the cells. The results of the p16/ Ki-67 DS studies varies, according to one group of authors (Schmidt et.al.2011), in the cytological diagnostic material of the cervix, p16/ Ki-67 DS immunocytochemistry for detecting CIN2+ lesions is characterized by high sensitivity, irrespective of the morphology of the stained cells. According to the study of the second group of authors (Prevodnik et.al. 2019), the high sensitivity of detecting high-grade dysplasia is manifested along with the presence of p16/Ki-67 DS along with cell atypia of stained cells. The results of studies on p16/ Ki-67 DS and high-grade cervical dysplasia by various authors together with the results of our study are shown in Table N13.



	Study	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV %	NPV %
p16/Ki-67 DS	Results of our study	100,0%	89,0%	85,0%	100%
	Gajsek, Ursula Salobir et al.	88,1%	65,2%	44,6%	94,5%
	Ikenberg, Hans et al.	86,7%	95,2%	15,6%	99,9%
	Luttmer, R., Dijkstra, M., Snijders, P. et al.	93,8%	51,2%	29,9%	2,6%
Pap test	Results of our study	9,0%	100,0%	85,0%	64,0%
	Nkwabong, Elie et al.	55,5%	75,0%	88,2%	33,3%
	Ikenberg, Hans et al.	68,5%	95,4%	13,3%	99,7%

**Table N13.**

Sensitivity, specificity, PPV and NPV of CIN2+ detection according to different studies.

In our study, the influence of female age on the expression of double p16/Ki-67 biomarker in cervical cytological material was not revealed ( $p>0.05$ ). However, within our study, the frequency of expression of dual biomarkers was more frequent in women over 40 years of age. The correlation of female age and the expression rates of double p16/Ki-67 biomarkers in cervical cytology was also not revealed in the study of Luttmer and colleagues (Luttmer et.al. 2016).

In our study, when examining cervical cytological material by double immunocytochemistry, correlation between overexpression of individual p16 or Ki-67 proteins in cells and the presence of cellular

atypia doesn't revealed ( $p>0.05$ ). Results of studies on different immunostaining categories and morphology in double-stained cytological material are scarce. In this direction, the results of large sized randomized studies are interesting. According to various studies, p16 immunocytochemistry in combination with morphological markers improves the ability to detect high-grade cervical dysplasia (Wentzensen et al 2005).

Studying the correlation between immunostaining indicators of epithelial cells and inflammatory infiltrates on a cytological slide during immunocytochemical staining revealed that there is no statistically significant relationship between these two indicators ( $p > 0.05$ ). Studies on the effect of inflammatory infiltrates on the expression of p16 and Ki67 biomarkers in cervical diagnostic material are scarce, however, according to different authors (Regauer et.al. 2007;Sobecki et.al. 2017) the expression of p16 positive cells and Ki-67 positive cells occurs during metaplasia and regeneration of cervical epithelial cells, respectively.

In our study, while studying the expression of p16/Ki-67 biomarkers in the cytological material of the cervix, it was revealed that the simultaneous expression of two proteins, the cell cycle inhibitor p16 protein and the cell proliferation indicator, Ki-67 protein, is associated with the presence of histopathologically confirmed high-grade dysplasia of the cervix. It was also revealed that the presence of different categories

of staining in the cells is not affected by the age of the woman and the presence of inflammatory infiltrates. It was also revealed that the morphological change of the cell nucleus in the cytological material does not affect the overexpression of p16 and Ki-67 protein separately. According to different authors, regarding the expression of p16 and Ki-67 proteins: protein p16 stops cell division and participates in the physiological aging process of the cell (Selvam et.al. 2018); Overexpression of p16 protein occurs in cells with persistent HR-HPV infection by viral E7 oncoprotein through inhibition of cellular protein Rb (Duncan et.al. 2013); Chronic overexpression of p16 protein in epithelial cells activates cell proliferation and promotes tumor development by paracrine stimulation of Wnt signaling cascade (Azazmeh et.al. 2020); Among gynecological malignancies, overexpression of p16 protein is observed in high-grade serous tumors of the endometrium (Yemelyanova et.al. 2009), as well as in high-grade serous tumors of the ovary (Vang et.al. 2009); Ki-67 expression is observed in mitotically active both normal and neoplastic cells (Gerdes et.al. 1983); Simultaneous expression of two proteins p16 and Ki-67 in cervical cytological diagnostic material was detected in Pap negative HPV positive women with histologically high grade dysplasia (Petry et.al. 2011). In our study, overexpression of p16 and Ki-67 biomarkers appear in cervical cytological material of women with repeated pathological

results by Pap test. Despite the results of the existing studies, it is a matter of further study to determine the molecular mechanisms that cause the overexpression of both proteins simultaneously in the cell, including whether the overexpression of the cell proliferation indicator Ki-67 protein is related to the overexpression of the cell cycle inhibitor p16 protein.

In our study, sensitivity of Pap cytology to detect high grade cervical precancerous lesions (CIN2+) was low (Table N3). Out of all cytology diagnosis, abnormal pap test results were detected in 80.9% of cases, from which LSIL, ASC-US, ASC-H and HSIL results were distributed as follows: 71%, 20.7%, 3.8% and 4.5%, respectively. Data on the prevalence of Pap test results vary according to the scientific literature. According to the research of one group of authors (Arslan et.al. 2018), the prevalence of pathological Pap test results is 4.7%, while the prevalence of ASC-US, LSIL, ASC-H and HSIL categories are 65%, 27%, 3.4%, 2.4%, respectively.

In our study, by studying the expression of dual biomarkers p16/Ki-67 in the cervical cytological material of women with repeated pathological SIL and ASC results during cytological screening, the possibility of detecting underlying high-grade dysplasia was revealed with high reliability. The sensitivity and specificity of detecting CIN2+ by p16/Ki-67 DS in the LSIL category are 100% and 91%, respectively, and

in ASC (ASC-US and ASC-H) category it is 100% and 86% respectively. According to the research results of one group of scientists (Schmidt et al. 2011), the sensitivity and specificity of detecting CIN2+ with p16/Ki-67 DS in the LSIL category are 94.2% and 68.0%, respectively, and in the ASC group, the sensitivity and specificity are 92.2% and 80.6%, respectively.

In our study, a low level of concordance was revealed between p16/Ki-67 DS immunocytochemistry and Pap cytological results to detect high-grade cervical dysplasia in cervical smear (Table N11).

long-term observation of screening results among woman with p16 /Ki-67 DS negative results, doesn't reveal any pathologic results after 3-10 years of follow-up. Due to the small amount of data and low reproducibility of cytological screening, it is impossible to establish a correlation with the long-term results of negative cervical cytology with p16 /Ki-67 DS. However, according to our results, negative p16 /Ki-67 DS results of the cervical smear, may be encouraging in terms of increasing the screening interval. According to modern screening guidelines, the screening interval is 5 years if the screening result is normal. The results of subsequent randomized trials are interesting in this regard. According to a study by one group of authors (Clarke et. al. 2019), women with negative p16/Ki-67 DS on cervical cytology have a significantly lower risk of developing CIN2+ within the next 5 years.

In our study, two cases of p16/Ki-67 DS positive immunocytochemistry results, which corresponding H&E histopathology results CIN1, were of particular interest. In one case, double-stained cells were with atypia, in the other case without atypia (Table N5). Due to insufficient data, our study could not determine whether a positive p16/Ki-67 DS immunocytology result is a predictor of the development of high-grade dysplasia in women with CIN1 histology. At this stage, there is little information regarding this, which gives motivation to continue similar type of research.

The use of p16 IHC has been recommended since 2012 for ambiguous histopathological diagnosis of the cervical precancerous lesion, according to the relevant guideline (Darragh et.al. 2012). In our study, the use of p16IHC in the case of 3 equivocal histopathological samples, made it possible to differentiate cervical precancerous lesions from non-neoplastic lesion. We think that, similar to the improvement of diagnostics by the integration of markers in the histological diagnostic material, the integration of biomarkers in the cervical cytological material creates an opportunity to differentiate neoplastic cellular changes from non-neoplastic cellular changes by clear, objective criteria, and this possibility is provided by the simultaneous detection of the expression of p16/Ki-67 biomarkers in cytology material.

During our study period, given the opportunistic nature of screening and the limited availability of HPV PCR testing, only 33 case of HPV PCR test results could be retrieved (Table N12, Diagram N10). In our study the prevalence of HR-HPV is 24%, of which HPV16 and 18 genotypes accounted for the majority of cases (>50%). Among our data, only for one case (LSIL by Pap cytology) with a diagnosis of CIN3 histopathology and corresponding p16/Ki-67 DS positive result, HR-HPV PCR result was positive. Due to the small amount of data, it was not possible statistically to compare the results of double immunocytochemistry and HR-HPV, although in our study it was revealed that, out of 8 HR-HPV PCR positive cases only in one case p16/Ki-67DS was positive. Taking into account, that p16/Ki-67 DS has high sensitivity of detection of cervical high grade interepithelial neoplasia, it is possible to assume high prevalence of HR-HPV infection and low specificity of HR-HPV PCR in detecting of precancerous lesions in screening population. although the results of randomized studies are important in this regard. Detection of HPV genotypes is important for better planning of preventive measures by adopting corresponding vaccines in the country. According to various authors, a particularly high prevalence of HR HPV genotypes is observed in developing countries.

Based on the study of the medical histories of the women involved in our study, various comorbid conditions were identified, although the

relationship between the mentioned conditions and cervical pathologies could not be studied.

Limitations of our study were the size of the study group and possible selection bias. The study group consisted of opportunistic cervical cancer screening participants with a history of abnormal Pap test results. p16/Ki-67 DS immunocytochemical testing of cervical material was performed in Georgia in three laboratories from March 2011 to December 2013. The following factors may have influenced the size of the study material and the selection bias: 1) Immunocytochemical examination was offered to all the women mentioned above. However, despite repeated abnormal Pap cytology results or non-identical cytology results from different medical centers, not all had access to p16/Ki-67 DS testing. During 2.5 years, we were able to study the cytological material of only 169 women on a double immunocytochemical study, of which 7 were unsatisfactory for study. 2) Cervical biopsy was performed on the recommendation of gynecologists. Not all women were offered a biopsy in accordance with the cervical cancer screening protocol, and despite the recommendation, not all women underwent a biopsy. In some cases, cryodestruction of cervical lesions was performed without collection of histological material. Therefore, only 29 H&E histopathology results of 29 biopsy materials were available to us, of which we performed p16 IHC examination based on available paraffin block material in 3 cases. Unfortunately, after the



mentioned period, we did not have the opportunity to extend study group further in Georgia. Despite the limitations discussed above, we decided to process the collected material, study it and publish the analysis.

The strength of our study lies in the fact, that cervical material collection and examination were performed simultaneously with pathomorphological and immunostaining methodologies. Immunostaining and collection of pathomorphological results were performed on cervical material collected from different medical centers, and diagnosis was performed by independent experts in the field of clinical pathology.

The results of our research revealed, that the simultaneous overexpression of two proteins, the cell cycle inhibitor p16 protein and the cell proliferation indicator Ki-67 protein, is associated with cell oncotransformation. Overexpression of only one of the proteins in the cells does not affect the presence of dysplastic changes in the cells. The obtained results give us the opportunity to prove that biochemical changes precede cyto-morphological changes. On the other hand, cyto-morphological changes are not always related to neoplastic processes. Therefore, we can conclude, that the integration of relevant biomarkers in the cell improves the diagnostic value of cytology. This finding, among other factors, may explain why there may be high rate of LSIL or ASC-US

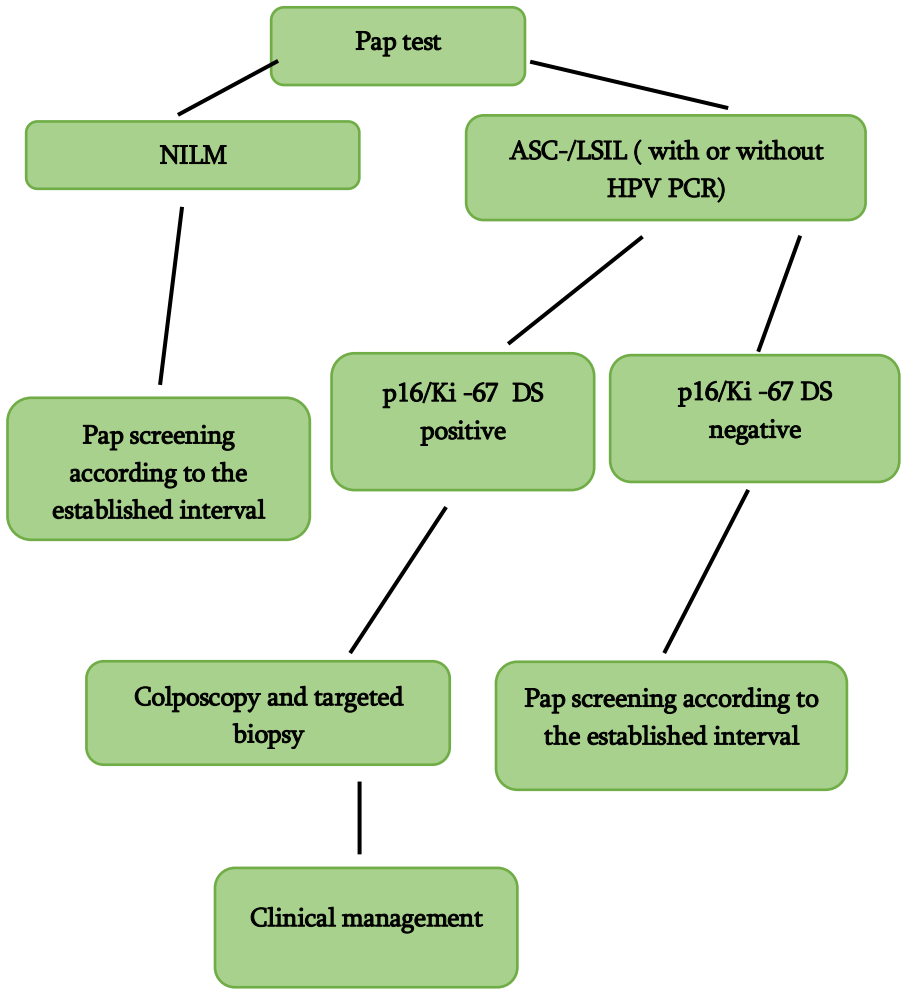
during screening or low reproducibility of the results, while SIL is not confirmed during histopathology examination of biopsy material at all.

Despite the relatively small size of a sample in our and others' studies, an important benefit lies in the fact, that the results give opportunities to make revision of the existing classification of precancerous lesions of the cervix, based on etiopathogenesis and immunophenotype of the lesions, by the integrating of dual p16/Ki-67 biomarkers in cytology (Table N14). The results of our study may give opportunities to make revision of an screening algorithm to build up enduring cervical cancer screening guideline (Diagram N12), which may improve outcomes of cytological screening, especially during triage of ASC/LSIL category.

Etiopathogenesis	HR-HPV infection		Cell cycle dysregulation related to the persistent HR-HPV infection	Invasive carcinoma
Pap test	NILM	ASC-US/LSIL	HSIL	Invasive carcinoma
p16/Ki-67DS immunocytochemistry	p16/Ki67DS negative		p16/Ki-67 DS positive	Invasive carcinoma
H&E histopathology	N	LSIL (CIN 1)	HSIL (CIN 2+)	Invasive carcinoma
P16 IHC	p16 IHC negative		p16 IHC positive	Invasive carcinoma

**Table N14.**

Classification of cervical precancerous lesions based on etiopathogenesis and morphological/immunophenotypic characteristics.



**Diagram N11.**

A possible management algorithm for cervical cancer screening with the use p16/Ki-67 DS.

Based on the results of our research, we can conclude, that the of integration of p16/Ki-67 dual biomarkers in cervical cytological material has greater diagnostic value during cervical cancer screening compared to cytological screening alone. Current methodologies of cervical cancer screening significantly decrease cancer burden, however, the low sensitivity of the pap test, the complexity and less compliance of the existing cyto-histological classification, as well as the low specificity of PCR HPV and the high prevalence of HPV infection remain challenges for both clinicians and patients. The integration of biomarkers in cytology may be important in improving the diagnostic role of cytology to overcome the difficulties associated with differentiating between neoplastic and non-neoplastic processes in cytology. The use of p16/Ki-67 DS in cervical cancer screening can improve the clinical management of women involved in screening, which, on the one hand, by early detection and subsequent appropriate treatment of precancerous lesions will reduce the burden caused by cancer, on the other hand, it will reduce reproductive health problems related to the unnecessary cervical interventions in young women. Despite the high cost of per test, p16/Ki-67 DS may be effective in the long term, especially in the triage of ASC/LSIL categories of pap cytology screening. In low-income countries, where screening is opportunistic and there is a paucity of pathological diagnostic referral centers, p16/Ki-67 DS can be considered as a quality control tool for cytological screening.

## Conclusions

1. In the Georgian cervical cancer screening population, study of p16 and Ki67 biomarker in cervical cytology material of woman with previous SIL or ASC pap test results, revealed the following staining categories: 1) p16 staining, 2) Ki-67 staining, 3) p16 and/or Ki-67 staining 4) p16/Ki-67 dual positive 5) negative for staining. According to the expression of biomarkers, the most frequent categories was p16 and/or Ki-67 negative cytology category and the rarest was Ki-67 positive cytology category. In parallel mode, in the same group of women, when studying the cervical cytological diagnostic material by Pap test, according to the Bethesda system classification, the following categories were identified: NILM, LSIL, HSIL, ASC-US(H); LSIL was the most common, and ASC-H was the rarest category; According to the histopathological results (control group) of the cervical biopsy material of the same group of women N, LISL and HSIL (WHO classification) categories were identified (N, CIN1 and CIN2+ by Koss classification, respectively), of which the HSIL(CIN2+) category was the most frequent.

2. Expression of p16/Ki-67 dual biomarkers in epithelial cells of cervical cytology material, irrespective of morphology of stained

cells, is statistically significant to detect high grade cervical intraepithelial lesion, with considering histopathology as a gold standard of diagnosis.

3. Simultaneous expression of p16/Ki-67 dual biomarkers in epithelial cells of cervical cytological diagnostic material, shows significantly improved sensitivity, accuracy and reproducibility of detection of precancerous lesions compared to Pap cytology.

4. The age of woman does not affect the expression of p16/Ki-67 dual biomarkers in cervical cytology material during cervical cancer screening, although the expression of p16/Ki-67 dual biomarkers is more common in women older than 40 years of age.

5. Overexpression of individual p16 or Ki-67 proteins during dual immunostaining of cervical cytology material does not affect the morphology of stained epithelial cells (biomarker expression vs cell morphology).

6. The presence of inflammatory infiltrates in cervical cytological material does not affect the overexpression of p16 and/or Ki-67 proteins in epithelial cells (biomarker expression vs inflammatory infiltration).

7. Observation of the long-term, an average 6,5 years, follow-up of cervical cytology-based screening of the woman with negative p16/Ki-67DS, revealed no abnormal screening results.

8. Integration of p16/Ki-67 biomarkers in cervical cytology diagnostic material fills the gap between molecular biology and cytopathology and improves the diagnostic value of cytology.



## **Practical recommendations**

### **For experts:**

The integration of p16 and Ki-67 biomarkers in cervical cytological diagnostic material reveals the possibility of improving cyto-histological classification of cervical precancerous lesions and recommendations in cervical cancer screening and management guidelines.

### **For clinical pathologists:**

Integration of p16/Ki-67 biomarkers in cervical cytological diagnostic material improves the ability of objective detection high-grade dysplasia and objective differentiate precancerous from non-dysplastic cytology.

### **For clinicians:**

Identification of high-grade dysplasia is the most important issue in the screening population, especially in the ASC-US/LSIL group, that determines the effectiveness of the clinical and economic outcomes of screening, therefore, considering the expression rate of dual biomarkers p16/Ki-67 as an indicator of high-grade precancerous lesion, will help gynecologists in making effective decisions during clinical management.

**For women involved in screening:**

Use of p16/Ki-67 DS during cervical cancer screening will reduce stress related to the repeated confusing pap cytology results (ASC, SIL) and unnecessary medical interventions. Objective methodology will increase confidence during screening, the number of women involved in the screening may rise, which in turn will contribute to the early detection of pre-cancer and reduction of cervical cancer related burden.

**For health system organizers:**

lack of reference medical centers for pathology diagnostics and restriction in sharing of pathomorphological results between laboratories, especially in the case of ambiguous results of cervical cancer screening, advocates the use biomarker-based diagnostic capacity as a tool of quality control, and therefore very important to promote this process.

**For researchers:**

Improving the fundamental knowledge related to dysplastic changes in cytological diagnostic material, will help to plan and develop molecular research on the development of invasive carcinoma from the high-grade dysplasia.

## List of Publications:

1. S. Kakaliashvili, Kharzeishvili, G, Didava, T.Saluqvadze, I.Getzadze, N.Lomtadze. „p16/Ki-67 double staining results in women with different types of dysplasia Based on pap-test results in Georgia“ Actual Topics on Women Health, 2012 vol. 1; p.65-69.
2. Sopio Kakaliashvili-Dzagnidze, Omar Khardzeishvili, Giorgi Didava. "Expression of p16/Ki-67 biomarkers during atypical Pap test results." First SYSSA Conference. D. Tvildiani Medical University Press; Tbilisi. Georgia. 2013, p. 4-7.
3. S. Kakaliashvili-Dzagnidze, A. Dzagnidze. "Prevalence of HPV infection among Woman Enrolled in Cervical cancer screening in Georgia". GAU International Scientific Conference. 2022, p.90-94.
4. Sopio Kakaliashvili-Dzagnidze, Omar Khardzeishvili, Sergo Tabagari. "The role of dual p16<sup>INK4a</sup>/ Ki-67 biomarkers integration in cytology". Journal of the Georgian Critical Care Medicine Institute, Georgian Academy of Medical Sciences, Georgian Association of Catastrophe & Critical Care Medicine, N7, 2023.
5. Kakaliashvili-Dzagnidze S, Khardzeishvili O, Tabagari S. Diagnostic Accuracy of p16 INK4a/Ki-67 Dual Immunostaining for

Detection of High-Grade Cervical Intraepithelial Neoplasia in Women Involved in Cervical Cancer Screening in Georgia. Analytical Cellular Pathology. 2023 Jun 5; 2023.

6. Kakaliashvili-Dzagnidze S. The role of the morphologic categorization of p16INK4a/Ki-67 dual stained cytology in detecting of high grade cervical intraepithelial neoplasia (CIN2+). Translational and Clinical Medicine-Georgian Medical Journal. 2023 Dec 21;8(2).