Peer Review File

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Reviewer A

The authors have examined the role of DNA ploidy analysis in detecting high-grade squamous intraepithelial lesions (HSIL) and cervical cancer in women with negative results for high-risk HPV types 16/18 and ThinPrep cytology (TCT). Using a retrospective cross-sectional design, the study evaluated diagnostic outcomes in 335 women who underwent HPV, TCT, and DNA ploidy testing, with 48 cases of HSIL or cervical cancer identified. The results indicate that DNA ploidy analysis alone has the highest sensitivity (93.8%) and specificity (92.7%) for detecting HSIL+. The combined use of all three methods demonstrated high sensitivity, suggesting that DNA ploidy analysis may enhance cervical cancer screening, particularly in cases where HPV and TCT are negative.

The claims are properly placed in the context of the previous literature. The experimental data support the claims. The manuscript is written clearly enough that most of it is understandable to non-specialists. The authors have provided adequate proof for their claims, without overselling them. The authors have treated the previous literature fairly. The paper offers enough details of methodology so that the experiments could be reproduced.

Comments

1. Cervical cytology (TCT) demonstrated a notably low sensitivity of 29.2% (14/48) in this study, which is substantially lower than the typical sensitivity reported in most studies, approximately 50%. This unexpected finding warrants further investigation to understand the discrepancy.

Reply 1

The sensitivity of TCT in this study is relatively low, but the specificity is consistent with other studies. The reviewer's comment reminds us that the results of different studies, in which even the same methods were used, can still vary due to different testing conditions and environments. In our study, although the same technician was responsible for sample collection and TCT, it was hard to guarantee the experiment conditions were the same as other studies.
In addition, the sample size of our study is relatively limited. All of these suggested us that the further investigation will be needed.

No change in text.

2. In the Methods section, the authors define a positive TCT outcome as HSIL or SCC, which is an unusual classification. Typically, in most studies, any cytology result of ASC-US or higher (ASC-US+) is considered abnormal (a positive TCT test), not solely HSIL+ cytology. This deviation from standard practice could affect the interpretation of the study's results and their comparability with other literature.

Reply 2
Thanks for pointing out this typo. Actually, we calculate the outcome of TCT-positive as none NILM. As we can see in the table 2, the positive TCT cases is 48, which is the sum of ASCUS, LSIL, HSIL and SCC.

Change in text
Edited in Line 143.

3. I am unclear on how accuracy was calculated in this study. Typically, accuracy is computed as the average of sensitivity and specificity, expressed as Accuracy = (Sensitivity + Specificity) / 2. However, the values in Table 2 do not reflect this formula. Could you please clarify the method used for calculating accuracy in this context?

Sørbye SW, Suhrke P, Revå BW, Berland J, Maurseth RJ, Al-Shibli K. Accuracy of cervical

cytology: comparison of diagnoses of 100 Pap smears read by four pathologists at three hospitals in Norway. BMC Clin Pathol. 2017 Aug 29;17:18. doi: 10.1186/s12907-017-0058-8. PMID: 28860942; PMCID: PMC5576325.

Reply 3

We used the formula accuracy = (true positive + true negative)/(true positive + true negative + false positive + false negative) for accuracy calculation in this study. Take the HPV 16/18 for example in the table 2. True positive = 17, true negative = 217, false positive = 68, false negative = 31. Therefore, the accuracy of HPV 16/18 is 70.27%.

Change in text

We added the formula as a footnote under the table 2.

4. When combining two different tests, there are typically three outcomes: both positive, both negative, or one positive and one negative. It appears that the study defines an outcome as at least one positive test, which enhances sensitivity but reduces specificity. Combining all three tests (TCT + HPV 16/18 + DNA ploidy) achieves the highest sensitivity (97.9%) but the lowest specificity (62.2%). In a screening context where the prevalence of HSIL+ is low, specificity becomes more critical than sensitivity. Referring all women with at least one positive result from three combined screening tests for colposcopy and biopsy would overwhelm colposcopy capacities in most settings.

Reply 4

This is a very critical question. As we can see the combination of three tests achieves highest sensitivity but lowest specificity. The only DNA ploidy method could achieve both high sensitivity and specificity (over 90%). So we believe the DNA ploidy test alone has higher clinical prevalence, but if participant could afford, the combinational test is recommend.

No change in text.

5. In Table 2, the group labeled 'DNA ploidy (patients with HPV 16/18- & TCT -)' appears highly

selective and potentially biased. This group includes only 19 cases of HSIL+, and assessing the specificity of DNA ploidy in this subset may not be relevant for broader screening strategies. Typically, the combined specificity of HPV 16/18 and TCT would influence the screening outcomes before a third test like DNA ploidy is introduced. Thus, evaluating DNA ploidy in this context doesn't reflect a typical screening scenario where the primary tests significantly filter the population first.

Reply 5

We agree that the cases number of different subset with HSIL+ is relatively small, which may cause some bias. However, DNA ploidy analysis helped retrieved 18 (8.7%) out of 207 participants who had negative HPV-16/18 and TCT result and further validated by pathological confirmation. If HPV and TCT is introduced before, still DNA ploidy could retrieve certain population of HSIL+. So based on this study, DNA ploidy showed higher sensitivity and specificity, we suggest the usage of DNA ploidy earlier, right after HPV test.

No change in text.

6. In Table 2, the combination of HPV 16/18 and DNA ploidy results in the highest Youden index, suggesting it might be the most effective balance of sensitivity and specificity among the test combinations evaluated. However, in populations with a low prevalence of HSIL+, the DNA ploidy test alone could be preferable. It offers high sensitivity and specificity, making it particularly suitable for use as a single test in such contexts.

Reply 6

WHO's recent 2021 guidelines recommended the general population of women to perform HPV-DNA detection as the primary screening method for cervical cancer prevention. The clinical evidence is higher. The high risk HPV subtypes is most essential, should be test upfront. So we still recommend the upfront test of HPV, and preferred the combination with DNA ploidy test.

No change in text.

7. The WHO guidelines recommend HPV-DNA detection as the primary screening method for cervical cancer prevention in the general population of women, beginning at age 30, with repeat testing every 5–10 years. However, this study includes women aged 18 and older. It is important to clarify why the study included women younger than 30 years, given that young women often have transient HPV infections and the risk of cervical cancer in this age group is very low. Including younger women could potentially skew the relevance and application of the study's findings to the general screening population.

Reply 7

We admit that women over 30 years old are the major population who would be at the high risk of developing cervical cancer and should be recommended with regular screening. However, there is a trend that the incidence age of cervical cancer is getting younger in the developed countries or regions. Besides, women with age between 21 and 29 are at a high risk of precancerous lesions and some of them would like to do the tests. To explore the application of those three screening tests on a broader population including young women who may be potential screening candidates in the future, we did not restricted women at 30 or above years old. There were 37 women range from 21 to 29 years old in this study.

Ref: Benard VB, Watson M, Castle PE, et al. Cervical carcinoma rates among young females in the United States. Obstet Gynecol. 2012; 120 (5): 1117-1123.

No change in text.

8. The WHO guidelines recommend HPV-DNA detection as the primary screening method for cervical cancer prevention. However, in this study, the authors have utilized an HPV mRNA test (HPV E6/E7 mRNA detection, Health Gene Technologies, Inc.). It would be beneficial to discuss how using an mRNA test, which targets active viral gene expression rather than the presence of viral DNA, might influence the study's outcomes compared to DNA-based testing. This is particularly relevant in terms of sensitivity and specificity, as well as the potential for detecting clinically significant infections.

Thanks for rising the comment. Study has revealed that HPV mRNA obtained similar results with HPV DNA test.

Ref: Maggino, T., Sciarrone, R., Murer, B. et al. Screening women for cervical cancer carcinoma with a HPV mRNA test: first results from the Venice pilot program. Br J Cancer 115, 525–532 (2016). https://doi.org/10.1038/bjc.2016.216

No change in text.

9. Most clinically validated HPV tests recommended for use in primary screening detect 14 high-risk HPV types. In this study, the HPV test (Health Gene Technologies, Inc.) used multiplex polymerase chain reaction (PCR) and capillary electrophoresis to detect 25 different HPV types. This includes high-risk HPV genotypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82, along with low-risk types 6, 11, 42, 43, 44, 81, and 83. It is important to discuss why an HPV test that detects a broader range of HPV types and is not clinically validated for primary screening was chosen for this study, considering the potential implications for the accuracy and applicability of the results.

Study showed that HPV types 16 and 18, types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 should be considered carcinogenic, or high-risk, types, and types 26, 53, and 66 should be considered probably carcinogenic.

Ref: Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003 Feb 6;348(6):518-27. doi: 10.1056/NEJMoa021641. PMID: 12571259.

No change in text.

10. In the manuscript, the presentation of percentages varies inconsistently, sometimes with one decimal and sometimes with two. For ease of reading and to streamline both the text and tables, I

recommend uniformly rounding these percentages to one decimal place. For example, change 25.53% in Table 2 to 25.5%. This will help maintain a cleaner and more consistent format throughout the document.

Reply 10

Thanks for raising up the issues.

Change in text

Highlighted in yellow in table 2 and in text at line 236 and 251.

11. In the manuscript, there is a reference to "Pathology confirmed HSIL or cervical cancer" in Table 2. It is important to specify the type of samples evaluated. When discussing biopsies, the correct terminology should be "Histologically confirmed HSIL or cervical cancer." This distinction ensures clarity, as it differentiates histological confirmation from cytological findings.

Reply 11

Thanks for the comment. Reworded in the table.

Change in text

Highlighted in yellow of the second column name in table 2.

12. Table 1: "Thinprep cytology test (TCT)" => "Cervical cytology using ThinPrep (TCT)"

Reply 12

Thanks for the suggestion. Edited in the table.

Change in text

Highlighted in yellow in table 1 where “TCT” locates.

13. Table 1: "Pathologically confirmed worst lesion" => "Histological diagnosis of cervical biopsy"

Reply 13

Thanks for the suggestion. Edited in the table.

Change in text

Highlighted in yellow in table 1 where “biopsy” locates.

Minor revisions

Lines 1-2, Title, "Triage of Women with Positive HPV Tests: Comparing DNA Ploidy Analysis with HPV 16/18 Genotyping and Cervical Cytology"

Reply minor revision 1

Thanks for the suggestion. Edited in the title at line 2-3.

Change in text

Highlighted in yellow in title.

Line 4, Running title, "DNA Ploidy vs. HPV 16/18 Genotyping and Cytology in the Triage of HPV-Positive Women"

Reply minor revision 2

Thanks for the suggestion. Added the running title.

Change in text

Highlighted in yellow at line 16-17.

Lines 6-25, Abstract, "Background: Cervical cancer screening primarily utilizes the Human papillomavirus (HPV) test with partial genotyping (HPV 16/18) and liquid-based cytology by ThinPrep (TCT) for triaging women with a positive HPV test. Although DNA ploidy quantitative

analysis has shown reliability, its integration into screening guidelines as a triage test, compared with partial genotyping and TCT, is not fully established.

Reply minor revision 3

Thanks for the suggestion. Reworded in the abstract.

Change in text

Highlighted in yellow at line 25-29.

Methods: We retrospectively analyzed data from 335 women aged 18 and older who participated in a cervical cancer screening program at xxx Hospital and underwent triage using HPV 16/18, TCT, and DNA ploidy testing. The sensitivity and specificity of these methods, both individually and in combination, were evaluated.

Reply minor revision 4

Thanks for the suggestion. Reworded in the abstract.

Change in text

Highlighted in yellow at line 30-33.

Results: Individually, the tests showed sensitivities and specificities of 35.4% and 76.1% for HPV 16/18, 29.2% and 88.2% for TCT, and 93.8% and 92.7% for DNA ploidy, respectively. Combining the tests improved outcomes, with DNA ploidy plus HPV 16/18 genotyping showing enhanced sensitivity and high specificity. Notably, DNA ploidy alone identified high-grade lesions (HSIL) and cervical cancer with a higher detection rate and a lower positivity rate in triage compared to HPV 16/18 and TCT.

Reply minor revision 5

Thanks for the suggestion. Reworded in the abstract.

Change in text

Highlighted in yellow at line 34-38.

Conclusions: DNA ploidy analysis demonstrated superior specificity and sensitivity in the triage of women with a positive HPV test, offering a more effective alternative for detecting high-grade lesions and cervical cancer. These findings support the potential for integrating DNA ploidy into current cervical cancer screening protocols to enhance triage effectiveness and reduce unnecessary colposcopy referrals."

Reply minor revision 6

Thanks for the suggestion. Reworded in the abstract.

Change in text

Highlighted in yellow at line 40-43.

Lines 31-34, Key Findings, "DNA ploidy analysis offers superior specificity and sensitivity in triage of women with a positive HPV test compared to HPV 16/18 genotyping and TCT.

Reply minor revision 7

Thanks for the suggestion. Reworded in the “Key Findings”.

Change in text

Highlighted in yellow at line 46 “Key Findings” part.

Reduces unnecessary referrals: DNA ploidy's lower positivity rate in triage compared to other methods suggests it could decrease unnecessary colposcopy referrals, optimizing resource use in cervical cancer screening programs."

Reply minor revision 8

Thanks for the suggestion. Reworded in the “Key Findings”.

Change in text

Highlighted in yellow at line 46 “Key Findings” part.

Lines 36-39, What is known and what is new?, "What is Known:

Reply minor revision 9

Thanks for the suggestion. Reworded the title.

Change in text

Highlighted in yellow at line 46 “What is known” part.

WHO guidelines recommend HPV-DNA detection as the primary screening method for cervical cancer prevention in the general population of women, starting at the age of 30, with regular testing every 5–10 years. For women with a positive HPV test, triage with partial genotyping (HPV 16/18) and cytological testing is recommended.

Reply minor revision 10

Thanks for the suggestion. Reworded in the “What is known” part.

Change in text

Highlighted in yellow at line 46 “What is known” part.

What is New:

Improving Triage: This study reveals that DNA ploidy analysis offers superior specificity and sensitivity compared to the current triage methods of HPV 16/18 genotyping and cytological testing, which are often limited by subjective interpretation and human error.

Resource Optimization: By demonstrating a lower positivity rate in triage, DNA ploidy analysis could reduce unnecessary colposcopy referrals, suggesting its potential to optimize resource use in cervical cancer screening programs."

Reply minor revision 11

Thanks for the suggestion. Added a new title “What is New” and reworded in the corresponding part.

Change in text

Highlighted in yellow at line 46 “What is new” part.

Lines 47-49, What is the implication, and what should change now?, "Enhancing Triage Accuracy: Based on its demonstrated higher specificity and sensitivity, DNA ploidy analysis should be considered as an alternative to partial genotyping and cervical cytology in the triage of women with a positive HPV test in primary screening. This change could significantly improve the accuracy of detecting high-grade lesions and cervical cancer.

Optimizing Screening Protocols: Implementing DNA ploidy analysis as a standard triage tool could reduce unnecessary colposcopy referrals, thus optimizing resource use and potentially leading to better patient outcomes in cervical cancer prevention programs."

Reply minor revision 12

Thanks for the suggestion. Reworded in the “What is the implication, and what should change now” part.

Change in text

Highlighted in yellow at line 46 “What is the implication, and what should change now” part.

Lines 52-58, Introduction, "Cervical cancer remains a significant public health concern globally, as the fourth most common cancer affecting women worldwide, after breast, colorectal, and lung cancers. It accounts for approximately 660,000 new cases and around 350,000 deaths annually. It arises from the cervix and is primarily driven by persistent infection with high-risk human

papillomaviruses (HPV). Early detection through effective screening programs, such as those utilizing HPV testing to detect oncogenic HPV DNA, has proven crucial in reducing both the incidence and mortality of cervical cancer. In 2019, China alone reported 109,759 new cases, underscoring the widespread impact of this disease. The shift towards HPV testing has enhanced the precision of screening programs compared to cytology-based methods alone. This has been pivotal in identifying women at risk earlier and more reliably, allowing for timely intervention and management. Moreover, advancements in HPV vaccination have begun to alter the landscape of cervical cancer incidence, promising a future decrease in its prevalence. Nonetheless, challenges remain, including the integration of these tools into coordinated screening efforts worldwide, particularly in regions where access to healthcare is limited and the disease burden is highest."

https://www.who.int/news-room/fact-sheets/detail/cervical-cancer

Wu S, Jiao J, Yue X, Wang Y. Cervical cancer incidence, mortality, and burden in China: a time-trend analysis and comparison with England and India based on the global burden of disease study 2019. Front Public Health. 2024 Mar 6;12:1358433. doi: 10.3389/fpubh.2024.1358433. PMID: 38510348; PMCID: PMC10951371.

Reply minor revision 13

Thanks for the suggestion. Reworded in the first paragraph of introduction part.

Change in text

Highlighted in yellow at line 49-62.

Lines 59-68, Introduction, "The risk of cervical cancer or precancerous lesions is closely associated with the type and duration of Human papillomavirus (HPV) infection. Persistent infection with high-risk HPV types is responsible for over 99% of cervical cancer cases. HPV16 and HPV18 are of particular concern, accounting for more than 70% of all cervical cancer cases. The progression of cervical cancer is marked by a series of cellular and molecular alterations. Cervical cytology using the ThinPrep test (TCT) aids in the collection of exfoliated cells from the cervix through a liquid-based method and an automated processing system, facilitating cytological examination to

detect potential cancerous changes. Currently, the integrated use of HPV testing and TCT constitutes the foundation of cervical cancer screening strategies."

Reply minor revision 14

Thanks for the suggestion. Reworded in the second paragraph of introduction part.

Change in text

Highlighted in yellow at line 63-71.

Lines 69-74, Introduction, "The updated guidelines recommend that negative HPV screening results be followed up at intervals of 5 years, reflecting the low 5-year risk of CIN3+ in HPV-negative and co-screening-negative cases, which are recorded at 0.14% (95% CI 0.13% to 0.15%) and 0.12% (95% CI 0.12% to 0.13%) respectively. These risks fall below the 0.15% threshold. However, the primary challenge in cervical screening is not the risk of CIN3+ among women with a negative HPV DNA test, but the test's low specificity, which necessitates further triage. Current strategies, such as partial genotyping for HPV 16/18 and cytological triage for women with non-HPV 16/18 positive tests, are hampered by the limited sensitivity and specificity of genotyping, and the subjective and error-prone nature of cytological assessments. It is crucial to develop more accurate and reliable triage methods to effectively identify women at significant risk after a positive HPV DNA test, thereby enhancing the efficacy of cervical cancer screening programs."

Reply minor revision 15

Thanks for the suggestion. Reworded in the third paragraph of introduction part.

Change in text

Highlighted in yellow at line 72-82.

Lines 75-85, Introduction, "At the molecular level, cervical carcinogenesis involves mutations in multiple genes, including the activation of oncogenes and the inactivation of tumor suppressor genes. Concurrently, alterations in signaling pathways related to cell growth, apoptosis, and DNA damage repair disrupt the regulation of cell proliferation, facilitating the unchecked growth and

survival of cancer cells. DNA ploidy Quantitative Analysis, which detects abnormal DNA content in the nuclei of cervical epithelial cells, provides insight into the proliferative potential of tumor cells and has emerged as a valuable diagnostic tool for identifying cervical cancer and its precursors. Given the need for more objective biomarkers in the triage of women with a positive HPV test in primary screening, to better balance benefits and harms, this study aims to evaluate the clinical utility of DNA ploidy analysis as a triage test for women with a positive HPV test in primary screening, comparing it to HPV 16/18 genotyping and TCT."

Reply minor revision 16

Thanks for the suggestion. Reworded in the fourth paragraph of introduction part.

Change in text

Highlighted in yellow at line 83-93.

Lines 88-93, Methods, "This study employed a retrospective cross-sectional design. We collected demographics, diagnoses, colposcopy, and biopsy results, along with other laboratory tests, including HPV, TCT, and DNA ploidy, which were performed as part of the cervical cancer or precancerous lesion screening program at xxx hospital. The data collection period spanned from November 2021 to February 2022, as detailed in Supplementary Table 1. Logistic regression was used to evaluate the association between the screening tests and the detection rate of high-grade lesions (HSIL) and cervical cancer, as confirmed by colposcopy and biopsy."

Reply minor revision 17

Thanks for the suggestion. Reworded in the fifth paragraph of introduction part.

Change in text

Highlighted in yellow at line 94-100.

Lines 134-139, Methods, "Colposcopy was performed using an electronic colposcope by experienced gynecologists. Examinees were positioned in the lithotomy position. After cervical exposure using a vaginal speculum, cervical secretions were removed with dry cotton balls for

preliminary observation. Subsequently, 3% acetic acid was applied to the cervix, and colposcopic images were carefully observed, recorded, and archived. For cases where abnormalities were suspected, cervical biopsies were collected under colposcopic guidance and fixed in 1% formalin solution for subsequent histological examination."

Reply minor revision 18
Reworded in the “colposcope and pathological examination” part.

Change in text
Highlighted in yellow at line 154-160.

Lines 156-177, Results, "Between November 1, 2021, and February 28, 2022, 388 women visited the Department of Gynecology at xxx Hospital for screening. After excluding 16 for non-standard TCT results, 3 with intrauterine adhesions, 19 with a history of cervical cancer, and 15 with incomplete colposcopy or screening test information, 335 women remained for analysis. Their mean age was 43.5 years (SD 12.3). Of these, 48 (14.3%) were diagnosed with cervical cancer or HSIL via biopsy (Table 1). Among the participants, 20 (6%) had a history of smoking, 79 (23.6%) were postmenopausal, and the majority (205, 61.2%) had been pregnant 1-3 times. Miscarriages occurred in 171 (67.6%) of those who had been pregnant, predominantly through induced abortion (154, 90.0%). High-risk HPV 16/18 was found in 85 (25.4%) participants, while 303 (90.4%) tested positive for other high-risk HPV types, with some co-infected. DNA ploidy testing identified abnormalities in 66 (19.7%). TCT results showed that 85.7% of participants had no intraepithelial lesions or malignancy (NILM), while 22 (6.5%) had LSIL and only one (0.3%) had HSIL."

Reply minor revision 19
Reworded in the result “overall description” part.

Change in text
Highlighted in yellow at line 178-189.

Lines 179-200, Results, "HPV 16/18 infection, TCT, and DNA ploidy tests are critical for

predicting cervical cancer or precancerous lesions. Individually, these predictors showed varying sensitivity and specificity (Table 2, Figure 2). HPV 16/18 and TCT demonstrated low sensitivity at 35.4% and 29.2%, respectively, but had relatively high specificity at 76.1% and 88.2%. The positive predictive values (PPV) were modest at 20.0% for HPV 16/18 and 29.2% for TCT, while the negative predictive values (NPV) were close to 90%. Their classification accuracies were 70.3% and 79.7%, with Youden Indices ranging from 11% to 18%. In contrast, the DNA ploidy test exhibited high sensitivity and specificity, exceeding 90%, with PPV and NPV of 68.2% and 98.9%, respectively, resulting in a predictive accuracy of 92.8% and a Youden Index of 86.4%.

Reply minor revision 20
Reworded in the first paragraph of “classification model” part.

Change in text
Highlighted in yellow at line 191-199.

When combining HPV 16/18 and TCT, sensitivity increased to 60.4% but specificity decreased to 65.7%. The combined model’s overall accuracy was only 65.0%, with a Youden Index of 13.7%."

Reply minor revision 21
Reworded in the second paragraph of “classification model” part.

Change in text
Highlighted in yellow at line 200-202.

Lines 201-214, Results, The section can be streamlined to emphasize the critical findings without the redundancy in describing each combined test's performance, focusing instead on the comparative results. Here's a suggested reformulation:

"We also evaluated the performance of combinations involving the single predictors HPV 16/18 and TCT with the better-performing DNA ploidy test (Table 2, Figure 3). The combined DNA

ploidy and TCT model showed good sensitivity (93.8% [95% CI: 86.9%, 100%]) and specificity (82.6% [95% CI: 78.2%, 87.0%]), but it did not perform better than DNA ploidy alone. Similarly, the DNA ploidy and HPV 16/18 model, as well as the tri-combination with all three predictors, yielded high sensitivity (97.9% [95% CI: 93.9%, 100%]) but lower specificity, particularly in the three-way combination (62.2% [95% CI: 56.6%, 67.9%]). When applying DNA ploidy alone to classify cervical cancer or precancerous lesions in participants negative for HPV 16/18 and TCT, both sensitivity and specificity reached 94.7%, with an accuracy and Youden Index of 94.7% and 89.4%, respectively. This approach identified 18 additional cases (8.7% of 207) initially negative for HPV-16/18 and TCT, subsequently confirmed by pathology."

Reply minor revision 22

Reworded in the third paragraph of “classification model” part.

Change in text

Highlighted in yellow at line 203-213.

Lines 216-224, Results, "The final section on risk factors can be rewritten for clarity and conciseness while maintaining the integrity of the statistical data presented. Here's a suggested reformulation:

Risk Factors for Cervical Cancer or Precancerous Lesions

Univariate analysis initially estimated the crude odds ratios (OR) for key predictive factors among 48 HSIL or cervical cancer cases. HPV 16/18 did not show a statistically significant association with increased risk (OR: 1.75; 95% CI: 0.90 – 3.32), unlike the other factors where DNA ploidy demonstrated a strong association (OR: 190.0; 95% CI: 62.9 – 830.6) and TCT showed moderate significance (OR: 3.06; 95% CI: 1.46 – 6.21). Multivariable logistic regression, adjusted for age, number of induced abortions, menstrual status, and squamocolumnar junction (SCJ) results, indicated that the influence of DNA ploidy remained robust (OR: 290.4; 95% CI: 7.9 – 1913.7). However, the associations for HPV 16/18 and TCT were not significant in this adjusted model

(HPV OR: 1.9; 95% CI: 0.6 – 6.2; TCT OR: 1.7; 95% CI: 0.5 – 5.8) (Table 3)."

Reply minor revision 23

Reworded in the “risk factors of cervical cancer or precancerous lesions” part.

Change in text

Highlighted in yellow at line 215-223.

Lines 227-235, Discussion, "The WHO's 2021 guidelines recommend HPV-DNA detection as the primary screening method for cervical cancer prevention. In this study, the detection rate for high-risk HPV-16/18 subtypes was 25.5%, which is significantly higher than the 5-15% typically reported for first-time screenings. This discrepancy may be attributed to the nature of our study population, which primarily consisted of symptomatic individuals rather than healthy participants routinely screened based on age. The sensitivity and specificity of HPV-16/18 testing in our study were 35.4% and 76.1%, respectively. These figures are lower than those reported in other studies but may more accurately reflect real-world performance. A notable strength of our study is the consistency in sample collection, which was performed by the same clinician, minimizing biases associated with sample preparation across the three tests."

Reply minor revision 24

Reworded in the first paragraph of discussion part.

Change in text

Highlighted in yellow at line 226-235.

Lines 245-257, Discussion, "In this study, we evaluated DNA ploidy as a triage test for women with a positive HPV DNA test, comparing it with partial HPV 16/18 genotyping and TCT triage. DNA ploidy demonstrated higher sensitivity and specificity for HSIL+ than the combination of HPV 16/18 and TCT (93.8% vs. 60.4% and 92.7% vs. 65.7%, respectively). Additionally, its lower positivity rate (19.7% vs. 37.9%) suggests a reduced referral rate for colposcopy, which is highly advantageous in a triage setting.

Reply minor revision 25

Reworded in the third paragraph of discussion part.

Change in text

Highlighted in yellow at line 246-251.

These findings may be linked to the biological processes associated with HPV infection. Previous research has indicated that abnormal DNA ploidy is observed in women with squamous intraepithelial lesions (SIL) infected by high-risk HPV types. The severity of SIL is positively correlated with the proportion of heteroploid cells. This correlation could stem from HPV DNA fragments integrating into the host's nuclear DNA within cervical epithelial cells, leading to aberrant cell division processes such as multipolar mitosis and unbalanced chromosome division. These processes result in changes in chromosome number and structure, increases in nuclear area, and alterations in DNA content and ploidy status. Consequently, DNA ploidy analysis could serve as an effective triage method for women with a positive primary HPV test."

Reply minor revision 26

Reworded in the fourth paragraph of discussion part.

Change in text

Highlighted in yellow at line 252-260.

Lines 258-267, Discussion, "This study has several limitations. Firstly, the sample size is modest, with only 335 women participating, including 43 diagnosed with high-grade lesions (HSIL) and cervical cancer. Secondly, the detection of DNA ploidy requires a certain period post-infection by high-risk HPV before changes in the host's cells become detectable. All tests in this study were conducted within a brief two-month period, thus the optimal timing for follow-up testing from HPV detection to DNA ploidy assessment remains unclear. Due to its cross-sectional retrospective design, future longitudinal multi-center studies are needed to address this limitation. Thirdly, the study's participants were sourced from a single hospital, which may not adequately represent the broader population, thus limiting the generalizability of the findings. Lastly, the limited number of

covariates collected restricted our ability to explore potential subgroups that might respond differently to the screening tools."

Reply minor revision 27
Reworded in the fifth paragraph of discussion part.

Change in text
Highlighted in yellow at line 261-273.

Lines 23-26, Conclusions, "This study demonstrated that DNA ploidy analysis offers superior specificity and sensitivity in the triage of women with a positive HPV test for detecting high-grade lesions (HSIL) and cervical cancer compared to triage using partial genotyping (HPV 16/18) and TCT. Notably, DNA ploidy analysis results in a lower positivity rate in triage than both HPV 16/18 and TCT. These findings suggest that DNA ploidy testing could serve as a more effective triage test than those currently recommended by guidelines."

that might respond differently to the screening tools."

Reply minor revision 28
Reworded in the fifth paragraph of discussion part.

Change in text
Highlighted in yellow at line 276-281.

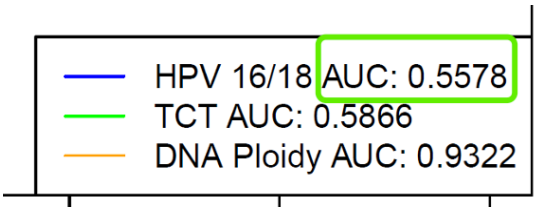
Reviewer B

1. Figures and tables
    - All abbreviations in figures/tables and legends should be explained. ROC, HPV, TCT, NILM in Figure 2 for example. Please check all abbreviations and provide the full names in the corresponding legends/footnote. E.g., Figure 1. xxx. Abbreviations: xxx, xxxx.

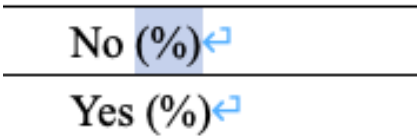
- All abbreviaions in the tables should be defined. SD, LEEP, HPV, TCT, SCJ, NILM, ASC-US, ASC-H, LSIL, HSIL in Table 1 for example. Please check all tables and provide the full names in the corrsponding foot.
- Table 1: Please provide a header of the first column.

Age (in years)	
Mean (SD)	

- Figure 2-4: It is suggested to add round bracket in each AUC value. For example, HPV 16/18 (AUC: 0.5578)".



- Table 1: ", n (%)" would be better.



- Table 2-3: Please indicate the meaning of + and – in the foot.
- The following highlighted data in Table 3 is inconsistent with that in the main text. Please check and revise.

HPV 16/18 (+ vs -)	1.75 (0.90 - 3.32)	1.90 (0.65 - 5.84)	1.87 (0.59 - 6.15)
TCT (+ vs -)	3.06 (1.46 - 6.21)	1.50 (0.48 - 4.81)	1.65 (0.49 - 5.82)
DNA ploidy (+ vs -)	190.00 (62.90 - 830.60)	177.20 (57.90 - 783.20)	290.40 (7.87 - 1913.70)
Age (per 10 years increase)	1.00 (0.97 - 1.03)	-	1.02 (0.52 - 1.99)

influence of DNA ploidy remained robust (OR: 290.4; 95% CI: 7.9–1913.7). However, the association between HPV 16/18 and TCT was not significant in this adjusted model (HPV OR, 1.9; 95% CI: 0.6–6.2; TCT OR, 1.7; 95% CI: 0.5–5.8) (Table 3).

Response:

The header and abbreviations are provided.

Round bracket was added in Figure 2-4. Table 1 added ", n (%)" inside.

Table 2-3 has provided the footnote of "+" and "-".

The context was kept consistent in the maintext and table 3.

2. Please check if more references should be cited since you mentioned **studies**.
    - The specificity of the TCT results in our study was 88.2%, which is consistent with other **studies**, ranging from 86–100%.[17]
    - Previous **studies** have indicated that abnormal DNA ploidy is observed in women with squamous intraepithelial lesions (SIL) infected with high-risk HPV types.

    Response: Reference17 is a systematic review. The studies indicated abnormal DNA ploidy is observed in women with squamous intraepithelial lesions (SIL) infected with high-risk HPV types were added inside the reference part.