

Immunohistochemical biomarkers for high-grade squamous intraepithelial lesions of the cervix: a systematic review with meta-analysis

Biomarcadores imunohistoquímicos para lesões intraepiteliais escamosas de alto grau no colo uterino: revisão sistemática com metanálise

Biomarcadores imunohistoquímicos para lesiones intraepiteliales escamosas de alto grado del cuello uterino: una revisión sistemática con metaanálisis

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ABSTRACT

Objective: to identify the main immunohistochemical markers for differentiating high-grade squamous intraepithelial lesions in the cervix and to analyze the different interpretation methods. **Method:** a systematic review with meta-analysis. After searching the Cochrane, Embase, Scopus, and Web of Science databases, conducted in March 2021, 51 case-control articles describing immunohistochemical biomarkers of high-grade squamous intraepithelial lesions of the cervix in cervical biopsies, published between 2000 and 2021, were included. **Results:** the biomarkers p16INK4 and Ki-67 were prominent in high-grade squamous intraepithelial lesions in the cervix. Regarding the articles that studied only p16INK4 and/or Ki-67, seven different methods for immunohistochemical interpretation of p16INK4 expression and three methods for Ki-67 were described. **Conclusion:** among the methods found, nuclear or cytoplasmic staining by epithelial layers (no staining or focal staining = negative; diffuse staining = positive) appears to be the most significant for interpreting the p16INK4 biomarker (OR = 13.97; 95%CI: 7.93 – 24.63; Z = 9.12; p < 0.00001) and the Ki-67 biomarker (OR = 8.47; 95%CI: 3.75 – 19.14; Z = 5.13; p < 0.00001) in high-grade squamous intraepithelial lesions of the cervix, when compared to low-grade lesions. Therefore, it is concluded that this is the most reliable immunohistochemical method for interpreting these lesions. PROSPERO registration: CRD42020210747.

Descriptors: Squamous intraepithelial lesions; Cervix uteri; Biomarkers; Immunohistochemistry; Systematic Review.

RESUMO

Objetivo: identificar os principais marcadores imunohistoquímicos para diferenciação de lesões intraepiteliais escamosas de alto grau no colo uterino e analisar os diferentes métodos de interpretação. **Método:** revisão sistemática com metanálise. Após busca nas bases Cochrane, Embase, Scopus e Web of Science, realizada em março de 2021, incluíram-se 51 artigos de caso-controle que descreveram biomarcadores imunohistoquímicos de lesões intraepiteliais escamosas de alto grau no colo uterino em biópsia cervical, publicados entre 2000 e 2021. **Resultados:** os biomarcadores p16INK4 e Ki-67 foram destaques nas lesões intraepiteliais escamosas de alto grau no colo uterino. Em relação aos artigos que estudaram apenas p16INK4 e/ou Ki-67, foram descritos sete diferentes métodos para interpretação imunohistoquímica para expressão de p16INK4 e três métodos para Ki-67. **Conclusão:** entre os métodos encontrados, a coloração nuclear ou citoplasmática por camadas de epitélio (sem coloração ou coloração focal = negativa; marcação difusa = positiva) se apresenta como o mais significativo para interpretação do biomarcador p16INK4 (OR = 13,97; IC95%: 7,93 – 24,63; Z = 9,12; p < 0,00001) e do biomarcador Ki-67 (OR = 8,47; IC95%: 3,75 – 19,14; Z = 5,13; p < 0,00001) em lesões intraepiteliais escamosas de alto grau no colo uterino, quando comparados à lesão de baixo grau. Conclui-se, portanto, que este é o método imunohistoquímico mais seguro para interpretar essas lesões. Registro PROSPERO: CRD42020210747.

Descritores: Lesões intraepiteliais escamosas; Colo do útero; Biomarcadores; Imunohistoquímica; Revisão sistemática.

RESUMEN

Objetivo: identificar los principales marcadores inmunohistoquímicos para diferenciar las lesiones intraepiteliales escamosas de alto grado en el cuello uterino y analizar los distintos métodos de interpretación. **Método:** revisión sistemática con metaanálisis. Tras la búsqueda en las bases de datos Cochrane, Embase, Scopus y Web of Science en marzo de 2021, se incluyeron 51 artículos de casos y controles que describían biomarcadores inmunohistoquímicos de lesiones intraepiteliales escamosas de alto grado en biopsias cervicales, publicados entre 2000 y 2021. **Resultados:** los biomarcadores p16INK4 y Ki-67 se destacaron en lesiones intraepiteliales escamosas de alto grado del cuello uterino. En los artículos que estudiaron únicamente p16INK4 y/o Ki-67, se describieron siete métodos diferentes para la interpretación inmunohistoquímica de la expresión de p16INK4 y tres métodos para Ki-67. **Conclusión:** entre los métodos encontrados, la tinción nuclear o citoplasmática de las capas epiteliales (sin tinción o tinción focal = negativo; tinción difusa = positivo) parece ser el más significativo para la interpretación del biomarcador p16INK4 (OR = 13,97; IC95%: 7,93-24,63; Z = 9,12; p < 0,00001) y del biomarcador Ki-67 (OR = 8,47; IC95%: 3,75-19,14; Z = 5,13; p < 0,00001) en lesiones intraepiteliales escamosas de alto grado del cuello uterino, en comparación con las lesiones de bajo grado. Por lo tanto, se concluye que este es el método inmunohistoquímico más seguro para la interpretación de estas lesiones. Registro PROSPERO: CRD42020210747.

Descriptores: Lesiones intraepiteliales escamosas; Cuello del útero; Biomarcadores; Inmunohistoquímica; Revisión sistemática.

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Introduction

Squamous intraepithelial lesions of the cervix are classified into low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). These lesions are associated with Human Papillomavirus (HPV), with subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 considered high-risk for oncogenic cancer. LSIL usually regresses; however, HSIL is considered a precursor to cervical cancer and can progress to carcinoma in situ if not treated appropriately. Cervical cancer is one of the leading causes of death in women worldwide, with some variations between countries and regions.¹⁻⁴

To identify the characteristics of these lesions, histological assessment is necessary and, especially in cases of HSIL, assessment of immunohistochemical characteristics. Identifying these alterations and their characteristics is essential for the study of differential diagnoses and innovative therapeutic interventions, as several factors can contribute to a negative outcome in conization.^{3,5}

Among the reasons that explain the absence of lesions on the cervix, the most frequent were colposcopy errors, spontaneous lesion regression, complete removal of small lesions during biopsy, errors in pre-conization material, false-negative results, and excisional error. Review of pathological specimen and the application of immunohistochemical biomarkers p16INK4 (or p16) and Ki-67 appeared to improve accuracy and help in the challenge of differential diagnosis.⁵ Thus, it is already well established that p16 and Ki-67 are surrogate biomarkers for the presence of HPV. However, there are reports of difficulties for pathologists in the differential diagnosis between cervical intraepithelial lesions, especially HSIL.⁶

For this purpose, the aim was to identify the main immunohistochemical markers for differentiating HSIL and to analyze the different methods of interpretation.

Method

This systematic review, registered in PROSPERO (International Prospective Register of Systematic Reviews) under number CRD420202107477, was conducted in March 2021 in the Cochrane, Embase, Scopus, and Web of Science databases using the following strategy: (("Squamous Intraepithelial Lesions of the Cervix" OR "High-grade squamous intraepithelial lesion" OR "cervical intraepithelial neoplasia") AND ("Biomarkers" OR "Marker" OR "inflammatory mediators" OR "inflammatory markers") AND ("Histology" OR "Immunohistochemistry" OR "Immunofluorescence")). The systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses protocol.⁸

This review aimed to answer the following question: what are the main immunohistochemical markers for differentiating HSIL and the different methods of interpretation? Descriptors were defined using the PICOS method,⁹ configuring the systematic review study, with Population (P): women with HSIL of the cervix; Intervention (I): immunohistochemical biomarker expression identification; Comparison or Control (C): women with LSIL in the cervix; Outcome (O): presence or absence of the association between immunohistochemical biomarker expression and HSIL of the cervix; Study Design (S): case-control, comparing immunohistochemical biomarker expression between HSIL and LSIL.

Original articles describing HSIL immunohistochemical biomarkers in cervical biopsies from 2000 to 2021 were included. Review articles, case reports, articles with animal studies, articles with in vitro studies, duplicate articles, and letters to the editor were excluded. Due to the secondary nature of the data collected and analyzed in the study, it was not submitted to a Research Ethics Committee.

Using the Rayyan platform, titles, abstracts, and full articles were assessed separately by two researchers to select studies for review, and in case of differences in selection between the two, a third researcher performed the assessment.

Two researchers independently extracted the data, carefully including them in the meta-analysis. The data collected were title, DOI, authors, year of publication, immunohistochemical biomarker, and immunohistochemical method for interpreting biomarker expression.

Meta-analysis was performed using Review Manager 5.3 software. For this purpose, the De Moura *et al.* method was used.¹⁰ Pooled Odds Ratio (OR) values with 95% Confidence Intervals (CI) were calculated to assess the association between immunohistochemical biomarker expression and HSIL of the cervix. Pooled ORs were determined by the Z-test, where a p-value <0.05 was considered significant.

The heterogeneity among studies was analyzed using the chi-square (Q2) and Higgins (I2) statistical tests.¹¹ To calculate the pooled ORs, the fixed-effects model was used when p-value for heterogeneity was >0.05 or I2 < 50%, and the random-effects model was used when p-value for heterogeneity was <0.05 or I2 > 50%.¹²

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Results

A total of 157 articles emerged from the database search (Figure 1). These showed 130 biomarkers for HSIL of the cervix.

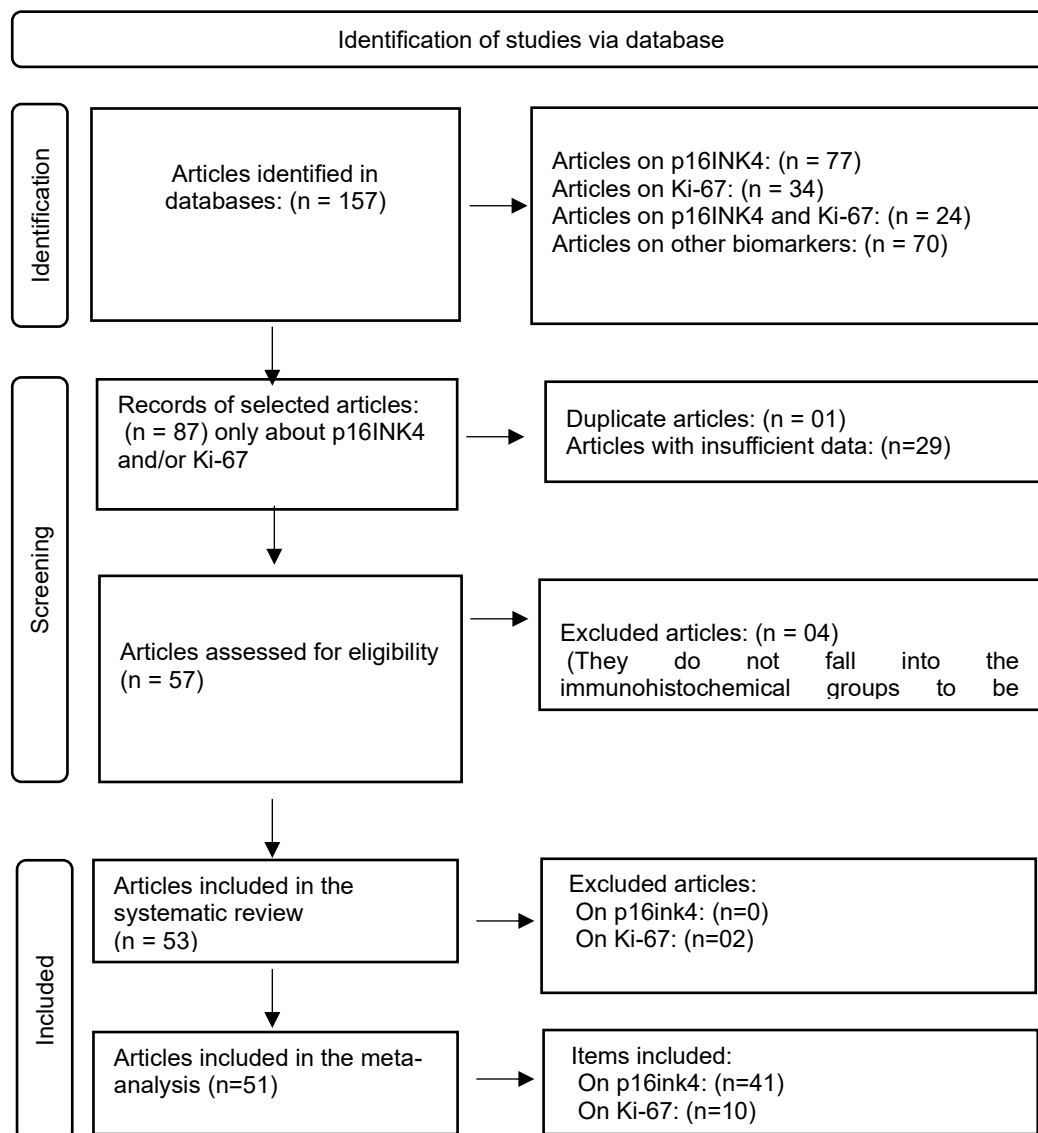


Figure 1 – Selection process for studies for systematic review and meta-analysis. Recife (PE), Brazil, 2023.

The p16INK4 (or p16) biomarker was the most studied in HSIL of the cervix, present in 77 (55.79%) of the articles found. Subsequently, the Ki-67 biomarker appears in 34 (24.63%) of articles, followed by the other 128 biomarkers studied in up to seven (5.07%) of the articles resulting from the search. It is noteworthy that some articles studied more than one biomarker. For instance, 25 articles studied the p16 and Ki-67 biomarkers simultaneously.

Based on the most studied biomarkers in HSIL of the cervix, 57 articles were initially selected for this study. However, 53 articles were included, grouped according to the seven different immunohistochemical interpretation methods for p16 expression and three for Ki-67 expression: method "A" (nuclear or cytoplasmic staining by epithelial layers: no staining or focal staining = negative; diffuse staining = positive); method "B" (nuclear staining by epithelial layers: no staining = negative; focal and diffuse staining = positive); method "C" (staining by % of cells: 0% = negative; >0% = positive); method "D" (staining by % of cells: <1% of cells stained = negative; > or equal to 1% = positive); method "E" (staining by % of cells: <5% of cells stained = negative; > or equal to 5% = positive); method "F" (staining by %: <10% = negative; > or equal to 10% = positive); and method "G" (staining by %: < or equal to 25% = negative; > 25% = positive).

In addition to the different immunohistochemical interpretation methods mentioned, other methods were found that used more than one interpretation parameter and were therefore eliminated from this study.

P16 expression in cases of HSIL of the cervix was analyzed in 41 articles out of 53 included in this systematic review. These 41 articles were grouped into the seven different methods found in the studies, with 14 articles in method "A", six in method "B", three in method "C", nine in method "D", three in method "E", four in method "F", and two in method "G".

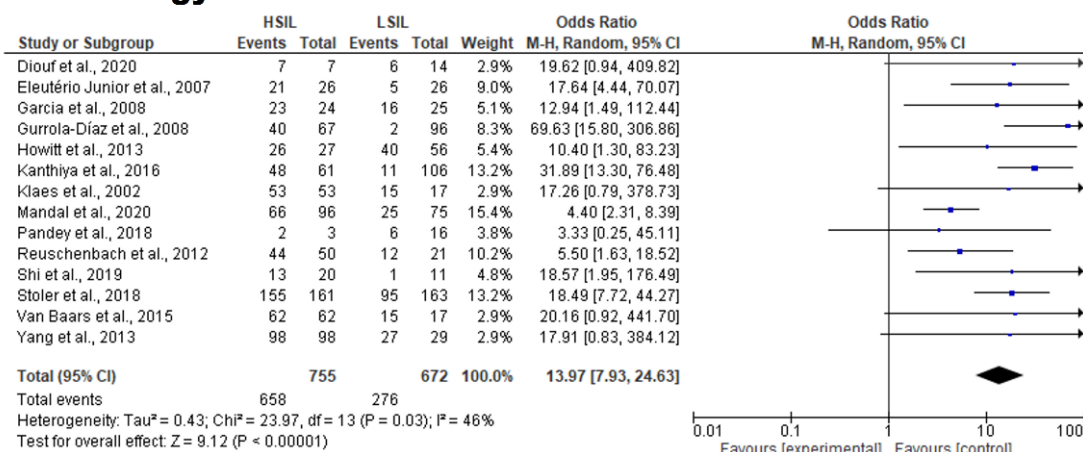
Ki-67 expression in cases of HSIL of the cervix was analyzed in 12 articles out of 53 included in this study. These articles were grouped between two immunohistochemical interpretation methods found in the studies, with five articles using method "A", five using method "F", and two using method "G".

The selection of outcome data for the meta-analysis was performed considering p16 and Ki-67 expression in cases of HSIL of the cervix compared to the control defined as LSIL in the cervix. This analysis was performed for each of the six immunohistochemical interpretation methods (from "A" to "F") used to interpret p16 and/or Ki-67 expression individually. Only method "G" was not possible to generate a meta-analysis due to the insufficient number of studies. Thus, a total of 51 articles were included in the meta-analysis: 41 for p16 and ten for Ki-67.

Concerning the p16 biomarker, methods "A" and "D" were the most studied for immunohistochemical interpretation, while the meta-analysis reveals that method "A" represented the highest OR, with OR = 13.97 (95%CI: 7.93 - 24.63; Z = 9.12; p < 0.00001). This indicates that method "A" (nuclear or cytoplasmic staining by epithelial layers: no staining or focal staining = negative; diffuse staining = positive) is more efficient for

interpreting p16 expression in HSIL and that women with positive p16 expression are 13.97 times more likely to develop HSIL compared to those with LSIL and those without positive p16 expression. Method “B” has an OR = 6.35 (95%CI: 4.18 – 9.65; $Z = 8.67$; $p < 0.00001$) for the p16 biomarker (Figure 2).

Methodology A



Methodology B

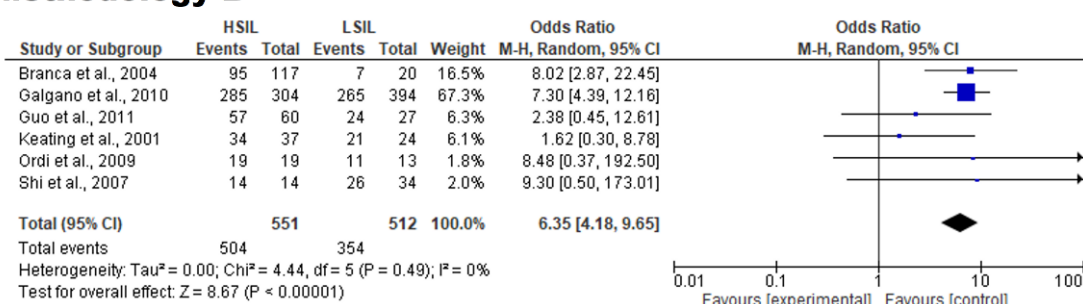


Figure 2 – Forest plots comparing methods “A” and “B” to interpret p16 expression in high-grade squamous intraepithelial lesions of the cervix. Recife (PE), Brazil, 2023.

Figure 3 shows that method “C” has an OR = 5.06 (95%CI: 2.31 – 11.07; $Z = 4.05$; $p < 0.0001$) for the p16 biomarker, method “D” has an OR = 8.60 (95%CI: 3.71 – 19.72; $Z = 5.02$; $p < 0.0001$) and method “E” has an OR = 11.99 (95%CI: 2.80 – 51.34; $Z = 3.35$; $p = 0.0008$) for the p16 biomarker. Figure 4, in turn, shows that method “F” has an OR = 6.93 (95%CI: 2.92 – 16.45; $Z = 4.39$; $p < 0.0001$) for the p16 biomarker.

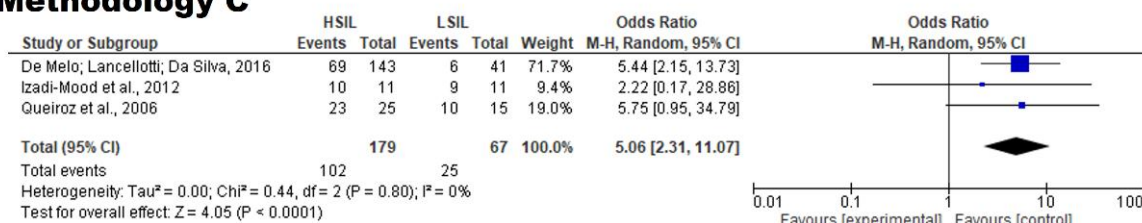
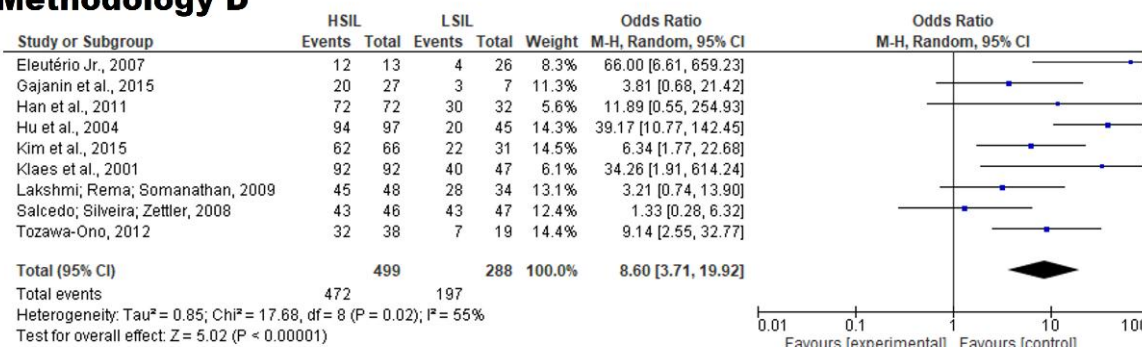
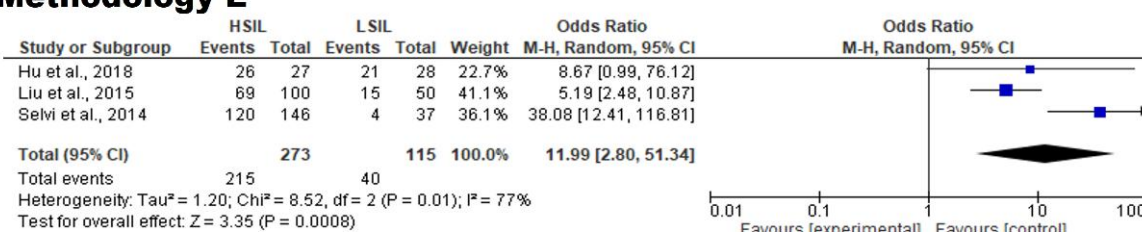
Methodology C**Methodology D****Methodology E**

Figure 3 - Forest plots of comparisons that used methods “C”, “D”, and “E” to interpret p16 expression in high-grade squamous intraepithelial lesions of the cervix. Recife (PE), Brazil, 2023.

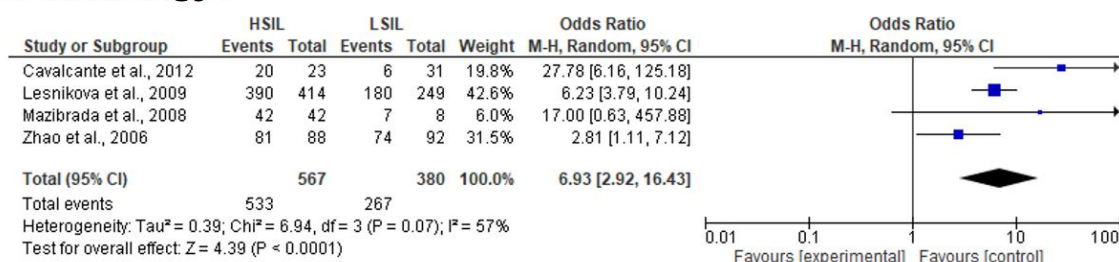
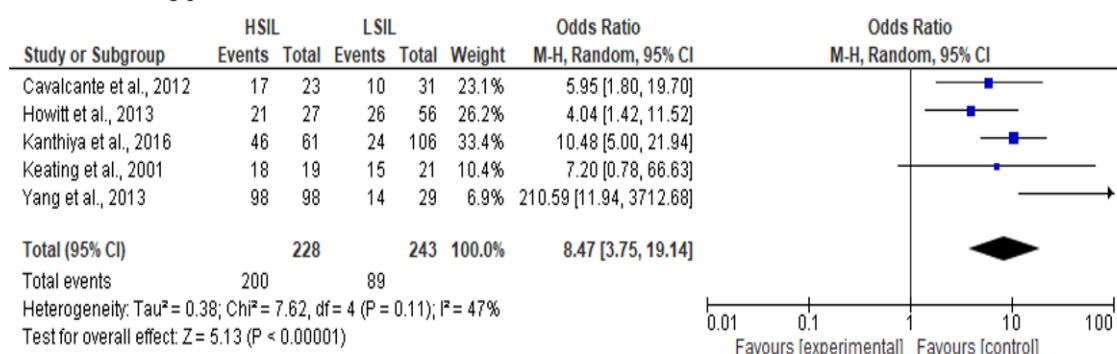
Methodology F

Figure 4 - Forest plots of comparisons that used the “F” method to interpret p16 expression in high-grade squamous intraepithelial lesions of the cervix. Recife (PE), Brazil, 2023.

Methods “A” and “F” were equally studied for the Ki-67 biomarker (Figure 5), but the meta-analysis outcome reveals that method “A” represented the highest OR, with OR = 8.47 (95%CI: 3.75 – 19.14; $Z = 5.13$; $p < 0.00001$). This indicates that method “A” is also more efficient for interpreting Ki-67 expression in HSIL, and that women with positive Ki-67 expression are 8.47 times more likely to develop HSIL compared to LSIL, when compared to those without positive Ki-67 expression. Figure 5 also shows that method “F” has an OR = 6.44 (95%CI: 2.11 – 19.66; $Z = 3.27$; $p = 0.001$) for the Ki-67 biomarker.

Methodology A



Methodology F

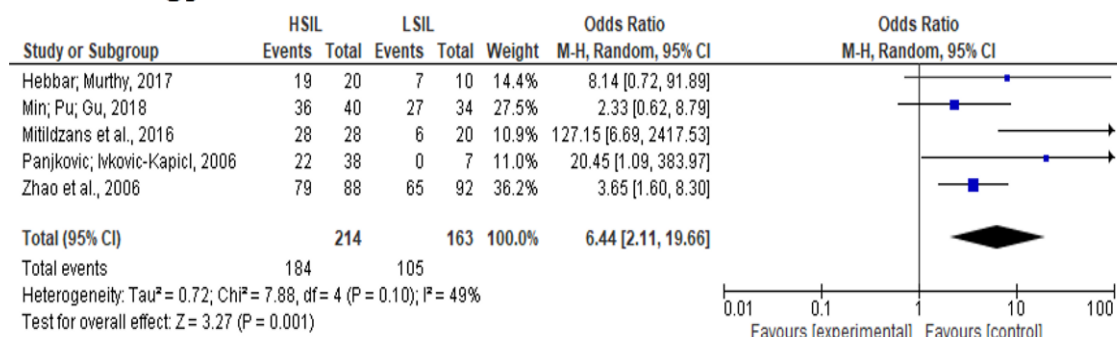


Figure 5 - Forest plots of comparisons that used methods “A” and “F” to interpret Ki-67 expression in high-grade squamous intraepithelial lesions of the cervix. Recife (PE), Brazil, 2023.

Furthermore, heterogeneity was found in 14 comparisons that used method “A” to interpret the p16 expression ($I^2 = 47\%$; $P = 0.03$). On the other hand, in five comparisons of Ki-67 expression interpretation using method “A”, no heterogeneity was detected ($I^2 = 47\%$; $P = 0.11$).

Discussion

Given the difficulty in differential diagnosis between cervical lesions based solely on morphology, immunohistochemistry (IHC) becomes an alternative to be applied for the identification of specific biomarkers for each type of lesion.¹³ In clinical practice, IHC has already been widely used for different types of cancer, and many studies have indicated the importance of its application not only for diagnosis but also for the indication of personalized therapies.¹⁴

This systematic review indicates that p16 and Ki-67 are the main biomarkers for HSIL of the cervix. In fact, biomarkers are more effective in confirming the presence of major anomalies, such as HSIL, than minor alterations such as LSIL.¹³ Several studies have already highlighted the strength of the expression of these two biomarkers in HSIL of the cervix. P16 expression has been widely applied and studied in HSIL of the cervix diagnosis, while, secondarily, Ki-67 expression has also proven important for this diagnosis.¹⁵⁻²⁰

These results may be related to HSIL pathogenesis, because although LSIL and HSIL are related to HPV infection, HSIL has been identified as being associated with high-risk HPV types, while LSIL mainly presents with low-risk HPV. One of the most common high-risk HPV types found among HSIL patients is HPV-16, whose greater persistence in the body increases the chances of progression to malignant neoplasms due to the production of oncogenes such as E7.²¹⁻²⁴

To understand the relationship between p16 and Ki-67 expression and HSIL pathogenesis, it is necessary to consider the biology of the cell cycle. The p16 protein (also known as p16INK4A) is a tumor suppressor protein that regulates cell cycle progression by inhibiting cyclin-dependent kinases, more specifically CDK4/6. This inhibition prevents the phosphorylation of retinoblastoma protein (pRB), which then continues to block the transcription factor E2F, preventing the transcription of genes related to cell cycle progression.²⁵ In HPV-16 infection, the viral oncoprotein E7 is produced, which binds to pRB, inactivating it, and E2F is released and then acts on the expression of genes that will lead to cell cycle progression. Consequently, p16 is upregulated, resulting from a negative feedback mechanism in the cell.²⁶

Furthermore, as a consequence of the deregulated cell cycle progression resulting from the action of the viral oncoprotein E7, there will be an increase in the number of mitoses. Ki-67 is a nuclear protein present in the cell cycle phases, and is therefore used as a marker of cell proliferation for the investigation of neoplasm diagnoses. The importance of histopathological assessment using hematoxylin and eosin together to assess the presence of atypical mitoses is highlighted.^{13;27}

Therefore, the diffuse p16 and Ki-67 expression is associated with HSIL pathogenesis, which is related to the prevalence of high-risk HPV and, thus, greater action of the E7 oncogene. This leads to cell cycle dysregulation, with consequent overexpression of p16, resulting from the negative feedback mechanism of the cell, as described previously, and also an increase in Ki-67 expression, resulting from the increased number of mitoses, when compared to LSIL.

A recent review study demonstrated the clinical importance of cervical cancer screening, emphasizing the need for and success of different tools that precisely guide clinical decision-making and the correct treatment for patients, such as dual labeling for p16/ki67.²⁸ Furthermore, other recent studies have shown the role of IHC for p16 and ki67 in distinguishing between HSIL and LSIL.²⁹⁻³¹ However, this study is the first systematic review with meta-analysis to rigorously demonstrate the relationship between the expression of these biomarkers as a complementary tool in the differential diagnosis of HSIL.

It is important to highlight that, in this systematic review, it was observed that immunohistochemical diagnosis for HSIL of the cervix has been performed using a variety of methods for analyzing tissue staining, with up to seven different immunohistochemical interpretation methods identified for interpreting p16 and Ki-67 expression. This result indicates a lack of standardization in this type of analysis, which may interfere with the final diagnosis. The study showed that difficulties still exist in the interpretation of IHC staining, especially for p16.¹⁹ Therefore, for the meta-analysis, subgroups were established according to the immunohistochemical interpretation method used on the extracted data, with the aim of reducing the influence of this factor on the heterogeneity of the effect size among studies.

Although all subgroup analyses showed a positive relationship between the presence of biomarkers and HSIL, it is noted that even different immunohistochemical interpretation methods with similar classification present an important difference in the reading of biomarker expression. Among the methods found, method "A", using nuclear or cytoplasmic staining by epithelial layers (no staining or focal staining = negative; diffuse staining = positive), shows greater significance for interpreting p16 expression in HSIL, with the highest OR among the subgroups analyzed (OR = 13.97; 95%CI: 7.93 - 24.63; Z = 9.12; p < 0.00001), when compared to LSIL. Similarly, this method showed greater significance for interpreting the Ki-67 biomarker, with a higher OR (OR = 8.47; 95%CI: 3.75 – 19.14; Z = 5.13; p<0.00001). Therefore, method "A", which considers nuclear or cytoplasmic staining by epithelial layers, with no staining or focal staining considered negative and diffuse

staining considered positive, showed the highest OR. This result indicates that the IHC analysis method can increase the chances of identifying clinical cases of HSIL and thus influence patient decision-making.

Some limitations of this systematic review and meta-analysis relate to the inclusion of only immunohistochemical biomarkers, but there are other markers to be studied in this context, such as immunological and genetic markers. Furthermore, the study design resulted in a prolonged time for data search, analysis, and interpretation. Therefore, it is possible that recent articles were not included in this review and meta-analysis.

Conclusion

HSIL diagnosis in the cervix can be made using different methods. To ensure the clinical safety of women, it is essential to follow scientific evidence with specific criteria. This systematic review with meta-analysis contributes to the validation of p16 and Ki-67 biomarkers as the most relevant in HSIL histopathological diagnosis compared to LSIL. Furthermore, it describes the different methods for interpreting p16 and Ki-67 expression, indicating that the method that classifies nuclear or cytoplasmic staining by epithelial layers (no staining or focal staining = negative; diffuse staining = positive) is more significant for interpreting both biomarkers in HSIL.

Authors' contributions

Study elaboration: Nirliane Ribeiro Barbosa, Luciana Xavier Pereira. **Data collection:** Nirliane Ribeiro Barbosa, Luciana Xavier Pereira, Salviane dos Santos Barbosa, Anderson Ferreira Barbosa. **Data analysis and interpretation:** Nirliane Ribeiro Barbosa, Luciana Xavier Pereira, Edilson Leite de Moura. **Manuscript writing:** Nirliane Ribeiro Barbosa, Luciana Xavier Pereira. **Manuscript critical review:** Edilson Leite de Moura, Elaine Virgínia Martins de Souza Figueiredo, Karol Fireman de Farias, Teresinha Gonçalves da Silva. **Approval of the final version of the text:** Nirliane Ribeiro Barbosa, Luciana Xavier Pereira, Edilson Leite de Moura, Elaine Virgínia Martins de Souza Figueiredo, Karol Fireman de Farias, Teresinha Gonçalves da Silva.

Conflict of interest

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