Original Article

Validation of CINtec® PLUS Cytology Kit in the Diagnosis of Persistent HPV Infections - Cohort Study in the Portuguese Population

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Abstract

Context: The use of the CINtec® PLUS Cytology Kit as a complementary method of diagnosis is intended to contribute to a risk assessment of CIN II+ cervical lesions development, thus avoiding unnecessary colposcopy in negative cases. This method has proved to be effective in the follow-up of cervical lesions, although there is need for consistency in the approach of equivocal or negative cytological results, with persistent positive High-Risk Human Papillomavirus (HR-HPV) test. **Aims:** The present study intends to retrospectively evaluate the capacity of the CINtec® to add information to the clinical practice within the screening and follow-up of cervical lesions. Additionally, this study intends to define criteria for CINtec® referral in order to maximize its utilization in the Portuguese context. **Materials and Methods:** For this purpose, 225 cases with CINtec® PLUS Cytology Kit and histology results were used, and data were analysed and compared according to the cytology and HR-HPV molecular test results. **Results:** The data of this study show that CINtec® PLUS Cytology Kit is a valid test for the diagnosis of persistent HPV lesions ($p = 0.0005$), with excellent sensitivity and reliable values of specificity. Relevant patient groups to apply the kit are in agreement with the manufacturer recommendations: women with ASC-US/LSIL result and NILM cytology with HPV-positive test, essentially over 30 years old. **Conclusions:** Although clinical data and previous cytological information must help guiding, authors considered that CINtec® is a really valid test for the prognosis of persistent HPV infections in the female population.

Keywords: Cervical cytology, cervical intraepithelial neoplasia, high grade, immunocytochemistry, triage

Introduction

In Portugal, two main sets of guidelines are consulted in Gynaecology,[1,2] but there is still ambiguity in the clinical follow-up of some cases. It is of utmost importance the application of tests with improved sensitivity and specificity for the effective screening of cervical cancer, essentially over 30 years old, where persistent infections rate increases.[3]

CINtec® PLUS Cytology Kit double positivity indicates oncogenic activity.[4-7] While molecular HPV detection methods does not differentiate transient from persistent infections, clinical utility of the Kit is high,[3,6,8-10] which contributes to the risk assessment of CIN II+ avoiding unnecessary colposcopies.

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Materials and Methods

This study comprises 225 samples of patients referred for the CINtec® PLUS Cytology Kit test (herein referred as CINtec®) at a laboratory in the north of Portugal, from January 2016 to September 2019. Inclusion criteria for the study were defined as cases with valid CINtec® test result, with colposcopy and histological information simultaneous or in 12 months after the CINtec® test. Information on cytology and molecular

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HR-HPV detection concurrent with the CINtec® test was also considered and collected whenever available. The ages of the patients considered are comprised between 18 and 72 years, with an average of 40. For a specific analysis in this study, women were grouped according to the age: women ≥30 years old (n = 203) and women <30 years-old (n = 22).

The study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. All data used were collected after patients' diagnosis analysis, with no further exams or tests performed for the present study. Data confidentiality was guaranteed. Procedures described in the methods section refer to the diagnosis laboratory procedures.

The materials for histological examination consisted of biopsies, curettages, and cone biopsies, which had been collected in 10% neutral buffered formalin and described through gross examination. The subsequent histological processing and embedding were performed, and 3 µm tissue sections were stained by HE (Hematoxylin and Eosin) and microscopically observed by an experienced pathologist. Figure 1 represents the histological outcomes of the samples, grouped according to their cytological result. Cases with morphological changes associated with HPV infection without dysplasia were grouped with CIN (Cervical Intraepithelial Neoplasia) I cases. The capacity of CINtec® test to translate high grade lesions was calculated using CIN II histological result (pre-invasive disease) as cut-off of disease.

For cytology, material sent in a ThinPrep® vial was processed on a ThinPrep 5000 or ThinPrep 5000 Autoloader, stained by the Papanicolaou technique and microscopically observed by an experienced cytotechnician, reviewed by the pathologist.

CINtec® PLUS Cytology kit is a qualitative immunocytochemical method used to detect cells under neoplastic transformation, showing positivity for both p16 and ki67 proteins. The method was performed on ThinPrep® cytological specimens or paraffin block tissues using BenchMark GX equipment (Roche, Germany) according to the kit instructions (CINtec® PLUS Cytology kit, reference 605-100). The signal detection occurs with two different chromogens, horseradish peroxidase-mediated conversion of 3,3'-Diaminobenzidine (DAB) chromogen, and alkaline phosphatase-mediated conversion of fast red chromogen, which result in brown and red colour at the p16 and Ki-67 antigen sites, respectively. The presence of at least one cervical epithelial cell with brown-labelled cytoplasm (and nucleus) and red-labelled nucleus is indicative of a positive test result. Microscopic results were carefully observed by an experienced pathologist.

The molecular HPV tests referred in this study regards to Aptima® HPV (Hologic, USA) and/or PapilloCheck® (Greiner Bio-one, Germany) genotyping assays. For the study, positivity for HPV was considered when a HR virus was detected in the sample concurrently with the CINtec® test performance.

For CINtec® results analysis, positivity rate, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. Prognosis capacity of CINtec® for CIN II+ lesions detection was determined through ROC (Receiving Operation Characteristic) Curve and respective AUC (Area Under the Curve) calculation. CINII was considered as a cut-off for disease.

Fisher's exact test was used to evaluate the correlation of CINtec® results among women with disease, as well as to

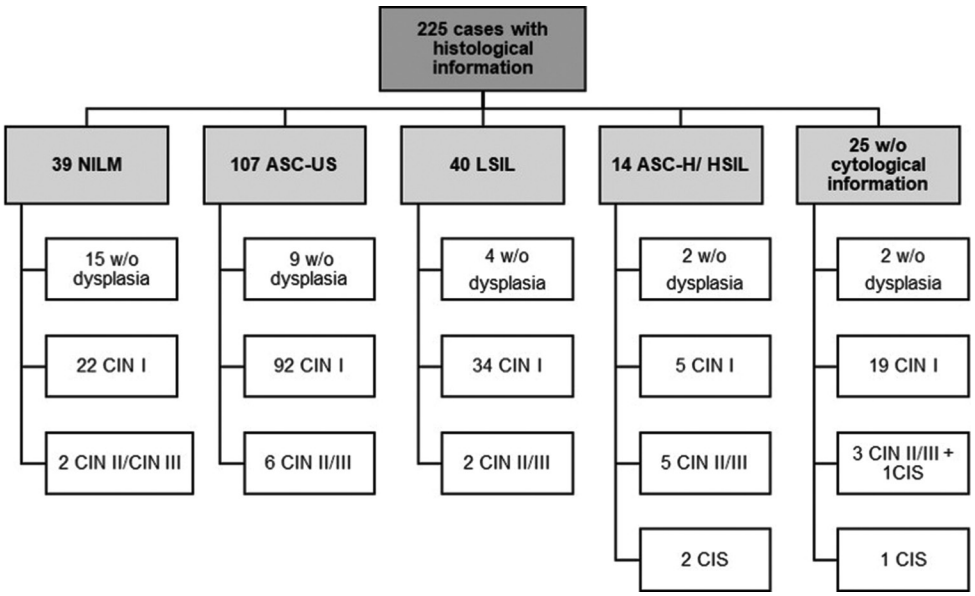

Figure 1: Schematic representation of the 225 cases based on histological diagnosis and cytological result. Abbreviations: ASC-H - Atypical Squamous Cells not excluding High-grade lesion; ASC-US - Atypical Squamous Cells of Undetermined Significance; CIN – Cervical Intraepithelial Neoplasia; CIS – Carcinoma In Situ; HSIL - High-grade Squamous Intraepithelial Lesion; LSIL - Low-grade Squamous Intraepithelial Lesion; NILM - Negative for Intraepithelial Lesion or Malignancy

determine the correlation between CINtec® and molecular HR-HPV test results.

All calculations and statistical analysis were performed using GraphPad Prism® 8.0 software (GraphPad Software, La Jolla CA, USA), considering a significance level of 0.05 (α) in all tests used.

Results

Among the 225 cases in study identified in Table 1, 39 (17%) were NILM (Negative for Intraepithelial Lesion or Malignancy), 107 (48%) had ASC-US (Atypical Squamous Cells of Undetermined Significance) result, 40 (18%) had LSIL (Low-grade Squamous Intraepithelial Lesion) cytology result, 14 (6%) had an ASC-H (Atypical Squamous Cells not excluding High-grade lesion) or HSIL (High-grade Squamous Intraepithelial Lesion) results and no information exists on 25 cases (11%). Table 1 also shows the proportion of CINtec® positive and negative results according to the histological and cytological category. This analysis reveals a global CINtec® positivity of 36% in this sample, and rates are expectably higher in women with cytological result of higher severity – 23% for NILM, 34% for ASC-US, 45% for LSIL, 79% for ASC-H/HSIL and 32% for those cases without cytological information. Positive cases for disease (CIN II+ lesions) were found in all cytological categories but only among CINtec® positive cases.

Sensitivity, specificity, positive and negative predictive values were calculated for the CINtec® test. The respective values were calculated for All Categories (regardless of the cytological results), as well as for specific groups of ASC-US/LSIL and NILM cytology resulted cases. Moreover, data analysis was done based on the age of the patients – over and under 30 years old. All information is depicted in Table 2.

CINtec® test demonstrated 100% sensitivity and 1.0 PNV in all situations considered, meaning that all CIN II+ lesions diagnosed were preceded by (or simultaneously detected with) a positive CINtec® test. PPV found in every groups were much lower (0.18-0.29) in association with lower specificity values (60-84%). The calculated specificity for the CINtec®, independently of the age, is 71% when all categories are considered, 68% in ASC-US/LSIL and 81% in NILM patient's group. When data were analysed according to the age of the patients, specificity and PPV values tend to be lower in the younger group women. For women ≥30 years old, specificity is 71% when all categories are considered, 69% in ASC-US/LSIL and 84% in NILM patient's group. Statistical significance using Fisher's exact test was achieved in all groups considered, except for women under 30 years of age.

A feasible value to assess the ability of a method to predict disease is the ROC curve and its AUC.[11] Concerning this tool, the present study confirms that CINtec® has an acceptable reliability to predict a CIN II+ histological outcome after a positive test. Here, AUC values range from 0.59 to 0.64 – reaching the statistical significance in all categories, independently of the age (AUC = 0.64; $p = 0.005$, in accordance with the abstract and the Table 2) and over 30 years old women group (AUC = 0.63; $p = 0.0012$), as shown in Table 2. The prognostic capacity in all women with ASC-US/LSIL result is almost statistically significant (AUC = 0.59; $p = 0.0617$). AUC values calculated are similar among groups under study.

Figure 2 shows the ROC curves calculated for NILM, ASC-US/LSIL and All categories groups, in women older than 30 years old, depicting the capacity of CINtec® to identify persistent HPV infections.

Among the 225 cases included in the study, a total of 138 had available information on molecular HR-HPV test performed. Sensitivity, specificity, ROC curves and AUC values were calculated for CINtec®, and the results obtained showed differences on the specificity and AUC of the kit based on the existence or absence of HR-HPV positive test [Table 3]. CINtec® prognostic capacity analysis showed very reliable values of AUC among the negative molecular test pool, reaching 0.70 in All Categories group and 0.75 ASC-US/LSIL group, with high specificity values (88% in All Categories and ASC-US/LSIL groups). Within positive HR-HPV cases specificity is lower (51% in All Categories, 47% in ASC-US/LSIL and 59% in NILM patients' group) and AUC values are within the range previously determined (0.57-0.63), reaching the statistical significance in the All categories group ($p = 0.0215$).

When correlating results of both molecular HR-HPV test and CINtec® there is a statistically significant correlation

Table 1: Information of Cytology and Histology Diagnosis According to CINtec® Plus Cytology Kit Result

Cytology/ Histology Result		CINtec® PLUS Cytology Kit⁻					CINtec® PLUS Cytology Kit⁺				
		w/o Lesion	CINI	CIN II/ CINIII	CIS	Total Negative (%)	w/o Lesion	CINI	CIN II/ CINIII	CIS	Total Positive (%)
39	NILM	14	16	0	0	30 (77%)	1	6	2	0	9 (23%)
107	ASC-US	9	62	0	0	71 (66%)	0	30	6	0	36 (34%)
40	LSIL	4	18	0	0	22 (55%)	0	16	2	0	18 (45%)
14	ASC-H/HSIL	1	2	0	0	3 (21%)	1	3	5	2	11 (79%)
25	w/o info	2	15	0	0	17 (68%)	0	4	3	1	8 (32%)
225	Total	30	113	0	0	143 (64%)	2	59	18	3	82 (36%)

ASC-H - Atypical Squamous Cells not excluding High-grade lesion; ASC-US - Atypical Squamous Cells of Undetermined Significance; CIN – Cervical Intraepithelial Neoplasia; CIS – Carcinoma In Situ; HSIL - High-grade Squamous Intraepithelial Lesion; LSIL - Low-grade Squamous Intraepithelial Lesion; NILM - Negative for Intraepithelial Lesion or Malignancy; w/o – without

Table 2: CINtec® Capacity to Predict CINII/CINIII Lesions According to Cytology Result and Age

	All Categories	ASC-US/LSIL	NILM
All patients (*n*, positivity %)	(*n*=225, 36%)	(*n*=147, 37%)	(*n*=39, 23%)
Sensitivity/Specificity	1.00/0.71	1.00/0.68	1.00/0.81
PPV/PNV	0.28/1.00	0.20/1.00	0.22/1.00
Fisher's test (*P*)	*P*<0.0001 (*)	*P*<0.0001 (*)	*P*=0.049 (*)
AUC/*P*	0.64/*P*=0.0005 (*)	0.59/*P*=0.0617 (ns)	0.61/*P*=0.3173 (ns)
Patients <30 years (*n*, positivity %)	(*n*=22, 36%)	(*n*=11, 45%)	(*n*=6, 33%)
Sensitivity/Specificity	1.00/0.70	1.00/0.60	
PPV/PNV	0.25/1.00	0.20/1.00	NC
Fisher's test (*P*)	*P*=0.1212 (ns)	*P*=0.4545 (ns)	
AUC/*P*	0.63/*P*=0.3393 (ns)	0.60/*P*=0.5152 (ns)	
Patients ≥30 years (*n*, positivity %)	(*n*=203, 36%)	(*n*=136, 36%)	(*n*=33, 21%)
Sensitivity/Specificity	1.00/0.71	1.00/0.69	1.00/0.84
PPV/PNV	0.28/1.00	0.18/1.00	0.29/1.00
Fisher's test (*P*)	*P*<0.0001 (*)	*P*<0.0001 (*)	*P*=0.0398 (*)
AUC/*P*	0.63/*P*=0.0012 (*)	0.59/*P*=0.076 (ns)	0.64/*P*=0.2522 (ns)

ASC-US - Atypical Squamous Cells of Undetermined Significance; AUC – Area Under the Curve; LSIL - Low-grade Squamous Intraepithelial Lesion; NILM - Negative for Intraepithelial Lesion or Malignancy; NC – not calculated due to the small number of cases; PPV – Positive Predictive Value; PNV – Negative Predictive Value; ns – without statistical significance; **P*<0.05 (statistical significance)

Table 3: CINtec® Capacity to Predict CINII/CINIII Lesions According to Molecular HR-HPV Test Result

	All Categories	ASC-US/LSIL	NILM
Positive HPV (*n*, positivity %)	(*n*=112, 56%)	(*n*=72, 57%)	(*n*=19, 47%)
Sensitivity/Specificity	1.00/0.51	1.00/0.47	1.00/0.59
PPV/PNV	0.25/1.00	0.15/1.00	0.22/1.00
Fisher's test (*P*)	*P*<0.0001 (*)	*P*=0.0335 (*)	*P*<0.0001 (*)
AUC/*P*	0.63/*P*=0.0215 (*)	0.57/*P*=0.2902 (ns)	0.61/*P*=0.4142 (ns)
Negative HPV (*n*, positivity %)	(*n*=26, 19%)	(*n*=18, 22%)	(*n*=4, 0%)
Sensitivity/Specificity	1.00/0.88	1.00/0.88	
PPV/PNV	0.40/1.00	0.50/1.00	NC
Fisher's test (*P*)	*P*=0.0308 (*)	*P*=0.0392 (*)	
AUC/*P*	0.70/*P*=0.1719 (ns)	0.75/*P*=0.1371 (ns)	

ASC-US - Atypical Squamous Cells of Undetermined Significance; AUC – Area Under the Curve; HPV – Human Papillomavirus; LSIL - Low-grade Squamous Intraepithelial Lesion; NILM - Negative for Intraepithelial Lesion or Malignancy; NC – not calculated due to the small number of cases; PPV – Positive Predictive Value; PNV – Negative Predictive Value; ns – without statistical significance; **P*<0.05 (statistical significance)

between them (p = 0.0009 for All Categories), according to Table 4. Similar results were found for ASC-US/LSIL group (p = 0.0161), although without statistical significance in NILM group (p = 0.1273). Indeed, 46% (All Categories and ASC-US/LSIL) and 39% (NILM) of the cases tested positive on CINtec® when HR-HPV test is also positive, 15% (All Categories and ASC-US/LSIL) and 17% (NILM) of the negative HR-HPV molecular test are also negative for CINtec®, and there is proportion 36% (All Categories), 34% (ASC-US/LSIL) and 43% (NILM) of the cases where a positive HR-HPV was found but resulted negative for CINtec®. This represents the important fraction of women where unnecessary colposcopies could be avoided. Additional Supporting Material (ASM) 1 represents schematically this information, including cases without available HR-HPV result.

Considering cases where HR-HPV information is available, (63/68) 93% of the positive CINtec® tests belong to the group that tested positive for HR-HPV molecular assay, and a small

Table 4: Correlation of CINtec® and HPV Molecular Test Results

HR-HPV/CINtec® result	CINtec® negative	CINtec® positive
Negative HPV (*n*, %)	21 (15,2%)	5 (3,6%)
Positive HPV (*n*, %)	49 (35,5%)	63 (45,7%)
Fisher's test (*P*)	0.0009 (*)	

HPV – Human Papillomavirus; **P*<0.05 (statistical significance)

fraction of 7% (5/68) had a previous negative HR-HPV molecular test, for All categories group. Similar values (91% vs 9%) were found for ASC-US/LSIL group and concerning NILM category, 100% of the CINtec® positive cases have a positive molecular HR-HPV result (ASM.1). Altogether, these results show the importance of a previous molecular HR-HPV assay as a triage test for the application of more accurate methods.

Altogether, data revealed a good capacity of CINtec® in predicting high grade cervical lesion in ASC-US/LSIL group, independently of the HR-HPV molecular result, and in positive

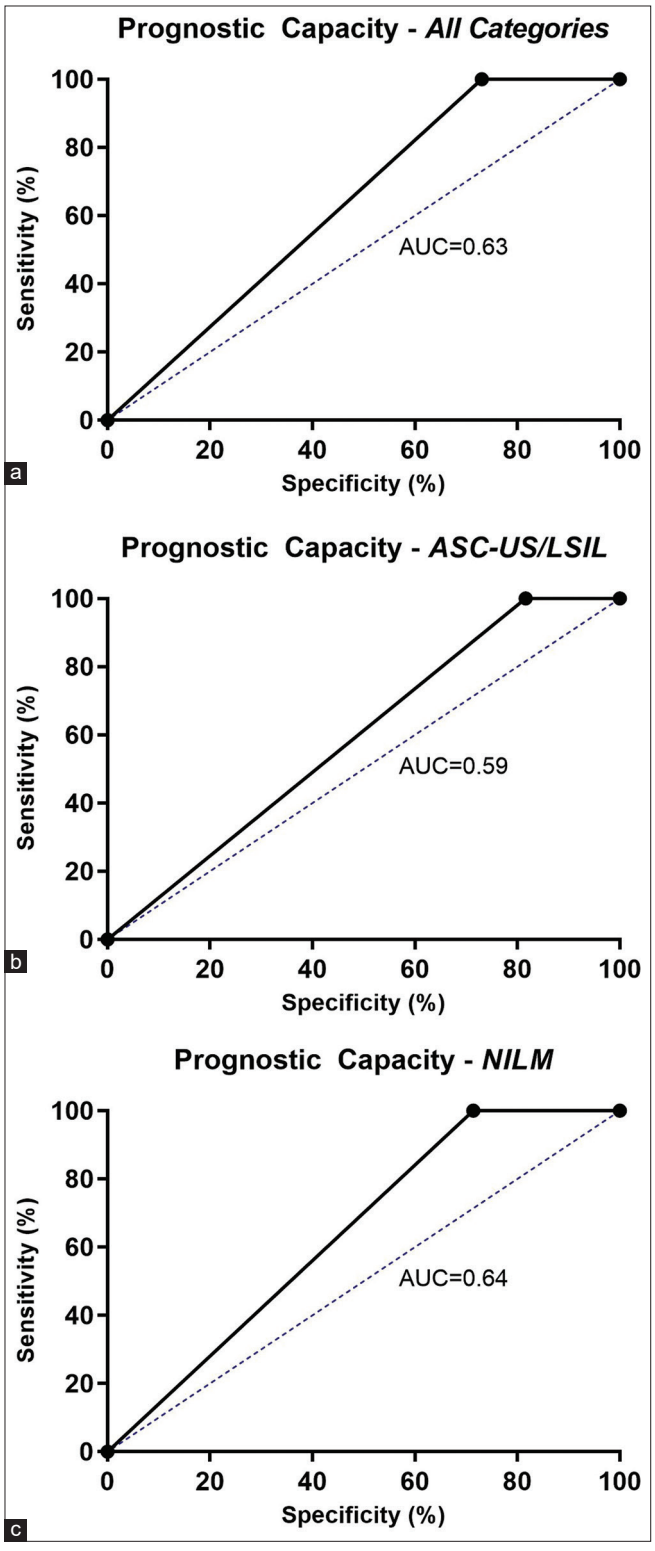

Figure 2: (a-c) ROC curves calculated for *All Categories*, *ASC-US/LSIL* and *NILM* groups, in women older than 30 years-old. Abbreviations: ASC-US - Atypical Squamous Cells of Undetermined Significance; LSIL - Low-grade Squamous Intraepithelial Lesion; NILM - Negative for Intraepithelial Lesion or Malignancy

HR-HPV/NILM-cytology, in women ≥30 years old. In younger women, results are not consistent due to the small number of cases. However, results obtained allows us to infer that cytological result and clinical information are still decisive to balance CINtec® prescription. Schematic representations are presented for both age groups (ASM.2 and ASM.3).

Although application of CINtec® on molecular HR-HPV positive cases may be feasible and necessary to decrease the assays performed, cytology is still crucial to define risk groups. Otherwise, some CIN II+ could be missed, as represented in ASM.4.

Discussion

Detection of p16 biomarker simultaneously with ki-67 has been used to identify persistent HPV infections, which are strongly associated with the development and progression of high-grade cervical intraepithelial lesions. Despite the existence of several studies on the application of CINtec® there are still no studies of major impact on the Portuguese female population.

Previously, the manufacturer of the kit recommended the CINtec® test as an adjunct to routine Pap smear test, essentially for the triage of women with ASC-US or LSIL cytology as well as women with NILM cytology and positive HPV test.[12] Moreover, studies indicate that the use of HPV testing as primary screening allows women with negative results to be routinely screened and safely increase the time interval between cytologies.[1,13,14,15]

However, a screening based on HPV molecular test, even with a reflex cytology, can result in an increased number of colposcopies for lesions that does not need any treatment. A possible strategy for effectively screening these patients would be their distinction according to the risk of developing CIN II+ lesions based on HPV genotype and CINtec® result.[16] This approach would certainly limit the early repetition of the HPV test and reduce the number of colposcopies. Although this approach seems desirable, a very judicious selection of patients need to be done and a very high expertise on CINtec® positive cells identification is needed.

The present work reveals that CINtec® sensitivity values are optimal in all groups considered, with reliable specificity and AUC values.

Data in the literature show high sensitivity and specificity of the double staining in the triage of women with ASC-US and LSIL, essentially among women positive for HPV, but is not consistent about the age of women where the kit is more effective younger or older than 30 years old.[3,6,8,10,16]

In agreement with the work carried out by *Possati-Resende et al.,*[3] in the present study results are more consistent among women older than 30 years old (p = 0.0012 for an AUC of 0.63, regardless of the cytology result). Results obtained for young women are not so robust and may have been influenced by the small number of cases under this category (22 women). However, due to the high rate of transient HPV infections in

young women,[17,18] it is for the authors understandable that a few numbers of tests are also prescribed on these cases, and that a positive CINtec® result might have more prognostic power of a CINII+ lesion progression in women older than 30 years.

Although within the range of published data,[19] specificity and AUC of CINtec® would be higher if other endpoint of analysis would be used. Indeed, this is a short period study with a small number of women with disease (cut-off defined as CIN II) and a follow-up was needed to assess the progression of lesions. Additionally, treatment of CIN I lesions during colposcopy decreases the number of cases where the neoplastic transformation would occur.

In agreement with published data, results show a positive correlation between the results of CINtec® and HR-HPV molecular tests.[3,6] In this study, 93% of the CINtec® positive cases belong to the positive HR-HPV molecular group, and 7% had a previous negative HR-HPV molecular test. Moreover, there were 36% cases in All Categories, 34% cases in ASC-US/LSIL and 43% cases in NILM group where results were surprisingly important: positive for molecular HR-HPV and negative for CINtec® (ASM.1). Those represent cases where CINtec® can reveal cost-effective, avoiding unnecessary colposcopies.

It is well known that most HPV infections does not necessarily translate into CIN II+ histology, essentially in young women. Besides, the HPV testing used is of utmost importance, since the biological basis of the virus detection is strongly variable among the existing methods, as well as it is the results translation to the clinical practice.[20-22] In Portugal, a negative HR-HPV result as a primary line screen implies that this woman would normally re-enter the screening scheme.[2,23] Women found in this study with CIN II+ lesions and positivity for CINtec® among negative HR-HPV cases, emphasizes the importance of the methodology selected to detect HPV. Moreover, our data suggest that maximal effectiveness of the CINtec® test is achieved when other methodology such as cytology is used as a guide, since a high percentage of disease would be missed if this HR-HPV test was used alone (ASM.4). Recently, in March 2020, Food and Drug Administration approved for the cervical cancer screening the combination of CINtec® Plus Cytology Kit as a biomarker-based triage test after the Cobas 4800 HPV test (Roche, Germany).[12] Regardless, the technical quality control is of great importance since those results will define the patient follow-up. In the present study information on molecular HR-HPV test was obtained by different methodologies. However, both have been strongly associated with persistent infections,[21,24] and they were used only as a source of information for the assessment of CINtec® performance.

Sensitivity and PNV achieved in the present study are excellent and emphasize the importance of the quality in the immunocytochemical technical procedure. The microscopic evaluation is strongly influenced by the technical procedures, namely the existence of a clean background and the quality of the double mounting technique for CINtec®. The interpretation of an unequivocal dual-stained cell is of utmost importance for a valid CINtec® positive test and requires expertise. For that, some aspects are relevant, such as: special attention to cell clusters is needed to evaluate the existence of a focal or diffuse colour; the evaluation and correct interpretation of the speckling pattern and bleeding of the red chromogen when occurring; and also, the recognition of artifacts and obscuring agents like mucus, blood and inflammatory cells.

Due to the lack of concrete information, CINtec® test is sometimes prescribed by clinicians in unexpected contexts. We assessed some CINtec® test results in negative HR-HPV/NILM women. In these cases, negative CINtec® test and negative histology confirms that it seems to be safe for a woman with this clinical profile to re-enter the screening scheme (ASM.1), according to Guidelines.[1,2] Importantly, the availability of actual data could be very helpful for clinicians when faced to equivocal cytology result, as well as to a HR-HPV positive/NILM result. CINtec® positivity rates calculated in this study are considerably high within negative, equivocal and mild abnormal cytologies, with a reliable specificity and 100% sensitivity. Therefore, it is possible to conclude that follow-up of women older than 30 years with positive HR-HPV and NILM result, as well as with ASC-US/LSIL cytology independently of the HPV molecular result, would strongly benefit from a CINtec® test (ASM.3). Concerning younger women (ASM.2) where a high incidence of transient infections occurs,[18] previous clinical information is crucial and may be decisive.

In conclusion, results of the present work corroborate data from the literature and reinforces that CINtec® test application with criteria would ensure its effectiveness. Authors consider CINtec® a really valid test for the prognosis of persistent HPV infections, and that its correct application would decrease the number of colposcopies, providing a more specific information and a better treatment orientation.

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Conflicts of interest

There are no conflicts of interest.

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