

Detection and genotyping of HPV-DNA through different types of diagnostic platforms in liquid-based cervical-cytology samples

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Key words

Cervical cancer • HPV test • Screening

Summary

Background. At present cervical cancer represents the second most common cancer in women worldwide and it reaches a global mortality rate of 52%. Only the early detection and the adequate treatment of pre-neoplastic lesions and early-stage cervical cancer decrease the mortality rate for this type of cancer. Cervical carcinoma screening, as a method of second prevention, is currently feasible through molecular research of high-risk HPV genotypes and in lots of organized screening programs the Pap-test is performed only in women with positive HPV-test. Currently, there are various diagnostic platforms detecting and molecular genotyping HPV, which are based on different procedures, determining uneven viral genotypes panels and using diverse type of vials to collect and store the samples. Previous studies have pointed out that DNA-HPV test can be negative in pre-neoplastic lesions, even of high grade, or in presence of cervical cancer. Therefore, it's important to assess the risk of false negative diagnoses using DNA-HPV molecular test, because in this circumstance women do not undergo immediately Pap-test, but they are submitted to second round screening with DNA-HPV test after 5 years: this protocol could increase the incidence of "interval cancers". The present study aims at comparing the results of HPV detection and genotyping on liquid based cervical cytology, using some of the most relevant diagnostic platforms in commerce.

Methods. The study is based on a group of patients which went to their private gynecologist in a contest of opportunistic screening. The vial used in the examined population has been EASY-PREP® preservative solution (YD Diagnostics CORP-Republic of Korea); liquid-based cervical cytology sampling has been done using a single device (plastic brush), allowing to collect simultaneously cytological material from exocervix and endocervix (Rovers® Cervex-Brush®). The diagnostic platforms employed have been the following: A) Digene HC2 HPV DNA Test, on RCS System (QIAGEN); B) BD Onclarity™ HPV test, on automate platform BD Viper™ LT (Becton Dickinson); C) Xpert® HPV, on

GeneXpert® Infinity Systems platform (Cepheid). Every platform researched high-risk HPV genotypes panels (hr-HPV). Part of the clinical records has also been analyzed through PCR and genes L1 and E6/E7 complete sequencing, in order to further typing the viral population.

Results. We have examined 1284 samples of women aged 16 to 73 years: 1125 have been tested using HC2 procedure, 272 samples with Onclarity method, 159 with Xpert® method and 55 samples have been analyzed using PCR and sequencing of gene L1 and gene E6/E7. HPV-DNA was detected with Onclarity method in 15,07%, with Xpert® method in 13,83% and using HC2 procedure in 12,27% of samples. The comparison between the three molecular methods revealed diagnostic discrepancies in 3,14% of our records between Onclarity test and Xpert® method and in 2,20% (6/272) between HC2 test and Onclarity test. Globally, in 431 tests, compared using different diagnostic platforms, discrepant diagnoses, referring to hr-HPV presence or to detected genotype, have been observed 11 times (2,55%). Genotype 16 appeared the most expressed in the positive samples (20,99%), whereas genotype 18 resulted the less expressed in the examined population (4,94%).

Discussion. The present study highlights the following: 1) Positive results' percentage for high-risk HPV-DNA genotypes, deriving from the three diagnostic platforms used and with the same vial to collect and store samples, does not significantly vary on the basis of the type of equipment and it is congruent with the Italian percentage already detected during organized screening programs. 2) Even the molecular diagnostic approach could give false negative results, preventing the detection in the screened population of cervical HPV-related lesions and theoretically endangering women to develop "interval cancer". 3) In the population examined, genotype 16 has been the most expressed, whereas genotype 18 was among the less frequently detected. Other genotypes often noticed have been: 56-59-66 (Onclarity P3 group), 31, 51 and 35-39-68

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(Onclarity P2 group). This remark emphasizes the importance of HPV infection and genotypes distribution's continuous monitoring, considering that HPV-vaccines planned in Italy in the "National vaccination prevention program 2017-2019" are not specific for the majority of these genotypes. 4) The necessity to improve the screening program to identify cervical carcinomas and pre-neoplastic cervical lesions is remarked by the detection during HPV-test of possible coinfection (present at least in 8,76% of our records). In fact, the risk of development of cervical cancer might be associ-

ated with type-specific interactions between genotypes in multiple infections and, in addition, other genotypes, not targeted by quadrivalent HPV-vaccine, can increase the risk of cervical carcinoma. 5) As there's a different combination of HPV-genotypes in diagnostic categories used by the HPV screening platforms, it's important that anyone who is in charge of this diagnostic analysis promotes among clinicians the adequate rendition of the laboratory's data in the patient records, reporting both the diagnostic result and the method through which it has been obtained.

Introduction

At present cervical cancer represents the second most common cancer in women worldwide ¹. More than 85% of cervical cancers develops in low-income or resource limited countries ², whereas in 2010, the invasive cervical cancer rate in the US was 7.5 per 100.000 women ³. This type of cancer reaches a global mortality rate of 52 ². Around 90% of these deaths affects low or medium income countries ⁴ and it is expected that by 2030 98% of cervical cancer deaths will occur in these same countries ⁵. The early detection and the adequate treatment of pre-neoplastic lesions and early-stage cervical cancer decrease significantly the mortality rate for this type of cancer ⁶. Indeed, still nowadays the healing chance is low when cervical cancer is diagnosed at a later stage of disease. In 2010 the 5-year survival rate in the US was 91% when the diagnosis of invasive cervical cancer was made at an early stage of disease, however the same survival rate decreased to 16% in late-stage cancer ⁷. The conventional Pap-test (Papanicolaou smear) has historically been the mainstay of pre-neoplastic lesions detection and cervical cancer screening; recently it has been introduced a new method, called "liquid-based cervical cytology" (LBCC) ⁸. The LBCC has one main convenience: it's possible to perform on a single sample both the Thin-Layer Cervical Cytology and the molecular HPV research. Many previous studies ⁹⁻¹⁶ have supported the usefulness of molecular research of specific DNA-HPV genotypes as a primary screening method, saving the LBCC just for women with positive HPV test. In this particular occurrence, it's convenient that the two tests (HPV-test and Pap-test) are realized from the same liquid-based cytological sample (co-testing), in order to reduce the number of false negatives of HPV-test or of Pap-test ¹⁷⁻²². Lately, the Government of Lombardy has approved ²³ a regional screening plan which establishes to perform the molecular HPV research in women aged between 34 and 64 years. The 12 high-risk HPV genotypes screened are the following: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, along with the possibility to include genotypes 66 and 68. Only women with positive molecular HPV test will undergo liquid based cervical cytology. Currently there are several types of diagnostic platforms detecting and molecular genotyping HPV, which are based on different procedures, determining uneven viral genotypes panels and using diverse type of vials to collect and store the samples. The present study

compares the results of HPV detection and genotyping on liquid based cervical cytology, using some of the most relevant diagnostic platforms in commerce. This comparison has two main goals: a) to verify the diagnostic homogeneity among the various platforms, especially as in the Lombardy cervical cancer screening program, women having a negative HPV molecular test do not undergo a Pap-test; b) to verify the most common high-risk HPV genotypes in this examined population, particularly referring to available cervical cancer vaccines.

Methods

The present study is not based on a population recruited through an organized screening program: all patients, during spontaneous access to their private gynecologist, underwent "liquid based cervical cytology" on which it has been performed the co-test Pap/Hpv or just the Pap test.

Our clinical records have been collected during the second half of 2016 and the first half of 2017.

The vial used in the examined population has been EASYPREP[®] preservative solution (YD Diagnostics CORP-Republic of Korea); liquid-based cervical cytology sampling has been done using a single device (plastic brush), allowing to collect simultaneously cytological material from exocervix and endocervix (Rovers[®] Cervex-Brush[®]). Within three months, in the patients who underwent Pap-test only it has been performed high-risk HPV-test, using the sample' stock. Likewise, within three months, part of the samples was analyzed through a second type of DNA-test, using a different diagnostic platform. The diagnostic platforms employed in the present study have been the following:

- BD Onclarity[™] HPV test, on automate platform BD Viper[™] LT (Becton Dickinson);
- Xpert[®] HPV, on GeneXpert[®] Infinity Systems platform (Cepheid);
- Digene HC2 HPV DNA Test, on RCS System platform (QIAGEN).

Table I compares high-risk genotypes panels detected by the various diagnostic platforms with the standard required from Lombardy for its screening program. The molecular and cytological diagnoses have been conducted independently by operators in a blind trial; when the same sample was tested with different platforms in order to identify HPV, operators were working ignoring

Tab. I. HPV genotypes determined with different diagnostic platforms.

HPV genotypes considered in Lombardy screening program	BD Onclarity™ HPV test	Xpert® HPV	Digene HC2 HPV DNA Test
16	X	X	X
18	X	X	X
31	X	X	X
33	X	X	X
35	X	X	X
39	X	X	X
45	X	X	X
51	X	X	X
52	X	X	X
56	X	X	X
58	X	X	X
59	X	X	X
66 (optional)	X	X	
68 (optional)	X	X	X

first analysis result. Pap-test diagnoses are not evaluated in the present study, because it is focused on HPV-test diagnostic concordance rate using different platforms: in fact, organized screening programs, which establish molecular test as the primary test to perform, do not prescribe Pap-test execution if molecular test is negative. Part of the clinical records has also been analyzed through PCR and direct sequencing of L1 and E6/E7 viral genes region, in order to further typing the viral population. Our Human Pathology Unit cooperates with external quality control for HPV screening (VEQ HPV Screening), organized by Lombardy Government. Results' statistical significance has been evaluated according to Chi-Squared test (one tailed). A p-value of ≤ 0.05 was considered as statistically significant.

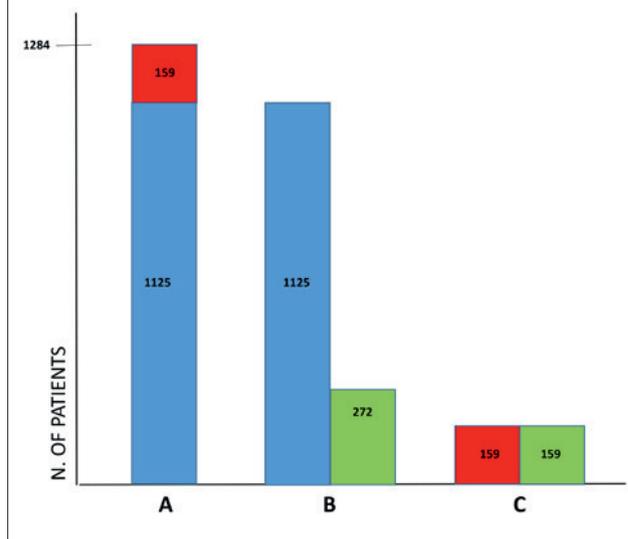
Results

We have examined 1284 samples of women aged 16 to 73 years. Table II displays the age distribution. Among these, 1125 have been tested using HC2 procedure, 272 samples have been analyzed with Onclarity method, 159 with Xpert® method (Fig. 1) and 55 samples have been analyzed using PCR and sequencing of gene L1 and genes E6/E7. HPV-DNA was detected with Onclarity method

Tab. II. Age distribution in the examined population.

Age	Number of patients	%
< 25	122	09,5%
25-33	327	25,5%
34-64	794	61,8%
> 64	41	3,2%
Total	1284	100

Fig. 1. Characteristics of the examined population. All samples have been collected with single device (plastic brush) "Rovers® Cervex-Brush®" and placed in the vial "EASYPREP® preservative solution" (YD Diagnostics CORP-Republic of Korea). A. Examined population: 1284 women; 1152 have been examined in the first place with Digene HC2 HPV DNA Test (blue column), and other 159 in the first place with Xpert® HPV test (red column). B. 272 of 1125 women analyzed using HC2 HPV DNA test have been reassessed with BD Onclarity™ HPV test (green column). C. All 159 women firstly examined with Xpert® HPV test (red column) have been re-analyzed with BD Onclarity™ HPV test (green column).



in 15,07% of samples, with Xpert® method in 13,83% of samples and using HC2 procedure in 12,27% of samples. These rates are not significantly different according to a statistical data analysis (HC2 versus Onclarity: $p = 1,54$; Onclarity versus Xpert: $p = 0.12$; HC2 versus Xpert: $p = 0.31$). The comparison between the three molecular methods revealed diagnostic discrepancies in 3,14% of our records (5/159) between Onclarity and Xpert (respectively 3 positive samples using Onclarity and negative using Xpert; and 2 positive samples using Xpert and negative using Onclarity) and it revealed discrepancies in 2,20% of our records (6/272) between HC2 test and Onclarity test (respectively 6 negative samples using HC2 and positive using Onclarity). The PCR analysis and L1, E6/7 viral genes sequencing revealed 1 positive sample for HPV-16 and 2 positive samples for HPV-18: this samples were negative according to HC2 method; in these three samples, all belonging to women older than 34 years, the Pap-test has always been positive for HPV-related lesions. Globally, referring to 431 tests evaluated with different methods, discrepant diagnoses of hr-HPV have been recorded 11 times (2,25%): HC2 High-Risk HPV DNA Test has diagnosed as "negative for hr-HPV infection" 6 of 272 samples (2,20%) co-tested with other platforms (Onclarity), Onclarity method has diagnosed as "negative for hr-HPV infection" 2 of 159 samples (1,26%) co-tested with Xpert and Xpert has diagnosed as "negative for hr-HPV infection" 3 of 159 samples (1,88%) co-tested with Onclarity. Among 81 HPV-test

Tab. III. Specific genotypes detected in 81 HPV-test positive samples.

HPV Genotypes	N° of cases	%	Prevalence ranking
16	17	20,99	1°
18	04	4,94	7°
31	12	14,81	3°
45	03	3,70	8°
51	10	12,34	4°
52	08	9,88	6°
P1	04	4,94	7°
P2	09	11,11	5°
P3	14	17,28	2°

Keys: P1 = genotypes 35,58. P2 = genotypes 35, 39,68. P3 = genotypes 56, 59, 66.

positive samples, it has been possible to identify the infecting genotype (Tab. III). The most detected genotype has been number 16 (20,99%), followed by P3 Onclarity group (including genotypes 56,59,66) (17,28%) and by genotype 31 (14,81%). Genotype 18 was observed to a lesser extent in our population (4,94%). Using the three HPV-screening methods, in 8,76% of the population co-infection with more hr-HPV genotypes has been detected. It was not possible to specifically characterize the genotypes determining these co-infections, because both Onclarity and Xpert join different genotypes in groups, which are not homogeneous and therefore not further analyzable. Onclarity individually reveals genotypes 16, 18, 31, 45, 51 and 52 positivity; in “P1” group it gathers genotypes 33 and 58; in “P2” group genotypes 35, 39, 68 and in “P3” group genotypes 56, 59 and 66. On the other hand, Xpert identifies as “P1” genotype 16, as “P2” genotypes 18 and 45, as “P3” genotypes 31, 33, 35, 52 and 58, as “P4” genotypes 51 and 59 and as “P5” genotypes 39, 68, 56 and 66. The PCR analysis and L1, E6/7 viral genes sequencing revealed that co-infection can be determined by high-risk HPV genotypes only, but also by concomitant presence of high-risk genotypes and intermediate or low risk ones (Tab. IV).

Tab. IV. HPV co-infection detected through PCR and genes L1 and E6/E7 complete sequencing.

	HPV Genotype	High risk	Intermediate risk	Low risk
Case				
1		16 + 31		
2		16		61
3		16	66	
4		18	66	
5		18	66	
6			53 + 67	
7		31		6
8				6 + 72
9			67	81

Discussion

The risk of invasive cervical cancer considerably decreases in women who periodically undergo Pap-test and/or DNA-HPV test²⁴⁻²⁵. The conventional Pap-smear has been proven to represent a critical tool to diagnose cervical pre-neoplastic lesions and cervical early stage cancer²⁶; its effectiveness has been further enhanced after the introduction in current clinical practice of LBCC²⁷⁻²⁸. The Pap-test remains, however, an exam whose diagnostic results are profoundly influenced by human subjectivity (high inter and intra operator variability referring to diagnostic criteria)²⁹⁻³⁰ and by the level of expertise/tiredness of screeners³¹⁻³³. Currently, molecular identification of high-risk HPV genotypes (hr-HPV test) is used as primary test in several cervical cancer screening programs, because its ability to identify high grade intra-epithelial cervical lesions is considered statistically superior than the cytological one³⁴⁻³⁵. However, the application of molecular HPV-test as primary cervical carcinoma screening tool unfolds several uncertainties. To date, several large available cervical cancer series have documented that HPV-test is negative in 10 to 19% of women with biopsy-confirmed cancer³⁶⁻⁴⁰. The test power to detect cervical adenocarcinoma varies from approximately 32 to 100%, depending on the geographic region and tumor subtype⁴¹⁻⁴⁶. Human papillomavirus DNA is detected in 80 to 100% of the 3 most common histological subtypes of cervical adenocarcinoma (endocervical, endometrioid and intestinal subtypes), whereas it's rarely detected in non-mucinous subtypes, such as clear cell, serous and mesonephric adenocarcinoma. In addition, the gastric type, which includes minimal-deviation adenocarcinoma, was shown to be unrelated to HPV infection⁴⁷. Cervical adenocarcinomas constitute about 5 to 27% of all cervical carcinomas: their number varies between different countries⁴⁸⁻⁵² and it is globally increasing⁵³⁻⁵⁴. Eventually it has been observed that Pap-test cervical carcinoma screening can occasionally detect endometrial carcinomas or endometrial atypical glandular cell (AGC)⁵⁵: these pathological entities are negative using HPV-test⁵⁶; therefore, primary HPV-test screening, instead of Pap-test, may result in losing the possible benefits of early diagnosis of endometrial cancer⁵⁷. According to several strategies of cervical cancer screening, the algorithm connecting cervical cytology and hr-HPV test is still debated and it's influenced by both economic available resources and patient characteristics (such as age; organized screening program versus spontaneous patient request of exam or occasional medical indication during gynecological visit – the so called “opportunistic screening” (OS)). Starting from a single liquid based cervical cytology sample, several diagnostic algorithms are possible:

- the two tests can be always and simultaneously performed (co-testing)⁴⁰⁻⁵⁸⁻⁶⁰;
- it's possible to initially search the virus presence (definition of “presence of infection”) and then it can

be performed LBCC on infected women, in order to verify ongoing cervical disease)¹⁶;

- c) there's the possibility to perform firstly the Pap-test and secondly the molecular HPV-test in women with positive Pap-test result (this algorithm is recommended in women younger than 34 years, due to high incidence of sub-clinical infections in young women)¹⁶;
- d) it can be performed firstly the Pap-test and then the HPV-test only in women presenting cytological atypia of undetermined significance (ASC-US; ASC-H; AGC referring to The Bethesda System)⁶¹, so to determine if these cytological atypia are HPV-related. The Pap/HPV co-test is considered the best strategy for cervical carcinoma screening in women aged from 30 to 65 years, because, compared with HPV-test only, co-testing is more sensitive for the detection of lesions \geq CIN3^{40 62-64}. Moreover, co-testing reduces the number of cervical carcinoma false negatives (particularly referring to glandular-type) which, instead, can occur using HPV-test as primary screening tool; co-testing allows to occasionally detect also endometrial adenocarcinoma or metastasis. Using HPV-test for primary cervical carcinoma screening exposes the patients to false negative results and non-detected patients HPV-infected do not undergo immediately Pap-test, but they are submitted to second round screening, always using HPV-test, after 5 years. This protocol might expose them to the risk of developing the so called "interval cancers"⁶⁵.

Our study, conducted on a population of women aged between 16 and 73 years, deriving from spontaneous screening, has demonstrated that there aren't statistically significant differences between HPV-test results from 3 different diagnostic platforms currently used for cervical carcinoma screening (HC2 versus Onclarity: $p = 1,54$; Onclarity versus Xpert: $p = 0,12$; HC2 versus Xpert: $p = 0,31$). Positive case percentage for high-risk HPV-DNA genotypes, acquired through the three diagnostic platforms used and with a single vial to collect and store the samples, varied from 12,27 to 15,07% ($\Delta = 2,8\%$, not statistically significant) and this percentage is comparable with that deriving from previous studies and with the use of different sample vials. Italian average rate of positive HPV-test result during organized screening programs in women aged from 25 to 64 years is between 6,7% and 7,9%^{66 67}, but variability range among different screening programs is between 4,3 and 13,9%⁶⁷. Besides, it has to be considered that the population of our study consisted of 35% women with less than 34 years and that in this age group HPV-test positivity rate can even reach 34,4%⁶⁸.

HPV-test is nowadays regarded as an "objective test", free from diagnostic errors, whereas it's stressed the intrinsic "subjectivity" of cytological diagnosis, strongly operator-dependent. This pervasive opinion has contributed to the decision of using HPV-test as the primary screening tool in women aged 34 to 64, using the Pap-test as a "clinical triage" only in HPV-test positive wom-

en. As far as our samples are concerned, when on the same sample different diagnostic platforms performing HPV molecular research are used, it's possible to notice discrepancies among the results: non-concordance diagnostic rate varied from 2,20 to 3,14%. Especially, in 11 women (2,55% of cases), one of the two diagnostic platforms compared has detected hr-HPV (representing a screening positive case), while the other has not detected hr-HPV (representing a screening negative case). Moreover, the re-analysis with PCR has revealed in other three cases, negative using HC2 test, the presence of hr-HPV genotypes: yet, this discrepancy could be due to the sensitivity of HPV-test methods committed to screening programs, which is deliberately inferior than PCR/gene sequencing, nevertheless it's notable that the Pap-test of each of the three women was positive for HPV-related lesions and that all the women were older than 35 years. These elements point out that even the molecular diagnostic approach could give false negative results, being sometimes unable to identify in the screened population women affected by cervical HPV-related lesions. A screening program based on hr-HPV-test performed on 100.000 women could in theory produce about 2.500 false negative diagnoses. This observation should lead to a serious consideration, that is to compare the costs of HPV screening test, which uses the Pap-test only as a clinical triage in positive patients, with the costs deriving from assisting and curing women with "interval cancers". These costs should also be examined in view of those of other screening strategies, such as: a) co-testing "DNA HPV-test/LBC Pap-test" screening; b) LBC-Pap test screening to identify patients with ongoing disease, and consecutive RNA-HPV test for prognostic evaluation and treatment protocol definition in women with cervical lesion. Recent studies have highlighted that Human Papillomavirus E6/E7 mRNA test has a significantly higher specificity and overall accuracy for HSIL or worse lesion than HPV-DNA test and that, therefore, it may be useful in clinical risk management⁶⁹, particularly in women younger than 35 years⁷⁰. It has also been observed that MiR-21-5p upregulation, MiR-34a downregulation and human telomerase RNA component (hTERC) amplification are associated with aggressive progression of CC⁷¹, suggesting that these genetic markers could be usefully employed in screening programs for the triage of LBC Pap-test positive patients.

In our selected population, the most detected genotype was genotype 16 (20,99%), while genotype 18 was among the less observed (4,94%). It has been noticed a prevalence higher than 10% for genotypes 56-59-66 (Onclarity "P3" group) (17,28%), for genotype 31 (14,81%), for genotype 51 (12,34%) and for genotypes 35-39-68 (Onclarity "P2" group) (11,11%). This remark stresses the importance of HPV genotypes' distribution continuous monitoring in the population, given that HPV vaccines planned in Italy in the "National vaccination prevention program 2017-2019" are the "bivalent one" (against genotypes 16 and 18) and the "quadrivalent one" (against genotypes 16, 18, 6 and 11)⁷². The

necessity to improve the screening program to identify cervical carcinomas and pre-neoplastic cervical lesions, even if there's an undergoing vaccination program, is remarked by the detection during HPV-test of coinfection from more genotypes. The risk of cervical cancer development might be associated with type-specific interactions between genotypes in co-infections and genotypes not targeted by quadrivalent vaccine confer 2.94-fold higher risk of cervical carcinoma⁷³. Among the samples analyzed with screening diagnostic platforms, our study observed 8,76% of prevalence of co-infection from more hr-HPV genotypes, but this percentage is surely underestimated, because only in a small proportion of HPV positive samples it has been possible to verify the single infectious genotypes. Moreover, the re-examination of 55 cases with PCR and sequencing of gene L1 and genes E6/E7 pointed out that co-infections can be determined by high-risk HPV genotypes but also by intermediate or low risk ones. The prevalence of multiple genotypes (double, triple and quadruple genotypes) in women older than 18, married and clinically symptomatic (abnormal vaginal bleeding/discharge, pain during coitus, lower abdominal pain and clinician suspicion of cervical malignancy) resulted noticeably higher (23,41% of 346 cases)⁷³ than the prevalence observed in our population. Even if in Italy, in the target population of organized screening programs, the incidence of coinfections is probably inferior (giving the fact that Italian screening programs do not include very young women), the possibility that coinfections might increase the risk of development of cervical carcinoma, also in women HPV-vaccinated, is real and represents another valid reason to perform organized screening programs in the population at risk. An ancillary point deriving from our study is related to the different "gathering method" in "P groups" of various genotypes detected by diagnostic platforms available to date in commerce. Just taking into consideration as an example BD Onclarity™ HPV test e Xpert® HPV (Cepheid), it's evident how it can be dangerous to report into clinical records of patients with positive HPV-test, only the "P group", without specifying the method used: in this particular example two women, one diagnosed using Onclarity method and the other using Xpert, who are both "P1" positive, are de facto infected by different genotypes: the first by genotype 33 or 58, the second by genotype 16. It's therefore important that anyone who is in charge of this diagnostic analysis promotes among clinicians the adequate rendition of the laboratory's data in the patient records, reporting both the diagnostic result and the method through which it has been obtained.

References

- World Health Organization. *Comprehensive cervical cancer control: a guide to essential practice*. 2nd ed. Geneva: WHO 2014.
- Bradford L, Goodman A. *Cervical cancer screening and prevention in low-resource settings*. Clin Obstet Gynecol 2013;56:76-87.
- Henley SJ, Singh S, King J, et al; Centers for Disease Control and Prevention. *Invasive cancer incidence United States, 2010*. MMWR Morb Mortal Wkly Rep 2014;63:253-9.
- Sowemimo OO, Ojo OO, Fasubaa OB. *Cervical cancer screening and practice in low resource countries: Nigeria as a case study*. Trop J Obstet Gynaecol 2017;34:170-6.
- Ferlay J, Shin HR, Bray F, et al. *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008*. Int J Cancer 2010;127:2893-2917.
- <https://www.cdc.gov/cancer/cervical/statistics/index.htm>
- Howlander N, Noone AM, Krapcho M, et al. *SEER Cancer Statistics Review, 1975-2011*. National Cancer Institute; Bethesda, MD: 2014.
- Wilbur DC, Cibas ES, Merritt S, et al. *ThinPrep processor. Clinical trials demonstrate an increased detection rate of abnormal cervical cytologic specimens*. Am J Clin Pathol 1994;101:209-14.
- Kitchener H, Canfell K, Gilham C, et al. *The clinical effectiveness and cost-effectiveness of primary human papillomavirus cervical screening in England: extended follow-up of the ARTISTIC randomised trial cohort through three screening rounds*. Health Technol Assess 2014;18:1-196.
- Ronco G, Dillner J, Elfstrom KM, et al. *Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of 4 European randomised controlled trials*. Lancet 2014;383:524-32.
- Wright TC, Stoler MH, Behrens CM, et al. *Primary cervical cancer screening with human papillomavirus: end of study results from the ATHENA study using HPV as the first-line screening test*. Gynecol Oncol 2015;136:189-97.
- Wright TC, Stoler MH, Behrens CM, et al. *Primary cervical cancer screening with human papillomavirus: end of study results from the ATHENA study using HPV as the first-line screening test*. Gynecol Oncol 2015;136:189-97.
- US Food and Drug Administration (FDA). *FDA approves first human papillomavirus test for primary cervical cancer screening*. Silver Spring, MD: FDA; 2014. Available at: www.eve-medical.com/fda-approves-first-human-papillomavirus-test-for-primary-cervical-cancer-screening/. Accessed January 1, 2016
- Simms KT, Hall M, Smith MA, et al. *Optimal management strategies for primary HPV testing for cervical screening: cost-effectiveness evaluation for the National Cervical Screening Program in Australia*. PLoS One 2017;12:e0163509.
- Cuschieri K, Ronco G, Lorincz A, et al. *Eurogin roadmap 2017: triage strategies for the management of HPV-positive women in cervical screening programs*. Int J Cancer 2018;143:735-45.
- L'implementazione del DNA-HPV come test primario nei programmi italiani di screening del cervicocarcinoma. Indicazioni dai risultati del Progetto MIDDIR Methods for Investments/Disinvestments and distribution of health technologies in italian regions*. <https://www.cpo.it/workspace/files/report-midir-aprile2016-571a18a7556a9.pdf>. In: Ministero della Salute. Piano Nazionale della Prevenzione 2014-2018, pp. 25-26.
- Blatt AJ, Kennedy R, Luff RD, et al. *Comparison of cervical cancer screening results among 256,648 women in multiple clinical practices*. Cancer Cytopathol 2015;123:282-8.
- Documento Gisci. *Indicazioni per il prelievo nello screening del carcinoma cervicale. Punto 6: Modalità di esecuzione e di identificazione del prelievo per test HPV e Pap test con dispositivi per raccolta in fase liquida*. 2016.
- Petry KU, Cox JT, Johnson K, et al. *Evaluating HPV-negative CIN2+ in the ATHENA trial*. Int J Cancer 2016;138:2932-9.
- Hui Y, Hansen K, Murthy J, et al. *Relevance of the Pap test: a report of HPV-DNA test-negative high-grade squamous intraepithelial lesions of the female lower genital tract*. Acta Cytol 2016;60:445-50.
- Zhou H, Mody RR, Luna E, et al. *Clinical performance of the Food and Drug Administration-approved high-risk HPV test for the detection of high-grade cervicovaginal lesions*. Cancer Cytopathol 2016;124:317-23.

- 22 Tracht JM, Davis AD, Fasciano DN, et al. *Discrepant HPV/cytology cotesting results: are there differences between cytology-negative versus HPV-negative cervical intraepithelial neoplasia?* Cancer Cytopathol 2017;125:795-805.
- 23 Delibera n° X/7013 del 31/07/2017. *Aggiornamento ed estensione del programma di screening organizzato del tumore della cervice uterina in Regione Lombardia*. <http://www.regione.lombardia.it>
- 24 Mille JW, Royalty J, Henley J, et al. *Breast and cervical cancers diagnosed and stage at diagnosis among women served through the National Breast and Cervical Cancer Early Detection Program*. Cancer Causes Control 2015;26:741-7.
- 25 Tsikouras P, Zervoudis S, Manav B, et al. *Cervical cancer: screening, diagnosis and staging*. J Buon 2016;21:320-5.
- 26 National Institutes of Health. *Cervical Cancer*. NIH Consensus Statement 1996;14:1-38.
- 27 Cox JT. *Liquid-based cytology: evaluation of effectiveness, cost-effectiveness, and application to present practice*. J Natl Compr Canc Netw 2004;2:597-611.
- 28 Tao X, Austin RM, Zhang H, et al. *Pap test reporting rates for conventional smear and liquid-based cervical cytology from the largest academic women's hospital in China: analysis of 1,248,785 Pap Test Reports*. Acta Cytol 2015;59:445-51.
- 29 Klinkhamer PJ, Vooijs GP, de Haan AF. *Intraobserver and interobserver variability in the diagnosis of epithelial abnormalities in cervical smears*. Acta Cytol 1988;32:794-800.
- 30 Bigras G, Wilson J, Russell L, et al. *Interobserver concordance in the assessment of features used for the diagnosis of cervical atypical squamous cells and squamous intraepithelial lesions (ASC-US, ASC-H, LSIL and HSIL)*. Cytopathology 2013;24:44-51.
- 31 Grohs DH, Dadeshidze VV, Domanik RA, et al. *Utility of the Trac-Cell system in mapping Papanicolaou-stained cytologic material*. Acta Cytol 1997;41:144-52.
- 32 Vooijs GP, Nauwelaers FA, van Aspert-van Erp AJ. *CytosafePLUS. A workstation for screening, supervision, reviewing, quality assurance and education in cytopathology*. Acta Cytol 1996;40:90-6.
- 33 Faraker CA, Boxer ME. *Rapid review (partial rescreening) of cervical cytology. Four years experience and quality assurance implications*. J Clin Pathol 1996;49:587-91.
- 34 Koliopoulos G, Nyaga VN, Santesso N, et al. *Cytology versus HPV testing for cervical cancer screening in the general population*. Cochrane Database Syst Rev 2017;8:CD008587.
- 35 Canfell K, Caruana M, Gebski V, et al. *Cervical screening with primary HPV testing or cytology in a population of women in which those aged 33 years or younger had previously been offered HPV vaccination: Results of the Compass pilot randomised trial*. PLoS Med 2017;14:e1002388.
- 36 Wu Y, Chen Y, Li L, et al. *Associations of high risk HPV types and viral load with cervical cancer in China*. J Clin Virol 2006;35:264-9.
- 37 Kang WD, Kim CH, Cho MK, et al. *Comparison of hybrid capture II assay with the human papillomavirus DNA chip test for the detection of high-grade cervical lesions*. Int J Gynecol Cancer 2009;19:924-8.
- 38 Moreira MA, Longato-Filho A, Taromus E, et al. *Investigation of human papillomavirus by hybrid capture II in cervical carcinomas including 113 adenocarcinomas and related lesions*. Int J Gynecol Cancer 2006;16:586-90.
- 39 Poljak M, Kovanda A, Kocjan BJ, et al. *The Abbott RealTime High Risk HPV test: comparative evaluation of analytic specificity and clinical sensitivity for cervical carcinoma and CIN 3 lesions with the Hybrid Capture 2 HPV DNA test*. Acta Dermatoven Alp Panonica Adriat 2009;18:94-103.
- 40 Blatt AJ, Kennedy R, Luff RD, et al. *Comparison of cervical cancer screening results among 256,648 women in multiple clinical practices*. Cancer (Cancer Cytopathol) 2015;123:282-8.
- 41 Pirog EC, Kleter B, Olgac S, et al. *Prevalence of human papillomavirus DNA in different histological subtypes of cervical adenocarcinoma*. Am J Pathol 2000;157:1055-62.
- 42 Pirog EC, Lloveras B, Molijn A, et al; RIS HPV TT Study Group. *HPV prevalence and genotypes in different histological subtypes of cervical adenocarcinoma, a worldwide analysis of 760 cases*. Mod Pathol 2014;27:1559-67.
- 43 Holl K, Nowakowski AM, Powell N, et al. *Human papillomavirus prevalence and type-distribution in cervical glandular neoplasias: results from a European multinational epidemiological study*. Int J Cancer 2015;137:2858-68.
- 44 Molijn A, Jenkins D, Chen W, et al; Chinese HPV Typing Group. *The complex relationship between human papillomavirus and cervical adenocarcinoma*. Int J Cancer 2016;138:409-16.
- 45 Quint KD, de Koning MN, Geraets DT, et al. *Comprehensive analysis of human papillomavirus and chlamydia trachomatis in in-situ and invasive cervical adenocarcinoma*. Gynecol Oncol 2009;114:390-4.
- 46 Pimenta JM, Galindo C, Jenkins D, et al. *Estimate of the global burden of cervical adenocarcinoma and potential impact of prophylactic human papillomavirus vaccination*. BMC Cancer 2013;13:553-65.
- 47 Pirog EC. *Cervical adenocarcinoma diagnosis of human papillomavirus-positive and human papillomavirus-negative tumors*. Arch Pathol Lab Med 2017;141:1653-67.
- 48 Davis JR, Moon LB. *Increased incidence of adenocarcinoma of uterine cervix*. Obstet Gynecol 1975;45:79-83.
- 49 Parazzini F, Lavecchia C. *Epidemiology of adenocarcinoma of the cervix*. Gynecol Oncol 1990;39:40-6.
- 50 Antonculver H, Bloss JD, Bringman D, et al. *Comparison of adenocarcinoma and squamous cell carcinoma of the uterine cervix - a population-based epidemiologic-study*. Am J Obstet Gynecol 1992;166:1507-14.
- 51 Wang SS, Sherman ME, Hildesheim A, et al. *Cervical adenocarcinoma and squamous cell carcinoma incidence trends among white women and black women in the United States for 1976-2000*. Cancer 2004;100:1035-44.
- 52 Adegoke O, Kulasingam S, and Virnig B. *Cervical cancer trends in the United States: a 35-year population-based analysis*. J Womens Health (Larchmt) 2012;21:1031-7.
- 53 Hemminki K, Li X, Mutanen P. *Age-incidence relationships and time trends in cervical cancer in Sweden*. Eur J Epidemiol 2001;17:323-8.
- 54 Smith HO, Tiffany MF, Qualls CR, et al. *The rising incidence of adenocarcinoma relative to squamous cell carcinoma of the uterine cervix in the United States: a 24-year population-based study*. Gynecol Oncol 2000;78:97-105.
- 55 Arbyn MAA, Jordan J. *European guidelines for quality assurance in cervical cancer screening*. 2nd edn. Luxembourg: Office for Official Publications of the European Communities, 2008:1-291.
- 56 Castle PE, Fetterman B, Poitras N, et al. *Relationship of atypical glandular cell cytology, age, and human papillomavirus detection to cervical and endometrial cancer risks*. Obstet Gynecol 2010;115:243-8.
- 57 Norman I, Hjerpe A, Dillner J. *Risk of high-grade lesions after atypical glandular cells in cervical screening: a population-based cohort study*. BMJ Open 2017;7:e017070.
- 58 Saslow D, Solomon D, Lawson HW, et al. *American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer*. CA Cancer J Clin 2012;62:147-72.
- 59 Committee on Practice Bulletins-Gynecology. *ACOG practice bulletin number 131: screening for cervical cancer*. Obstet Gynecol 2012;120:1222-38.

- ⁶⁰ Bouchard-Fortier G, Hajifathalian K, McKnight MD, et al. *Co-testing for detection of high grade cervical intraepithelial neoplasia and cancer compared with cytology alone: a meta-analysis of randomized controlled trials.* J Public Health (Oxf) 2014;36:46-55.
- ⁶¹ Nayar R, Wilbur DC. *The Pap test and Bethesda 2014.* Acta Cytologica 2015;59:121-32.
- ⁶² Katki HA, Schiffman M, Castle PE, et al. *Five-year risks of CIN 3+ and cervical cancer among women who test Pap-negative but are HPV-positive.* J Low Genit Tract Dis 2013;17(5 suppl 1):S56-S63.
- ⁶³ Katki HA, Kinney WK, Fetterman B, et al. *Cervical cancer risk for women undergoing concurrent testing for human papillomavirus and cervical cytology: a population-based study in routine clinical practice.* Lancet Oncol 2011;12:663-72.
- ⁶⁴ Gage JC, Schiffman M, Katki HA, et al. *Reassurance against future risk of precancer and cancer conferred by a negative human papillomavirus test [serial online].* J Natl Cancer Inst 2015;107;pii.dju153.
- ⁶⁵ Herbert A, Anshu, Gregory M, et al. *Invasive cervical cancer audit: a relative increase in interval cancers while coverage increased and incidence declined.* BJOG 2009;116:845-53.
- ⁶⁶ Altobelli E, Lattanzi A. *Cervical Carcinoma in the european union. an update on disease burden, screening program state of activation, and coverage as of march 2014.* Int J Gynecol Cancer 2015;25:474Y483.
- ⁶⁷ Ronco G, Giorgi-Rossi P, Giubilato P, et al. *A first survey of HPV-based screening in routine cervical cancer screening in Italy.* Epidemiol Prev 2015;39:77-83.
- ⁶⁸ Ma S, Stern JE, Feng Q, et al. *Incidence and risk factors for human papillomavirus infections in young female online daters.* J Med Virol 2017;89:2029-36.
- ⁶⁹ Ge Y, Christensen P, Luna E, et al. *aptima human papillomavirus e6/e7 mrna test results strongly associated with risk for high-grade cervical lesions in follow-up biopsies.* J Low Genit Tract Dis 2018 Jul;22(3):195-200.
- ⁷⁰ Granados R, Tellez-Safina H, Solis I, et al. *Cervical cancer screening cotesting with cytology and MRNA HPV E6/E7 yields high rates of CIN2+ lesions in young women.* Diagn Cytopathol 2017;45:1065-72.
- ⁷¹ Zhu Y, Han Y, Tian T, et al. *MiR-21-5p, miR-34a, and human telomerase RNA component as surrogate markers for cervical cancer progression.* Pathol Res Pract 2018;214:374-9.
- ⁷² Repubblica Italiana-Ministero della Salute. *Piano Nazionale Prevenzione Vaccinale 2017-2019.* 17.01.2017. http://www.salute.gov.it/imgs/C_17_pubblicazioni_2571_allegato.pdf
- ⁷³ Senapati R, Nayak B, Kar SK, et al. *HPV genotypes co-infections associated with cervical carcinoma: Special focus on phylogenetically related and non-vaccine targeted genotypes.* PLoS One 2017;21;12:e0187844. doi: 10.1371/journal.pone.0187844. eCollection 2017.