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NEW BIOMARKERS AND NEW RECOMMENDATIONS FOR HPV-BASED CERVICAL CANCER SCREENING

Submitted for the degree of doctor in Clinical and Experimental Medicine at the University of Modena and Reggio Emilia, to be defended by

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Summary

Background The new challenge in cervical cancer prevention is the reduction of over-diagnosis and over-treatment. These phenomena may increase with the introduction of the HPV test. We need triage tests to reduce colposcopy referral of HPV-DNA positive women, and we need appropriate follow up strategies for women who had a colposcopy and received a treatment for a high-grade cervical intraepithelial neoplasia (CIN2 or CIN3).

Objectives In the present PhD research project, we aimed, firstly, to assess the accuracy of biomarkers (HPV E6/E7 mRNA and p16/Ki67) as test of triage in HPV-DNA based screening protocols. Secondly, we aimed to assess the prognostic value of these biomarkers for the identification of regressive lesions. Finally, we developed evidence-based recommendations within the Multisociety Italian guidelines for cervical cancer prevention.

Methods In collaboration with the Epidemiology unit of the AUSL-IRCCS of Reggio Emilia, we coordinated the 12 months follow up and the analyses of data collected by all recruiting centres of the New Technologies in Cervical Cancer 2 (NTCC2) trial. NTCC2 aimed to measure the accuracy of mRNA and p16/Ki67 and their negative predictive value for CIN2 or more severe lesions (CIN2+), recruiting women who were invited for a new screening round based on HPV-DNA test within the screening programs (i.e. aged 25-59). Regarding the third objective, as member of the Italian Group for Cervical Cancer Screening (GISCi) we lead the Evidence Review Team involved in the Multisociety Italian guidelines for cervical cancer prevention development process at a national level. We coordinated the systematic reviews and the Grading of Recommendations Assessment, Development and Evaluation (GRADE) Evidence-to-Decision (EtD) process to update recommendations for the management of women treated for CIN2 or CIN3.

Results The NTCC2 12-month follow up ended and 40,509 women of which 3147 (7.8%) HVP-DNA positive were included the final analysis. Cumulatively, 174 CIN2+ (including 95 CIN3 and 1 Adenocarcinoma in situ) were found. As for cross-sectional accuracy, adjusted sensitivity for CIN2+ was 61.0% (95%CI 53.6-68.0), 94.4% (95%CI 89.1-97.3), and 75.2% (95%CI 68.1-81.6) for cytology, mRNA, and p16/ki67 respectively. The overall referral was 65.3%, 78.6%, and 63.7%, respectively. The Positive Predictive Value (PPV) was 9.5% for cytology, 8.3% for mRNA, and 10.1% for p16/ki67. As for prognostic value for regression of CIN2+ lesions and clearance of HPV DNA, in NTCC2 data regression was almost null in biomarker-positive women at baseline, while in biomarker-negatives was [-76% (95%CI from -97% to more than 100%) and -39% (95%CI from -72% to 33%) for E6/E7 mRNA and p16/ki67, respectively]. However, the test for interaction was not statistically significant in either case. Moreover, among HPV DNA+ /cytology- women, the clearance of HPV DNA after 12 months in mRNA-negative women was 1.9 times (95% CI 1.7, 2.2) that in mRNA+ women and clearance in p16/ki67- women was 1.9 times (95% CI 1.5, 2.5) that in p16/ki67+ women.

Finally, we published the first recommendation on the HPV vaccination of women treated for CIN2 or CIN3 in the Italian National System of Guidelines (SNLG) repository of the National Institute of Health. We also completed the adolopment process of six recommendations voted by a previous panel on the choice of follow up tests, number of episodes and intervals, and we completed the evidence synthesis and EtD process for the remaining clinical questions on the management of women according to the results of after-treatment follow up tests. Adopted and new recommendations are currently under review by SNLG assessment team for publication.

Conclusions The present PhD research project provided new evidence on the use of biomarkers in cervical cancer screening, identifying p16/ki67 as the most promising triage test. Moreover, we supported national recommendations development updating existing guidelines on the management of women treated for CIN2 or CIN3.

Keywords

Cervical cancer, Human papillomavirus, HPV Vaccination, Cervical cancer screening, prevention

Italian summary

Introduzione La nuova sfida nella prevenzione del cancro cervicale è la riduzione della sovra diagnosi e dei trattamenti non necessari. Questi fenomeni possono aumentare con l'introduzione del test HPV come test primario di screening. È pertanto necessario introdurre test di triage per ridurre l'invio in colposcopia di donne positive al test HPV-DNA. Sono inoltre necessarie strategie di follow-up appropriate per le donne che hanno avuto una colposcopia e hanno ricevuto un trattamento per una neoplasia intraepiteliale cervicale di alto grado (CIN2 o CIN3), attualmente non definite in maniera dettagliata dalle linee guida esistenti con conseguente rischio di inappropriatezza.

Obiettivi Gli obiettivi del progetto di ricerca di dottorato sono:

- valutare l'accuratezza dei biomarcatori (HPV E6 / E7 mRNA e p16 / Ki67) come test di triage nei protocolli di screening basati su HPV-DNA;
- valutare il valore prognostico di questi biomarcatori per l'identificazione di lesioni regressive;
- aggiornare le linee guida nazionali per la prevenzione del cancro della cervice uterina con raccomandazioni basate sulle evidenze disponibili per la gestione delle donne trattate per CIN2 e CIN3.

Metodi In collaborazione con l'Unità di Epidemiologia dell'Azienda USL-IRCCS di Reggio Emilia, abbiamo coordinato la raccolta e l'analisi statistica dei dati di follow up a 12 mesi raccolti da tutti i centri di reclutamento dello studio New Technologies in Cervical Cancer 2 (NTCC2). NTCC2 ha l'obiettivo di misurare l'accuratezza di mRNA e p16 / Ki67 e il loro valore predittivo negativo per CIN2 o lesioni più gravi (CIN2 +), reclutando donne invitate per un nuovo round di screening con test HPV-DNA all'interno dei programmi di screening organizzato (età 25-59 anni). Per quanto riguarda il terzo obiettivo, come membri del Gruppo Italiano per lo Screening del Cancro Cervicale (GISCi) guidiamo l'Evidence Review Team coinvolto nel processo di sviluppo di linee guida multi-societarie a livello nazionale. Abbiamo coordinato le revisioni sistematiche e il processo Evidence-to-Decision (EtD) secondo la metodologia Grading of Recommendations Assessment, Development and Evaluation (GRADE) per aggiornare le raccomandazioni relative alla gestione delle donne trattate per CIN2 o CIN3.

Risultati Il follow-up di 12 mesi NTCC2 è terminato e 40.509 donne di cui 3147 (7,8%) positive al HPV-DNA sono state incluse nell'analisi finale. Complessivamente, sono state identificate 174 lesioni CIN2+ (incluse 95 CIN3 e 1 adenocarcinoma in situ). Per quanto riguarda l'accuratezza *cross-sectional*, la sensibilità aggiustata per CIN2+ è stata 61,0% con Intervallo di Confidenza al 95% (IC95%) 53,6-68,0 per citologia, 94,4% (IC 95% 89,1-97,3) per mRNA e 75,2% (IC 95% 68,1-81,6) per p16 / ki67. L'invio in colposcopia complessivo è stato 65,3% per citologia, 78,6% per mRNA e 63,7% per p16 / ki67. Il valore predittivo positivo (PPV) era del 9,5% per la citologia, dell'8,3% per l'mRNA e del 10,1% per p16 / ki67. Per quanto riguarda il valore prognostico per la regressione delle lesioni CIN2 + e la clearance delle infezioni da HPV ad un anno, dai dati NTCC2 la regressione delle lesioni è risultata quasi nulla nelle donne positive al biomarker al baseline, mentre nelle donne negative al biomarker era di -76% (IC

95% da -97% a più rispetto al 100%) per mRNA E6 / E7 e -39% (IC 95% da -72% a 33%) per p16 / ki67. Tuttavia, il test per l'interazione non è risultato statisticamente significativo in entrambi i casi. Inoltre, tra le donne HPV DNA + / citologia negative, la clearance dell'HPV a 12 mesi nelle donne mRNA negative era 1,9 volte (95% CI 1,7 - 2,2) maggiore rispetto a quella nelle donne mRNA positive. Analogamente, la clearance delle infezioni nelle donne p16 / ki67 negative è risultata 1,9 volte (95% CI 1.5, 2.5) maggiore rispetto a quella nelle donne p16 / ki67 positive.

Infine, a Luglio 2019 abbiamo pubblicato la prima raccomandazione sulla vaccinazione HPV delle donne trattate per CIN2 o CIN3 nel database online del Sistema Nazionale Linee Guida (SNLG) dell'Istituto Superiore di Sanità. Abbiamo anche completato il processo di *adolopment* di sei raccomandazioni votate da un precedente panel sulla scelta dei test di follow-up, del numero di episodi e degli intervalli tra episodi. Abbiamo infine completato la sintesi delle evidenze ed il processo EtD per i restanti quesiti clinici sulla gestione delle donne in base ai risultati dei test di follow-up post-trattamento. Tali raccomandazioni sono attualmente in fase di revisione da parte del team di valutazione SNLG per la pubblicazione sul database nazionale.

Conclusioni Il progetto di ricerca di dottorato ha fornito nuove evidenze sul ruolo dei biomarcatori nello screening del cancro cervicale, identificando p16 / ki67 come il test di triage più promettente. Inoltre, abbiamo supportato lo sviluppo di raccomandazioni nazionali aggiornando le linee guida esistenti sulla gestione delle donne trattate per CIN2 o CIN3.

Acronyms

AGENAS:	Italian National Agency for Regional Health Services
ACS:	American Cancer Society
AIO:	Italian Association of Obstetrics (Associazione Italiana Ostetricia)
AIS:	Adenocarcinoma in situ
AOGOI:	Italian Association of Hospital Obstetricians and Gynaecologists (Associazione Italiana Ostetrici e Ginecologi Ospedalieri)
ASCCP:	American Society for Colposcopy and Cervical Pathology
ASC-H:	Atypical Squamous Cells - cannot exclude HSIL
ASCP:	**American** Society for Clinical Pathology
ASC-US:	Atypical squamous cells of undetermined significance
ASMR:	Age-standardized mortality rate
CI:	Confidence interval;
CIN:	Cervical Intraepithelial Neoplasia
CNEC:	National Centre for Clinical Excellence, Quality and Safety of Care (Centro nazionale per l'eccellenza clinica, la qualità e la sicurezza delle cure)
COI:	Conflict-of-Interests
CP:	Conventional Pap smear
CTS:	Technical Scientific Committee (CTS)
E6 / E7 mRNA:	Test for the detection of overexpression of messenger Ribonucleic acid of the Human Papillomavirus oncogene E6 and E7
EBM:	Evidence Based Medicine
ERT:	Evidence Review Team
EtD	Evidence-to-Decision framework
FOBT:	Faecal occult blood test;
GDG:	Guidelines Development Group
GISCi:	Italian group for cervical cancer screening (Gruppo Italiano per lo Screening del Cervicocarcinoma)
GRADE:	Grading of Recommendations Assessment, Development and Evaluation
HDI:	Human Development Index
HPV:	Human Papillomavirus
HPV-DNA:	Test for the detection of Human Papillomavirus Deoxyribonucleic acid
HR:	Hazard Ratio
hrHPV:	High risk Human Papillomavirus
H-SIL:	High Grade Squamous Intraepithelial Lesion
INAIL:	The National Institute for Insurance against Accidents at Work (Istituto nazionale Assicurazione Infortuni sul Lavoro)

ISS:	The Italian National Institute of Health (Istituto Superiore di Sanità)
ISTAT:	The Italian National Institute of Statistics (Istituto Nazionale di Statistica)
LBC:	Liquid-based cytology
LEA:	Essential Levels of Care (Livelli Essenziali di Assistenza)
L-SIL:	Low-Grade Squamous Intraepithelial Lesion
LSIL:	Low-grade squamous intraepithelial lesion
NILM:	Negative for intraepithelial lesion or malignancy
NTCC2:	New Technologies in Cervical Cancer 2 trial
ONS:	The National Centre for Screening Monitoring (Osservatorio Nazionale Screening)
p16 / Ki67:	Dual immunostaining of p16INK4a and Ki-67 proteins
PASSI:	The Italian Behavioral Risk Factor Surveillance System (Progressi delle Aziende Sanitarie per la Salute in Italia)
PICO:	Population, intervention, comparison, outcomes
PNP:	National Prevention Plan
PNPV:	National Immunization Plan
PRP:	Regional Preventon Plan
RR:	Risk Ratio
SIAPEC-IAP:	Italian Society for Pathological Anatomy and Diagnostic Cytology. Italian Division of the International Academy of Pathology (Società Italiana Anatomia Patologica e Citologia Diagnostica. Divisione Italiana dell'International Accademy of Pathology)
SICi:	Italian Society of Cytology (Società Italiana di Citologia)
SICPCV:	Italian Society for Colposcopy and Cervico-Vaginal Pathology (Società Italiana di Colposcopia e Patologia Cervico-Vaginale)
SIGO:	Italian Society of Gynecology and Obstetrics (Società Italiana Ginecologia e Ostetricia)
SItI:	Italian Society of Hygiene, Preventive Medicine and Public Health (Società Italiana di Igiene, Medicina Preventiva e Sanità Pubblica)
SIV-ISV:	Italian Society for Virology (Società Italiana di Virologia)
SNLG:	The Italian National System of Guidelines (Sistema Nazionale linee Guida)
SoF:	Summary of Findings table
USPSTF:	United States Preventive Service Task Force

1. Chapter 1: Dissertation outline

1.1. Background

1.1.1. Burden of cervical cancer

1.1.1.1. Incidence

Globally, cervical cancer or cancer of the uterine cervix (ICD-10 code C53) is the fourth cancer in terms of incidence among women of all ages, and second among women of reproductive ages. [1] Cervical cancer account for more than 6% of the global cancer burden in women, following only breast, colorectal and lung cancer. [2]

In 2018, an estimated 570 000 new cases were diagnosed resulting in an age-standardized incidence rate (ASIR) of 13.1 per 100 000 women worldwide. [1]

The incidence rate of cervical cancer differs across countries showing wide variations due to several factors, including cultural and health related behaviors, prevalence of biological risk factors, i.e. Human Papillomavirus (HPV) and Human Immunodeficiency Virus (HIV), and differences in the implementation of preventive strategies. [1] (Figure 1)

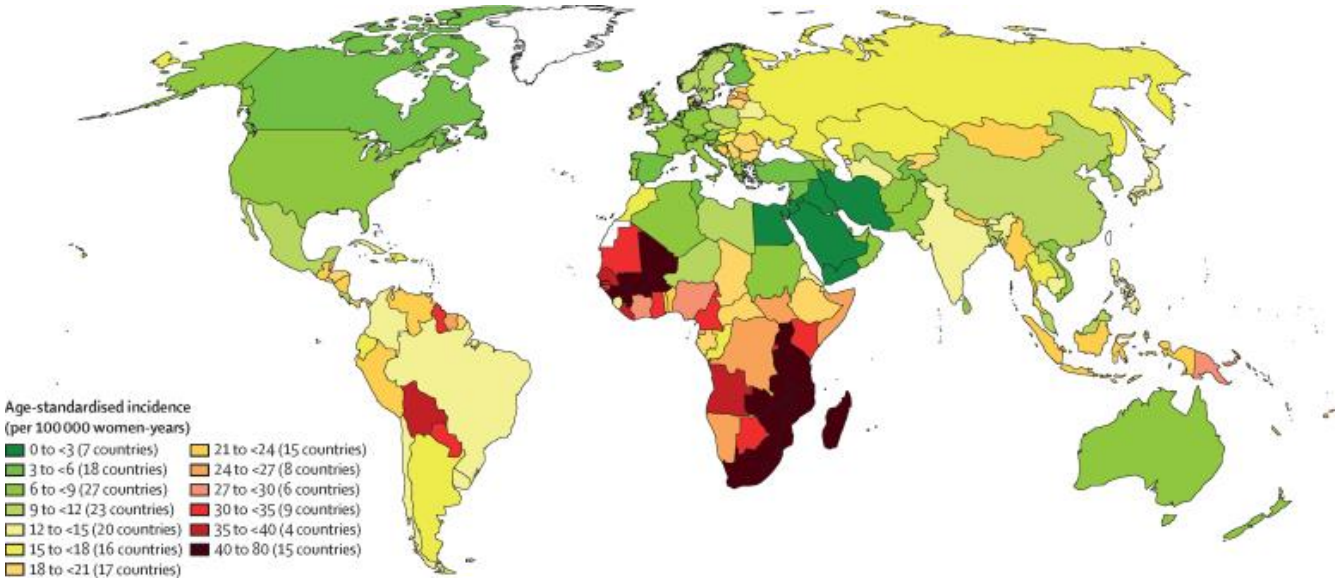


Figure 1 Geographical distribution of world age-standardized incidence of cervical cancer by country, estimated for 2018 [1]

The discovery of HPV role in the etiopathogenesis of cervical cancer was supported by data relating cervical cancer incidence to HPV prevalence in different countries. A meta-analysis including data on more than 2 million women without cervical lesions tested for HPV worldwide reported a pooled prevalence of 15.3% for any HPV infection, with 70% contribution from high-risk HPV types, with a wide range across countries. HPV 16 emerged as the most common oncogenic type in all regions, followed by HPV 18, HPV52, HPV 58, and HPV 31. [3]

Data from the Cancer Incidence in Five continents (CI5) database shows that also cervical cancer incidence trends differ across countries, providing insights on how they are influenced by context, culture, policies and practices. [4]

Decreasing trends are observed in most of countries, although the magnitude of the decline may vary largely. In most of these countries the reduction in incidence reflects the sustained implementation of population-based screening programs. While, in context with no organised screening programs, declines can be partly attributed to an improvement in social conditions allowing for a less exposure to risk factors, such as HPV, for girls and women (i.e. higher education, lower fertility, later age at marriage).[5]

At the same time, data from countries showing increasing trends of cervical cancer incidence, highlighted the impact of an early exposure to HPV in girls due to changes in sexual behaviours overtime and, mostly, the importance of quality assurance and population-wide coverage in the implementation of cervical cancer prevention strategies. As an example, Eastern Europe countries are affected by an increase in cervical cancer incidence despite the introduction of screening programs, which are characterised by limited population coverage and lacking in quality control procedures. [6, 7]

1.1.1.2. Mortality

As for incidence, cervical cancer is also the fourth cause of cancer related death among women of all ages, worldwide, and the second among women in reproductive ages. [1]

Globally, 311 000 cervical cancer related deaths were estimated in 2018, accounting for an age-standardized mortality rate (ASMR) of 6.9 per 100 000 women. [1, 2]

Mortality follows similar pattern to those observed for incidence, mostly affecting countries with the lowest levels of the Human Development Index (HDI), a composite measure including life expectancy, access to knowledge, and living standards. [8]

Moreover, differences in mortality were lower across countries with high HDI, compared to differences across low HDI countries, despite similar prevalence in HPV or other risk-factors, highlighting the impact of cervical cancer screening programs on both incidence and mortality. [1]

1.1.1.3. Survival

The impact of healthcare systems in the management of cancer diseases is well represented by population-based survival data. [9] Population-based survival presented as net survival is the probability to survive a cancer up to a given time since diagnosis, after controlling for competing causes of death. [10] It is estimated that approximately 1.5 million women alive at the end of 2018 had been diagnosed with cervical cancer in the preceding 5 years. [2]

Data from 295 population-based cancer registries in 64 countries on more than 660 thousand women with a diagnosis of cervical cancer between 2000-2014 were collected by CONCORD-3, a global

surveillance of cancer survival trends. [11] According to CONCORD-3, the 5-year age-standardised net survival of women diagnosed for cervical cancer in 2010-2014, ranged widely worldwide, from 70% in high HDI countries to 50% in low HDI countries. In 18 Western and Southern European countries, Canada, and the USA 5-years survival ranges from 60 to 69%. [11]

1.1.2. Etiopathogenesis and Human Papillomavirus

1.1.2.1. Human Papillomavirus and cervical cancer

The Nobel Prize in Physiology or Medicine in 2008 has been awarded for the discovery of connection between HPV and Cervical cancer, that posed the basis for the development of new effective preventive strategies. [12]

The HPVs are a group of more than 2 hundred double-stranded DNA viruses infecting human skin and mucosa. [13], The HPV genome includes six early genes (E) involved with viral replication and maintenance, and two late (L) genes encoding the self-assembling capsid proteins. [14]

Two early genes, E6 and E7, are the main responsible for the oncogenic activity of high-risk HPV types, inducing powerful cell-cycle disruption and anti-apoptosis activities, aimed to maintain virion production yet promoting genetic instability. [15]

The IARC classified 12 HPV types as carcinogenic to humans (Group 1), yet with different carcinogenic activity that outline four risk categories based on etiologic fraction of cancers. [16-19]

The more carcinogenic is HPV16, causing almost 60% of squamous cancers and half of adenocarcinomas of the cervix. A related group of HPV types including HPV31, HPV33, HPV35, HPV52, and HPV58 is responsible for an additional 15% of squamous cervical cancer. A 15% of squamous cancer was also related to HPV18 and HPV45 types, that are also responsible for almost half of cervical adenocarcinomas. Finally, the remaining carcinogenic types including HPV39, HPV59, HPV51, and HPV56 cause approximately 5% of squamous cancer. [17, 20, 21].

It must be said that most cervical HPV infections, even with carcinogenic types, typically cause no detectable cytologic alterations or benign wart, flat or raised (condylomas). Condylomas are generally caused by non-carcinogenic types, particularly HPV6 and HPV11. [15]

Thus, the understanding of the relative role of HPV types on carcinogenesis it's mandatory to estimate and optimize the impact of HPV vaccination and of HPV based screening programs in preventing cervical cancer.

1.1.2.2. HPV infection and multi-state cervical carcinogenesis

Cervical carcinogenesis is a not-completely understood process, including a set of necessary states and transitions from the normal cervix to cancer mortality. [14] This process commonly takes decades and understanding the role of each state and transition is the basis to implement effective and safe preventive strategies.

The states of the natural history process can internationally be described as: normal uninfected cervix, carcinogenic HPV infection, precancer and cancer. The two-way natural history transitions between following steps can be named as HPV appearance (and disappearance) and progression to (or regression of) precancer. The only one-way transition is cancer invasion. (Figure 2) [14]

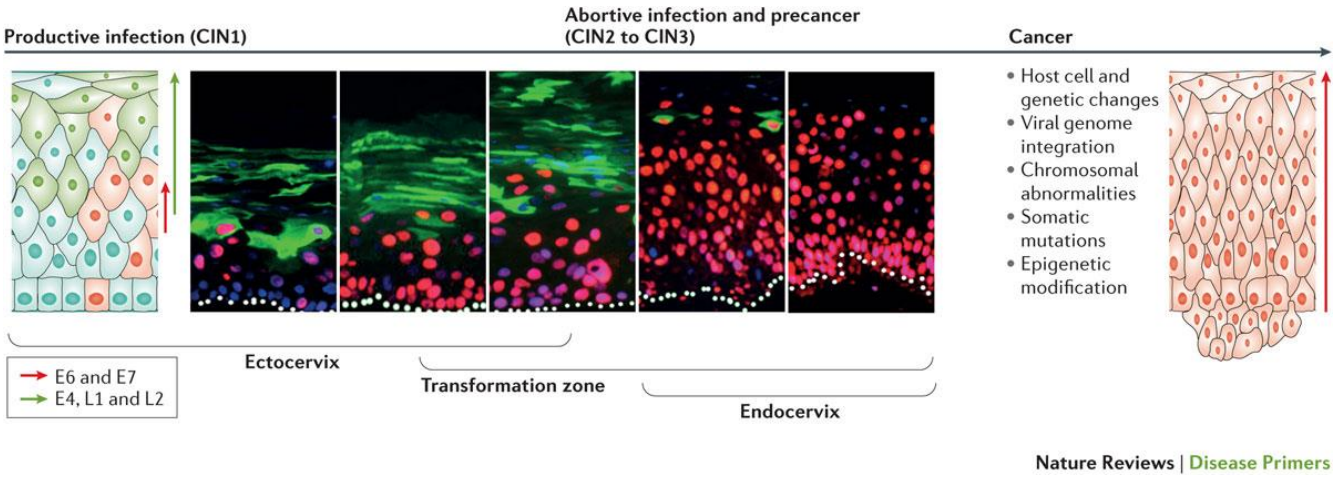


Figure 2 Molecular events during progression and invasion in the cervix [14]

A woman may encounter and control several HPV infections in her life, even with oncogenic types, without developing detectable cervical abnormalities. In a woman lifetime, HPV detection can represent a primary infection or a re-appearance after one or more episodes of disappearance (clearance) due to an effective immune response. [16] The median clearance of screen-detected HPV infections occurs in a year and its pattern is similar for all HPV types. [13, 19, 23] HPV clearance may represent a "latent" infection replicating in the basal epithelium under immune control, without full virion production, or a "real" viral eradication. Despite not distinguishable, clearance is relevant from a preventive perspective since only persistently apparent infections to HPV DNA tests confer a risk of progression to precancer and cancer. [24] Thus, HPV type and persistence of infections are the two main drivers for progression to precancer. [25]

The carcinogenic process starts when the immune response fails to control one of these infections, even if when and why it occurs in not yet well understood. Thus, cervical cancer results from a persistent carcinogenic HPV infection of a cervical cell, inducing clone expansion, somatic mutations, and immune escape. [14] The cells involved define two histologic types of cervical precancer and cancer, squamous and glandular, with different probabilities of progression in the natural history process and different detectability in cytology-based screening strategies.

1.1.2.3. Natural history and prevention of cervical cancer

The understanding of cervical cancer natural history provides necessary information for effective, safety and efficient preventive strategies.

The cytology-based screening, one of the gamechanger interventions in the history of women's health prevention, was based on the visual and microscopic alterations of cervical cells. With the identification of the HPV role, it needs to update and integrated with new emerging technologies. [26]

At the same time, the HPV prevalence emerging by HPV-DNA screening rounds makes not feasible to treat all women with HPV infections, having a low Positive Predictive Value. Moreover, the different risks of "progression to" and "regression of" precancer lesions pose a challenge in making cancer prevention more "precise", then safe and efficient. [26]

Thus, reducing HPV infections, especially from high-risk types, is the first step to be tackled through health promotion campaigns and HPV immunization strategies. [26]

Secondly, the distinction between productive HPV infections and abortive/transforming states of infections, typical of precancer lesions is the second step to be focused to increase the performance of screening strategies. During this shift, viral and cellular changes occur at a molecular level and represent potentially useful biomarkers for screening triage. Among viral changes, an overexpression of two oncogenes, E6 and E7, is found to be an important driver for transition to an abortive state. E6 and E7 interfere with the normal regulation of replicating basal cells cycle, abrogating apoptosis and disrupting the Rb tumours suppressor pathway. [12] Moreover, virion production per cell decreases in the abortive infections, with a increase in methylation of genomic sites, such as L1 and L2 genes. [27, 28] Among cellular changes, the E7 viral oncogene interference with Rb pathway led to an accumulation of p16 protein that is detectable by immunostaining. [29] The accumulation of p16 was found to be more predictive of sever precancer lesions when found together with the accumulation of ki67 protein, a largely studied cell-proliferation marker. [30]

To date, these are the most promising biomarkers to be used in cervical cancer screening, although the body of available evidence is not "mature" enough for implementation in real practice.

1.1.3. Cervical cancer prevention

1.1.3.1. Cervical cancer screening

Cytology-based screening completely changed the epidemiology of cervical cancer, reducing both incidence and mortality, as showed in several large cohort and ecological studies [31]

To date, strong evidence is available on the greater reduction in cervical cancer incidence and mortality from HPV DNA screening compared to cytology. [32] HPV DNA showed a high prospective sensitivity, reducing the 10-year risk of precancer lesions after a negative test compared to the risk after a negative cytology and allowing longer interval between screening rounds. Despite this, HPV DNA test showed a higher proportion of women testing positive, increasing colposcopy referral with a lower positive predictive value. This makes the screening not efficient and induce overdiagnosis and overtreatment.

Indeed, the rationale for cervical cancer screening is based on the availability of non-invasive and safe treatment options for precancerous lesions. [31, 33] Despite the side effects of treatments are limited

compared to the consequences of cervical cancer, they are not negligible in the reproductive age, impacting on obstetric outcomes. [34] Thus, to reduce undesirable effect it is important to reduce the referral of women with a very low probability of progression to cancer, such as HPV-negative women. [35] Moreover, all guidelines recommend treating Cervical Intraepithelial Lesions of Grade 3 (CIN3) and almost all also CIN2 precancerous lesions, with very few exceptions recommending strict follow up in younger women with higher probability of regression and slower progression to cancer. CIN1 or less severe lesions are not considered anymore pre-cancer lesions and should not be treated due to the extremely low probability of progression leading to an unfavourable balance of benefits and harms of treatment. [36-39]

Thus, to reduce screening harms, a triage of HPV positive women is required before referring to colposcopy. Nevertheless, the balance between reduction in cervical cancer incidence and mortality, and increase in referral, overdiagnosis and overtreatment favours HPV-based screening. For that reasons, the implementation of HPV DNA as primary test in cervical cancer screening is recommended by European [36, 37] and US guidelines [38, 39] from 2008 and 2012, respectively.

Despite recommended since the first decade of the XXI century, in 2017 HPV-based screening was not widely implemented, with differences even between European countries. [40] (Figure 3) Recently, also Belgium, Spain, Ireland, and Poland started a transition to HPV primary testing.

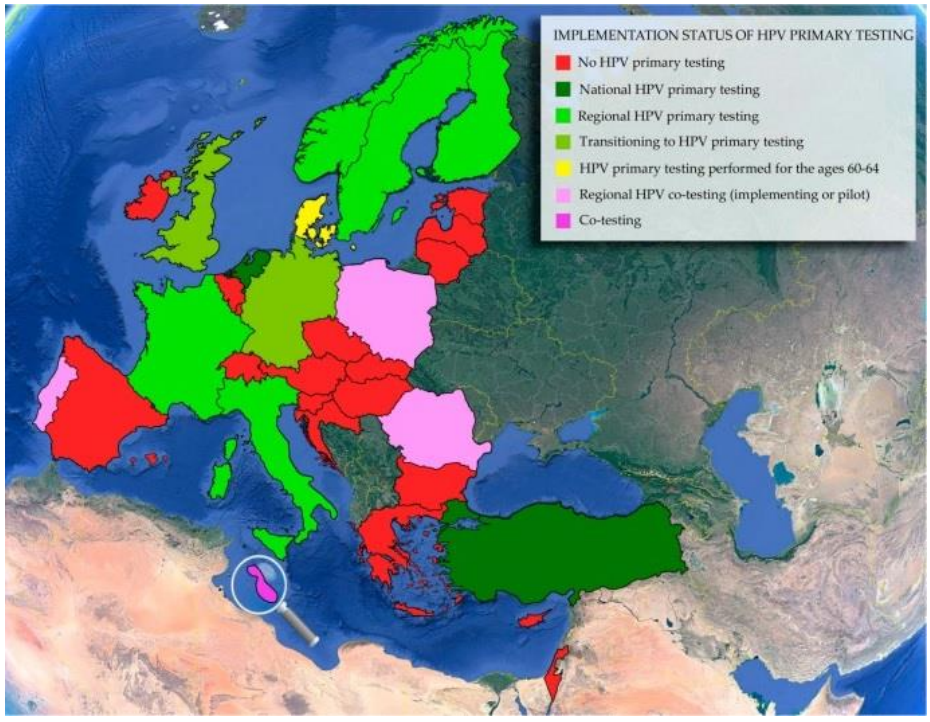


Figure 3: The implementation status of primary HPV testing in E.U. member states and some E.U. associated countries as of May 2018. The magnifying glass serves to enlarge the island of Malta. It is important to state that this is a rapidly changing field, and that the status of implementation could not be confirmed for all countries from two independent sources. [40]

As reported in Table 1, common recommendations across international guidelines are the target age for starting HPV-based screening (30-65 years) and at least 5-year interval between screening rounds, together with the needing of a triage test for HPV positive women. (Table 1) [41]

In Europe, European guidelines are implemented with different triage strategies in the screening algorithms between countries. (Table 2) [42]

The Italian screening protocol starts at age 25 with Pap test every three years up to age 30, with immediate referral to colposcopy for ASC-US or more sever lesions (ASC-US+). From 30 to 65 years, Italian women are invited to HPV-DNA test, to be repeated every 5 years, if negative. HPV-DNA positive women undergo triage with cytology and are referred to colposcopy if ASC-US+ lesions are detected. Triage negative women are invited to a second triage with HPV-DNA test at 1-year and immediately referred to colposcopy if HPV positive or to routine screening if HPV negative.

Finally, current international guidelines provide limited recommendation for the follow up of women diagnosed and treated for CIN2 or more sever lesions. The Multi-Society American Guidelines (ACS-ASCCP-ASCP) recommend two episodes of co-testing at 1- and 2-year intervals as test of cure [38], while European guidelines recommend at least one contesting after six months, with limited details on the following management. [36, 37]

Document (year)	screening target population	Use of HPV for primary screening	target age for HPV	interval for HPV negative	cotesting or triage	management of HPV+ cyto+	management of HPV+ cyto-	management of HPV- cyto+	HPV typing
USPSTF (2012) [39]	21-65	yes	30-65	5yy	Cotesting or stand-alone with triage	colposcopy	1y cotesting	not defined	not mentioned
ACS-ASCCP-ASCP (2012)[38]	21-65	yes	30-65	5yy	cotesting or stand-alone with triage	colposcopy	1y cotesting	ASC-US -> 3yy cotesting; L-SIL -> 1y cotesting or colpo; ASC-H or H-SIL -> colpo	HPV 16 or 18 may be referred to colposcopy even if cyto negative
European Guidelines (2015)[36, 37]	25-64	yes	30/35-64	>=5yy (up to a maximum of 10yy according to age and screening history)	stand-alone with triage	ASC-US or L-SIL -> 1y or colpo; ASC-H or H-SIL -> colpo	cytology at 6-12m; HPV followed by cytology triage at 1y; HPV only at 1y	Not applicable	only in research
ASCU-US: atypical squamous cell of undetermined significance; L-SIL: low grade squamous intraepithelial lesion									

Table 1. Summary of recommendations for HPV testing as primary screening for cervical cancer by the US Preventive Service Task Force [39], the Multisocietal American Guidelines (ACS-ASCCP-ASCP) [38] and the European Guidelines. [36, 37] Adapted from Giorgi Rossi P, Ronco G, 2018. [41]

Country	Screening policy	First triage	Second triage
England	HPV alone	Current pilot (6 sites) algorithm: cytology, if ASC-US+: colposcopy, if NILM: second triage. HPV16/18 typing also assessed in component of pilot sites where persistent 16/18 positivity at first and second triage = colposcopy	For pilot studies: At 12 M: hrHPV testing: If positive: colposcopy, If negative: routine screening TBD for roll out
Germany	Co-testing	If ASC-H, AIS, HSIL: colposcopy, if NILM-LSIL: second triage.	At 6–12 M: co-testing If positive (any test): colposcopy If negative : routine screening
Ireland	HPV alone	Cytology, if ASC-US+: colposcopy, if NILM: second triage. Inclusion of limited typing likely but not yet defined.	To be determined.
Italy[1]	HPV alone	Reflex cytology, if ASC-US+: colposcopy, if NILM: second triage.	At 12 M: hrHPV testing: If positive: colposcopy, If negative: routine screening
Netherlands	HPV alone	Cytology, if ASC-US+: colposcopy, if NILM: second triage.	At 6M: cytology If ASC-US+: colposcopy If NILM: routine screening
Norway[2]	HPV alone	Reflex cytology if ASC-US+: colposcopy. If reflex cytology NILM second triage.	At 12 M: hrHPV testing If positive: colposcopy, If negtive: routine screening
Scotland	HPV alone	Cytology, if ASC-US+: colposcopy, if NILM: second triage.	At 12 M: hrHPV testing: If positive: colposcopy, If negative: routine screening
Sweden[3]	HPV alone	Cytology, if ASC-US+: colposcopy	At 36M: cytology If ASC-US: colposcopy If NILM: routine screening
US	Co-testing	Immediate referral of HPV positive ASC-US, any LSIL1HPV1/NILM second triage.	Repeat HPV/cytology. If either HPV+ or ASC-US+, colposcopy If co-test negative, routine screening.
US	HPV alone	HPV16/18+: colposcopy hrHPV other: reflex LBC, if ≥ASCUS: colposcopy; if reflex LBC=NILM: second triage.	At 12M: repeat co-testing. If either HPV+ or ASC-US+, referral to colposcopy If co-test negative, routine screening.
Australia/ New Zealand	HPV alone	HPV16/181: colposcopy hrHPV other: LBC: if HSIL+: colposcopy if ≤LSIL: second triage	

Acronyms: ASCUS, atypical squamous cells of undetermined significance; CP, conventional Pap smear; hrHPV: High risk Human Papillomavirus; LBC: Liquid-based cytology; LSIL: low-grade squamous intraepithelial lesion; NILM: negative for intraepithelial lesion or malignancy.

[1]Cytology for women 25–33 years. HPV alone for women 34–64 years.

[2]Cytology for women 25–33 years. HPV alone for women 34–69 years.

[3]Cytology is offered to women age 23–29, HPV only interval = 3 years, age 30–49, with one co-test at age 41; HPV only, interval = 7 years, age 50–64. Nonparticipating continue to be invited yearly up to age of 70.

Table 2. Triage strategies in HPV-based screening algorithms of European countries, US, and Australia / New Zealand. Adapted from Cuschieri *et al.* 2018. [42]

1.1.3.2. HPV vaccination

HPV-based screening was one of the two technologies that changed cervical cancer prevention after the understanding of HPV role. The second is the development of vaccine against HPV useful to reduce infections and, thus, precancer and cancer with primary prevention.

To date, three HPV vaccines are registered and available on the market. The first was the bivalent Cervarix (GlaxoSmithKline GSK, London, UK) covering against HPV16 and 18. The second, Gardasil (Merck, Kenilworth, New Jersey, USA) includes HPV 16, 18 and the low-risk types 6 and 11. Finally, the most recent was the nonavalent Gardasil9 (Merck, Kenilworth, New Jersey, USA) including five high-risk types [31, 33, 45, 52 and 59] in addition to type 16, 18, 6, and type 11.

All the registered vaccines showed high effectiveness with full protection against persistent HPV-infections from the included types and precancer lesions in women aged 16-26. [43, 44] Moreover, nowadays a large body of evidence is also available demonstrating the safety profile of HPV vaccines [45]

Thus, HPV immunization programs became a strategic priority together with organised screening programs in cervical cancer preventive policy of almost all industrialized countries. (Figure 4)

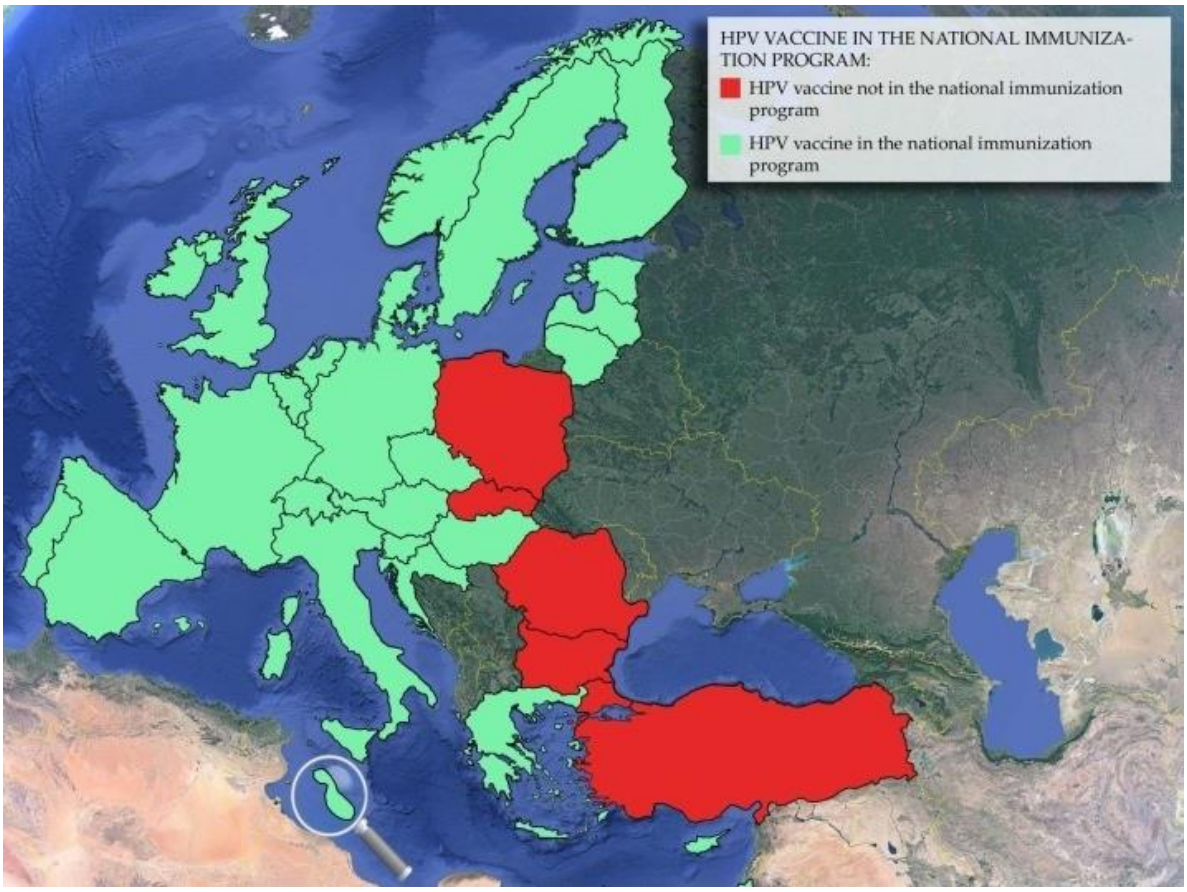


Figure 4. The implementation status of HPV vaccination in E.U. member states and some E.U. associated countries as of 15 May 2018, based on the World Health Organization (WHO) "Vaccine in National Immunization Program Update". [40]

The most widely implemented strategy targets women just before the age of start of sexual activity, around the 11th years of age in most countries. [46-48]. The rationale is to vaccinate cohort of women virtually HPV free and not to earlier to require no booster doses before the age of higher exposure. The first HPV immunization campaigns targeting 11th years old women started in 2007 and 2008 in Australia and in many European countries, such as in Italy that actively invited women from the 1997 birth cohort and provided HPV vaccine for free from 1996 birth cohort. [48, 49] This strategy with active invitation reached high coverage in target populations even if the reduction in precancer and cervical cancer incidence will be evident only several years after vaccination, when target women will reach screening age. [49] To accelerate HPV immunization impact in several countries catch-up strategies targeting girls aged 16 or older, although reaching limited impact with few exceptions. [50, 51]

1.1.3.3. Integration between cervical cancer prevention strategies

HPV-based screening and HPV immunization represent a unique integration of cancer preventive strategies, characterised by high effectiveness and safety, and feasibility for large scale implementation.

The coexistence of these strategies represents an excellent opportunity yet posing some new challenges especially when first vaccinated cohorts will reach screening age.

1.1.3.3.1. Equity

Equity is a relevant common issue since high coverage of both vaccination and screening are required to increase the value of cervical cancer prevention strategies. Fail to reach a large amount of target women increases the risk of losing screening opportunities in women not receiving HPV vaccination and vice-versa. Studies on the association between HPV vaccination uptake in the 11th years of age and proxies of screening participations shows that inequity in the access could reduce the overall impact on cervical cancer burden, especially in disadvantaged populations. [49, 52] Active invitation from healthcare services and population-based screening programs showed to be effective in reducing access inequity. [53]

1.1.3.3.2. Screening in HPV vaccinated women

Beside equity issues, the balance between benefits and harms of current screening algorithms in vaccinated women should be carefully assessed. HPV vaccination reduces persistent infections from high-risk HPV types and related precancer lesions, thus screening should be adapted to the new epidemiology of cervical cancer. [26] Indeed, the main changes will be a reduction of more than 60% of clinically relevant cervical lesions in vaccinated women [54, 55], reducing the positive predictive value of cytology [56] as much as cytology sensitivity biased by the low prevalence of lesion. [57, 58] Since cytology is the only test recommended for screening below age 30, the need of adapting screening algorithms is urgent. In HPV vaccinated women also HPV test will have a reduction in Positive Predictive Value even if in a lesser extent compare to cytology since not directly influenced by prevalence of the disease. [59] However, HPV16 and 18 persistent infections responsible for most of

cervical cancer, especially early onset cancers, will virtually disappear in vaccinated women. Thus, together with a lower prevalence of diseases, a longer lag between detected precancer lesions and cancer is expected in vaccinated cohorts. [26] To date, the only evidence-based recommendation for screening adaptation has been developed in Italy, suggesting delaying the age to start screening at 30 for vaccinated women, starting with HPV test. [60] Now the discussion of the scientific community is about the safety of longer interval between screening rounds, an opportunity to increase screening efficiency and reduce overtreatment. Finally, the debate of changing screening algorithms to all the women of vaccinated cohorts (one-size fits all strategy) or only for actually vaccinated women is ongoing. [60] Since local sexual habits and social norms have a relevant impact, the choice of which strategy implement according to immunization coverage will be country specific. [26, 61]

1.1.3.3.3. HPV vaccination in women treated for CIN2 and CIN3

Moreover, HPV vaccination of high-risk groups of women older than age 16 may add value to primary prevention interventions. Among these groups, women treated for precancer lesions (CIN2/3) represent a strategic target having an increased risk of recurrences and increased risk of obstetric adverse events due to repeated conservative treatments. [34, 62-64] Despite the already available evidence, up to 2020 no international guidelines included recommendations on HPV vaccination in women treated for CIN2 or CIN3, both in opportunistic or organised screening programs.

1.2. Gap of knowledge

In high-income countries cervical cancer is a well-controlled disease, thanks to the diffusion of Pap-test and organized screening programmes. Indeed, this is one of the few oncologic diseases that can be prevented through the identification and treatment of pre-cancerous lesions. Since persistent carcinogenic human papillomaviruses (HPV) infection has been identified as the necessary, but not sufficient, cause of cervical cancer, vaccination, and HPV-DNA test-based screening have become the main prevention strategies.

The current challenge in cervical cancer prevention is the reduction of over-diagnosis and over-treatment. These phenomena may increase with the introduction of the HPV DNA test, less specific than Pap-test, and in the delay in implementation of effective technologies within current screening protocols.

Thus, evidence-based guidelines defining the best management of HPV positive women to safely reduce colposcopy referral and overtreatment are needed.

Triage of HPV positive women is indeed the step with larger room for improvement. Nonetheless, a reduction in inappropriateness could be gained by the standardisation of follow up strategies for women who had a colposcopy and received a treatment for a high-grade cervical intraepithelial neoplasia (CIN2 or CIN3), currently not detailed in available international guidelines.

Firstly, evidence-based guidelines require robust evidence on the performance of new candidates for triage in HPV-based organised screening protocols.

Secondly, evidence-based guidelines require a transparent and rigorous methodology to be developed, allowing a timely implementation of emerging technologies.

1.3. Research aims

The PhD Research project aims are:

- To assess the accuracy of biomarkers (HPV E6/E7 mRNA and p16/Ki67) as test of triage in HPV-DNA based screening protocols.
- To assess the prognostic value of biomarkers (HPV E6/E7 mRNA and p16/Ki67) for the identification of regressive lesions.
- To update guidelines for the follow up of women treated for CIN2 and CIN3 with evidence-based recommendations.

1.4. Methods and chapters' contents

Chapter 2 reports an insight on the conceptual framework of cervical cancer screening programs and an overview of cervical cancer screening implementation in the Italian context.

The conceptual framework aimed to support the assessment of cancer screening programs explicating the causal chain linking actions to effects on population health. It was developed within a Multicentre project "Progetto di Supporto alla Valutazione del Piano Nazionale per la Prevenzione 2014 – 2018" funded by the Italian Ministry of Health within the Centre for Disease Control (CCM) 2014 funding programme.

The overview of the characteristics of women participating to cervical cancer in Italy, including socio-economic and health-related inequalities in screening uptake, was provided analysing data from more than 40,000 women in the screening target age interviewed within the Italian Behavioural Risk Factor Surveillance System (PASSI).

To achieve the first and the second research aims, the results of the New Technologies in Cervical Cancer 2 study (NTCC2) were reported in Chapter 3.

NTCC2 is multicentre randomised trial, funded by the Italian Ministry of Health in 2009, that involved 10 centres in 7 Italian regions. NTCC2 aims to measure the accuracy of E6/E7 mRNA and p16/Ki67 and their negative predictive value for CIN2 or more severe lesions (CIN2+). The project recruited more than 40,000 women who were invited for a new screening round based on HPV-DNA test within the organised screening programs (i.e. aged 25-59). HPV-DNA positive women were tested for cytology, E6/E7 mRNA and p16/Ki67. To assess specificity, a random sample of a thousand HPV-DNA negative women was tested with E6/E7 mRNA. In 2019, the baseline assessment data collection ended. Results were reported for cross-sectional accuracy of biomarkers in triage, for prognostic value for regressive lesions and HPV clearance.

To achieve the third research aim, the methodology of the "Multisociety Italian guidelines for cervical cancer prevention" development process and the first recommendation published in the Italian National System of Guidelines (SNLG) database are reported in Chapter 4.

The "Multisociety Italian guidelines for cervical cancer prevention" development is a project started by the Italian Group for Cervical Cancer Screening (GISCi) with the endorsement of the National Centre for Screening Monitoring (ONS) to produce Italian recommendations in the framework set by European guidelines. The project is conducted by all the nine Italian scientific societies involved in tackling cervical cancer. They set up a Panel of Experts including patients, clinicians, and healthcare decision makers. The Guidelines Development working group adopted the GRADE (Grading of Recommendations Assessment, Development and Evaluation) methodology according to the methodological requirements of the SNLG. SNLG already approved the "Multisociety Italian guidelines for cervical cancer prevention" project and published the first recommendation on HPV vaccination of women treated for CIN2 and CIN3 in the SNLG database.

1.5. Bibliography Chapter 1

1. Arbyn M, Weiderpass E, Bruni L, et al. Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. Lancet Glob Health. 2020;8(2):e191-e203. doi:10.1016/S2214-109X(19)30482-6
2. Ferlay JEM, Lam F, Colombet M, Mery L, et al. Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer (IARC). 2018. Available from: https://gco.iarc.fr/today/home (Accessed on March 2021)
3. Serrano B, Brotons M, Bosch FX, Bruni L. Epidemiology and burden of HPV-related disease. *Best Pract Res Clin Obstet Gynaecol*. 2018;47:14-26. doi:10.1016/j.bpobgyn.2017.08.006
4. IARC. Cancer Incidence in Five Continents, CI5plus: IARC CancerBase No. 9. International Agency for Research on Cancer (IARC). 2018 Available from: https://ci5.iarc.fr/Default.aspx (Accessed on March 2021)
5. Dhillon PK, Yeole BB, Dikshit R, Kurkure AP, Bray F. Trends in breast, ovarian and cervical cancer incidence in Mumbai, India over a 30-year period, 1976-2005: an age-period-cohort analysis. *Br J Cancer*. 2011;105(5):723-730. doi:10.1038/bjc.2011.301
6. Ojamaa K, Innos K, Baburin A, Everaus H, Veerus P. Trends in cervical cancer incidence and survival in Estonia from 1995 to 2014. *BMC Cancer*. 2018;18(1):1075. Published 2018 Nov 7. doi:10.1186/s12885-018-5006-1
7. Vaccarella S, Franceschi S, Zaridze D, et al. Preventable fractions of cervical cancer via effective screening in six Baltic, central, and eastern European countries 2017-40: a population-based study. *Lancet Oncol*. 2016;17(10):1445-1452. doi:10.1016/S1470-2045(16)30275-3
8. Ginsburg O, Bray F, Coleman MP, et al. The global burden of women's cancers: a grand challenge in global health. *Lancet*. 2017;389(10071):847-860. doi:10.1016/S0140-6736(16)31392-7
9. Allemani C, Coleman MP. Public health surveillance of cancer survival in the United States and worldwide: The contribution of the CONCORD programme. *Cancer*. 2017;123 Suppl 24(Suppl 24):4977-4981. doi:10.1002/cncr.30854
10. Perme MP, Stare J, Estève J. On estimation in relative survival. *Biometrics*. 2012;68(1):113-120. doi:10.1111/j.1541-0420.2011.01640.x
11. Allemani C, Matsuda T, Di Carlo V, et al. Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. Lancet. 2018;391(10125):1023-1075. doi:10.1016/S0140-6736(17)33326-3
12. Press release. NobelPrize.org. Nobel Media AB 2021. Sun. 21 Feb 2021. Available at: https://www.nobelprize.org/prizes/medicine/2008/press-release/ (Accessed on March 2021)

13. Bzhalava D, Eklund C, Dillner J. International standardization and classification of human papillomavirus types. *Virology*. 2015;476:341-344. doi:10.1016/j.virol.2014.12.028
14. Schiffman M, Doorbar J, Wentzensen N, et al. Carcinogenic human papillomavirus infection. *Nat Rev Dis Primers*. 2016;2:16086. Published 2016 Dec 1. doi:10.1038/nrdp.2016.86
15. Doorbar J, Quint W, Banks L, et al. The biology and life-cycle of human papillomaviruses. *Vaccine*. 2012;30 Suppl 5:F55-F70. doi:10.1016/j.vaccine.2012.06.083
16. Bouvard V, Baan R, Straif K, et al. A review of human carcinogens--Part B: biological agents. *Lancet Oncol*. 2009;10(4):321-322. doi:10.1016/s1470-2045(09)70096-8
17. Guan P, Howell-Jones R, Li N, et al. Human papillomavirus types in 115,789 HPV-positive women: a meta-analysis from cervical infection to cancer. *Int J Cancer*. 2012;131(10):2349-2359. doi:10.1002/ijc.27485
18. de Sanjosé S, Serrano B, Tous S, et al. Burden of Human Papillomavirus (HPV)-Related Cancers Attributable to HPVs 6/11/16/18/31/33/45/52 and 58. *JNCI Cancer Spectr*. 2019;2(4):pky045. Published 2019 Jan 7. doi:10.1093/jncics/pky045
19. Demarco M, Hyun N, Carter-Pokras O, et al. A study of type-specific HPV natural history and implications for contemporary cervical cancer screening programs. *EClinicalMedicine*. 2020;22:100293. Published 2020 Apr 25. doi:10.1016/j.eclinm.2020.100293
20. Clifford GM, Tenet V, Georges D, et al. Human papillomavirus 16 sub-lineage dispersal and cervical cancer risk worldwide: Whole viral genome sequences from 7116 HPV16-positive women. *Papillomavirus Res*. 2019;7:67-74. doi:10.1016/j.pvr.2019.02.001
21. Mirabello L, Yeager M, Cullen M, et al. HPV16 Sublineage Associations With Histology-Specific Cancer Risk Using HPV Whole-Genome Sequences in 3200 Women. *J Natl Cancer Inst*. 2016;108(9):djw100. Published 2016 Apr 29. doi:10.1093/jnci/djw100
22. González P, Hildesheim A, Rodríguez AC, et al. Behavioral/lifestyle and immunologic factors associated with HPV infection among women older than 45 years. *Cancer Epidemiol Biomarkers Prev*. 2010;19(12):3044-3054. doi:10.1158/1055-9965.EPI-10-0645
23. Plummer M, Schiffman M, Castle PE, Maucort-Boulch D, Wheeler CM; ALTS Group. A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion. *J Infect Dis*. 2007;195(11):1582-1589. doi:10.1086/516784
24. Doorbar J. Host control of human papillomavirus infection and disease. *Best Pract Res Clin Obstet Gynaecol*. 2018;47:27-41. doi:10.1016/j.bpobgyn.2017.08.001
25. Rodríguez AC, Schiffman M, Herrero R, et al. Longitudinal study of human papillomavirus persistence and cervical intraepithelial neoplasia grade 2/3: critical role of duration of infection. *J Natl Cancer Inst*. 2010;102(5):315-324. doi:10.1093/jnci/djq001
26. Giorgi Rossi P, Carozzi MF. Cervical Cancer: Screening, Vaccination, and Preventive Strategies. 2018. doi: 10.1016/B978-0-12-801238-3.65138-3 Reference Module in Biomedical Sciences. Encyclopedia of Cancer 3rd Edition. Elsevier. Eds. Boffetta P, Hainaut P. Published on October 2018.Book ISBN: 9780128124840 eBook ISBN: 9780128124857
27. Lorincz AT, Brentnall AR, Vasiljević N, et al. HPV16 L1 and L2 DNA methylation predicts high-grade cervical intraepithelial neoplasia in women with mildly abnormal cervical cytology. *Int J Cancer*. 2013;133(3):637-644. doi:10.1002/ijc.28050
28. Gradissimo A, Lam J, Attonito JD, et al. Methylation of High-Risk Human Papillomavirus Genomes Are Associated with Cervical Precancer in HIV-Positive Women. *Cancer Epidemiol Biomarkers Prev*. 2018;27(12):1407-1415. doi:10.1158/1055-9965.EPI-17-1051
29. Wentzensen N, Bergeron C, Cas F, Vinokurova S, von Knebel Doeberitz M. Triage of women with ASCUS and LSIL cytology: use of qualitative assessment of p16INK4a positive cells to identify patients with high-grade cervical intraepithelial neoplasia. *Cancer*. 2007;111(1):58-66. doi:10.1002/cncr.22420
30. Nam EJ, Kim JW, Hong JW, et al. Expression of the p16 and Ki-67 in relation to the grade of cervical intraepithelial neoplasia and high-risk human papillomavirus infection. *J Gynecol Oncol*. 2008;19(3):162-168. doi:10.3802/jgo.2008.19.3.162

31. International Agency for Research on Cancer. Cervix Cancer Screening. IARC Handbooks of Cancer Prevention Volume 10. 2005. Lion. ISBN-13 (Print Book) 978-92-832-3010-6. ISBN-13 (PDF) 978-92-832-3010-6
32. Ronco G, Dillner J, Elfström KM, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials [published correction appears in Lancet. 2015 Oct 10;386(10002):1446]. *Lancet*. 2014;383(9916):524-532. doi:10.1016/S0140-6736(13)62218-7
33. WHO. WHO guidelines for treatment of cervical intraepithelial neoplasia 2–3 and glandular adenocarcinoma in situ: Cryotherapy, large loop excision of the transformation zone (LEEP/LLETZ), and cold knife conization. 2014. Geneva: World Health Organization. ISBN 978 92 4 150677 9
34. Kyrgiou M, Athanasiou A, Paraskevaidi M, et al. Adverse obstetric outcomes after local treatment for cervical preinvasive and early invasive disease according to cone depth: systematic review and meta-analysis. *BMJ*. 2016;354:i3633. Published 2016 Jul 28. doi:10.1136/bmj.i3633
35. Dalla Palma P, Giorgi Rossi P, Collina G, et al. The risk of false-positive histology according to the reason for colposcopy referral in cervical cancer screening: a blind revision of all histologic lesions found in the NTCC trial. *Am J Clin Pathol*. 2008;129(1):75-80. doi:10.1309/EWYGWFRRM8798U5P
36. Arbyn M, Anttila A, Jordan J, Schenck U, Ronco G, Segnan N, et al (eds.). European guidelines for quality assurance in cervical cancer screening. Second edition. European Commission, Office for Official Publications of the European Communities, 2008 Luxembourg.
37. Anttila A, Arbyn A, De Vuyst H, Dillner J, Dillner L, Franceschi S, et al. (eds.). European guidelines for quality assurance in cervical cancer screening. Second edition, Supplements. Office for Official Publications of the European Union, 2015 Luxembourg, pp. XIII–XXIV.
38. 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. *Am J Clin Pathol*. 2012;137(4):516-542. doi:10.1309/AJCPTGD94EVRSJCG
39. Moyer VA; U.S. Preventive Services Task Force. Screening for cervical cancer: U.S. Preventive Services Task Force recommendation statement [published correction appears in Ann Intern Med. 2013 Jun 4;158(11):852. Ebell, Mark [added]]. *Ann Intern Med*. 2012;156(12):880-W312. doi:10.7326/0003-4819-156-12-201206190-00424
40. Chrysostomou AC, Stylianou DC, Constantinidou A, Kostrikis LG. Cervical Cancer Screening Programs in Europe: The Transition Towards HPV Vaccination and Population-Based HPV Testing. *Viruses*. 2018;10(12):729. Published 2018 Dec 19. doi:10.3390/v10120729
41. Ronco G, Giorgi Rossi P. Role of HPV DNA testing in modern gynaecological practice. Best Pract Res Clin Obstet Gynaecol. 2018;47:107-118. doi:10.1016/j.bpobgyn.2017.08.002
42. 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(4):735-745. doi:10.1002/ijc.31261
43. Lehtinen M, Paavonen J, Wheeler CM, et al. Overall efficacy of HPV-16/18 AS04-adjuvanted vaccine against grade 3 or greater cervical intraepithelial neoplasia: 4-year end-of-study analysis of the randomised, double-blind PATRICIA trial [published correction appears in Lancet Oncol. 2012 Jan;13(1):e1]. *Lancet Oncol*. 2012;13(1):89-99. doi:10.1016/S1470-2045(11)70286-8
44. Joura EA, Giuliano AR, Iversen OE, et al. A 9-valent HPV vaccine against infection and intraepithelial neoplasia in women. *N Engl J Med*. 2015;372(8):711-723. doi:10.1056/NEJMoa1405044
45. Arbyn M, Xu L, Simoens C, Martin-Hirsch PP. Prophylactic vaccination against human papillomaviruses to prevent cervical cancer and its precursors. *Cochrane Database Syst Rev*. 2018;5(5):CD009069. Published 2018 May 9. doi:10.1002/14651858.CD009069.pub3
46. Obel J, McKenzie J, Buenconsejo-Lum LE, et al. Mapping HPV Vaccination and Cervical Cancer Screening Practice in the Pacific Region-Strengthening National and Regional Cervical Cancer Prevention. *Asian Pac J Cancer Prev*. 2015;16(8):3435-3442. doi:10.7314/apjcp.2015.16.8.3435
47. Dorleans F, Giambi C, Dematte L, et al. The current state of introduction of human papillomavirus vaccination into national immunisation schedules in Europe: first results of the VENICE2 2010 survey. *Euro Surveill*. 2010;15(47):19730. Published 2010 Nov 25. doi:10.2807/ese.15.47.19730-en
48. De Vuyst, H., Howell-Jones, R., Levy-Bruhl, D., Giorgi Rossi, P., Franceschi, S. (2015). Implementation of vaccination against human papillomavirus in Europe. In European guidelines for quality assurance in cervical cancer screening.

Second edition, Supplements. Anttila, A., Arbyn, M., De Vuyst, H., et al. (eds.). Luxembourg: Office for Official Publications of the European Union, pp. 109-137.

49. Venturelli F, Baldacchini F, Campari C, et al. Association between mothers' screening uptake and daughters' HPV vaccination: a quasi-experimental study on the effect of an active invitation campaign. *BMJ Open*. 2017;7(9):e016189. Published 2017 Sep 25. doi:10.1136/bmjopen-2017-016189
50. Pollock KG, Kavanagh K, Potts A, et al. Reduction of low- and high-grade cervical abnormalities associated with high uptake of the HPV bivalent vaccine in Scotland. *Br J Cancer*. 2014;111(9):1824-1830. doi:10.1038/bjc.2014.479
51. Brotherton JM, Saville AM, May CL, Chappell G, Gertig DM. Human papillomavirus vaccination is changing the epidemiology of high-grade cervical lesions in Australia. *Cancer Causes Control*. 2015;26(6):953-954. doi:10.1007/s10552-015-0568-6
52. Malagón T, Drolet M, Boily MC, Laprise JF, Brisson M. Changing inequalities in cervical cancer: modeling the impact of vaccine uptake, vaccine herd effects, and cervical cancer screening in the post-vaccination era. *Cancer Epidemiol Biomarkers Prev*. 2015;24(1):276-285. doi:10.1158/1055-9965.EPI-14-1052
53. Giorgi Rossi P, Baldacchini F, Ronco G. The Possible Effects on Socio-Economic Inequalities of Introducing HPV Testing as Primary Test in Cervical Cancer Screening Programs. *Front Oncol*. 2014;4:20. Published 2014 Feb 10. doi:10.3389/fonc.2014.00020
54. Bruni L, Albero G, Serrano B, et al. ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the World. Summary Report 17 June 2019. Available at: https://hpvcentre.net/statistics/reports/XWX.pdf (Accessed on March 2021)
55. Li N, Franceschi S, Howell-Jones R, Snijders PJ, Clifford GM. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. *Int J Cancer*. 2011;128(4):927-935. doi:10.1002/ijc.25396
56. Kiviat NB, Hawes SE, Feng Q. Screening for cervical cancer in the era of the HPV vaccine--the urgent need for both new screening guidelines and new biomarkers. *J Natl Cancer Inst*. 2008;100(5):290-291. doi:10.1093/jnci/djn038
57. Franco EL, Mahmud SM, Tota J, Ferenczy A, Coutlée F. The expected impact of HPV vaccination on the accuracy of cervical cancer screening: the need for a paradigm change. *Arch Med Res*. 2009;40(6):478-485. doi:10.1016/j.arcmed.2009.06.003
58. Evans KK, Tambouret RH, Evered A, Wilbur DC, Wolfe JM. Prevalence of abnormalities influences cytologists' error rates in screening for cervical cancer. *Arch Pathol Lab Med*. 2011;135(12):1557-1560. doi:10.5858/arpa.2010-0739-OA
59. Giorgi-Rossi P, Franceschi S, Ronco G. HPV prevalence and accuracy of HPV testing to detect high-grade cervical intraepithelial neoplasia. Int J Cancer. 2012;130(6):1387-1394. doi:10.1002/ijc.26147
60. Giorgi Rossi P, Carozzi F, Federici A, et al. Cervical cancer screening in women vaccinated against human papillomavirus infection: Recommendations from a consensus conference. Prev Med. 2017;98:21-30. doi:10.1016/j.ypmed.2016.11.020
61. Baussano I, Lazzarato F, Brisson M, Franceschi S. Human Papillomavirus Vaccination at a Time of Changing Sexual Behavior. *Emerg Infect Dis*. 2016;22(1):18-23. doi:10.3201/eid2201.150791
62. Strander B, Hällgren J, Sparén P. Effect of ageing on cervical or vaginal cancer in Swedish women previously treated for cervical intraepithelial neoplasia grade 3: population based cohort study of long term incidence and mortality. *BMJ*. 2014;348:f7361. Published 2014 Jan 14. doi:10.1136/bmj.f7361.
63. Rebolj M, Helmerhorst T, Habbema D, et al. Risk of cervical cancer after completed post-treatment follow-up of cervical intraepithelial neoplasia: population based cohort study. *BMJ*. 2012;345:e6855. Published 2012 Oct 31. doi:10.1136/bmj.e6855
64. Onuki M, Matsumoto K, Sakurai M, et al. Posttreatment human papillomavirus testing for residual or recurrent high-grade cervical intraepithelial neoplasia: a pooled analysis. *J Gynecol Oncol*. 2016;27(1):e3. doi:10.3802/jgo.2016.27.e3

2. Chapter 2: Cancer screenings in context

2.1. Rationale

The appraisal of evidence on cervical cancer screening performance requires the knowledge of context specific cultural and healthcare related factors.

The first chapter provides an insight on cervical cancer screening programs organisation, monitoring and implementation in Italy.

2.2. Research article #01: Conceptual framework for interpretation of monitoring indicators of cancer screenings from the Italian National Prevention Plan

The findings of this research article were published in Italian in *Epidemiologia e Prevenzione*.

Zappa M, Bevere F, Braga M, Broccoli S, Cosentino M, Galeone D, Federici A, Marvulli M, Vasselli S, Venturelli F, Bellentani M, Giorgi Rossi P. Sviluppo di un modello concettuale di riferimento per l'interpretazione degli indicatori di monitoraggio degli screening oncologici nel Piano nazionale della prevenzione [Development of a conceptual model for interpretation of monitoring indicators of cancer screenings from the Italian National Prevention Plan]. Epidemiol Prev. 2019 Sep-Dec;43(5-6):354-363. Italian. doi: 10.19191/EP19.5-6.P354.105. PMID: 31659883.

ABSTRACT

Objectives The National Prevention Plan (PNP) posed the standard to be achieved by Regions for the implementation of cervical, breast and colorectal cancer screening: to invite all of the target populations and to increase the screening uptake up to 50%, 60% and 50% respectively, the standard defined by the Essential Levels of Care (LEA). Moreover, for cervical cancer screening it requires the implementation of HPV-DNA test and for breast cancer screening, the PNP demands for the definition of diagnostic and follow up pathways for high familial risk women. The PNP also set up a monitoring system to assess the impact of implemented policies. A conceptual framework has been defined to facilitate interpretation of variation in outcome indicators.

Design After a systematic review, the DPSEEA model (Driving forces, Pressure, State, Exposure, Effect, Actions) was identified as the more appropriate framework to assess the results of preventive policies. Factors for each component of the framework were identified and indicators that allow measuring the changing of each of these factors were defined.

Results Among the *Driving forces*, the trust in the Health Care System and the Social Capital were included. The presence of opportunistic screening, the competing private clinical activity, the commitment of General Practitioners and "medical" leaders, the attitude to cooperation and to patients' involvement, and the level of agreement between the positions of scientific societies and the recommendations implemented in organized screening programs were included in the *Pressures*. In the *State* were identified the availability of technological and human resources, the level of management skills and of accessibility. The *Exposure* was defined as the coverage of active invitation of the target population and the uptake of screening tests. The *Exposure* factors influence the *Effect*, described as the impact on anticipation of cancer diagnosis, on disease incidence (for cervical and colorectal cancer) and prognosis. The changing in screening programs performance modifies the impact of invitation coverage and test uptake (*Exposure*)

Conclusion DPSEEA model set up a logical conceptual framework which includes implementable actions and the mechanisms through which these actions should impact on the *Exposure* (invitation coverage and screening uptake) and on the screening performance (quality).

BACKGROUND

Since 2003, the European Commission recommends member states to implement organized screening programs for cervical, breast and colorectal cancer [1] following evidence-based guidelines. [2–7]

In Italy, organized cancer screening programs with active invitation of target populations were included in the Essential Assistance Levels (LEA) since 2001. [8] Each phase of the screening process is assessed with indicators and standards whose interpretation and validation derives from randomized studies. The population health-impact assessment is measured both through the monitoring of surrogate endpoints and early outcomes, and through large observational studies that directly measured the impact on mortality and incidence of cancer. [9-11] To date, in Italy every year more than 12 million people are actively invited to participate in screening programs. [12] The 2014-18 National Prevention Plan (PNP) [13] defined 10 macro-objectives, identified the risk factors / determining factors to be countered / promoted to improve the health of the population, outlined the strategies and central objectives that contribute to achieving macro-objectives and defined central indicators and related standards with which to evaluate the implementation of the PNP.

The Regions defined the Regional Prevention Plans (PRP) which identify: specific intermediate objectives oriented to the achievement of central objectives; actions for the implementation of PNP strategies; populations target of the actions; indicators for monitoring the implementation of programs and processes aimed to achieve intermediate and central objectives.

The Document on the assessment of the PNP [14] identifies over 130 central indicators and related performance standards for 2018, which the Regions must aim for through the PRPs. These indicators cover all the objectives of the PNP and are aimed to the evaluation of both process (how the Regions have implemented the policies planned by the PNP), and impact (how much these policies and the actions with which they have been implemented have improved population health). However, to assess the impact of policies, it is necessary that the causal link between actions and changes in indicators is plausible, even if outside the experimental context. The conceptual models are created with the purpose of facilitating the choice of appropriate indicators and the interpretation of their variations. [15,16]

The "2014-2018 PNP evaluation project" was funded by the Italian Ministry of Health-Centre for Disease Control (CCM) and coordinated by the National Agency for Regional Health Services (Agenas). This project aims to provide a logical framework for the interpretation of the causal relationships between the actions implemented by the National and the Regional Health Systems (SSR) and the observed variations in indicators identified by the PNP assessment document. [14]

The project started when the 2014-18 PNP assessment document had already been adopted- Thus, conceptual models were not used for the identification of indicators, but only for the interpretation and possible integration with context indicators. A conceptual model was designed for each specific

main area of PNP. A reliable interpretation of the causal links between actions and measured results should make decision makers more accountable for their work and allows the timely implementation of corrective measures. This article aims to present the rationale, methodology, and development process of a conceptual model to support the assessment of health planning in the context of cancer screening.

METHODS

Project structure

A scientific board was set up with experts from the main institutes dealing with the evaluation of health services and the monitoring of indicators in Health Care: Agenas, National Institute of Health, University of Turin, Institute for cancer research, prevention and oncological network (ISPRO) Tuscany, Azienda USL of Reggio Emilia-IRCCS, University Ca 'Foscari di Venezia, Italian National Institute of Statistics (ISTAT), National Institute for Insurance against Accidents at Work (INAIL), Scuola Superiore Sant'Anna di Pisa - Management and Health Laboratory, Ministry of Health) and an advisory committee in which the Regions and scientific societies were represented (Italian Association of Epidemiology, Italian Society of Hygiene and Preventive Medicine, Italian Society of Medical Statistics and Clinical Epidemiology). The board identified the PNP areas to develop conceptual models according to criteria of opportunity (presence of skills capable of guiding the process within the board), of relevance (impact on regional planning, burden of disease, innovative strategies) and representation (covering all macro-objectives).
The chosen areas were cancer screening, road accidents, workplace accidents, foodborne diseases surveillance, food intolerances, prevention of obesity in children and adolescents, smoking, air pollution. The board was divided in working groups, one for each area, elaborating the conceptual models. The committee was consulted during the definition of the specific objectives and areas of intervention, during the drafting of the methodology and the models' development.

Review of existing models

A review of the meta-literature was conducted to identify the existing models suitable for the identified areas.

A proposal has been found for the use of the PRECEDE-PROCEED model for the improvement of screening programs. [17] This model was born in the context of health promotion and has a pragmatic approach aimed at defining the necessary interventions to modify the behaviour of individuals. It includes an epidemiological and social evaluation phase similar to the construction of an interpretative model and it was already applied to screening in Italy. [18] However, it does not propose a defined theoretical framework for the analysis of determinants.

A previous systematic review [15] assessed different frameworks used in the literature according to prespecified criteria: 1. design that allows the identification of indicators, 2. inclusion of

environmental and health factors, 3. use of a causal chain approach, 4. inclusion of distal factors, 5. inclusion of an explicit process of exposure to risk and protective factors, 6. explicit inclusion of actions / interventions, 7. explicit inclusion of multiple targets for interventions and actions in the various part of the model, 8. possibility of using the model for measuring and monitoring the impact of changes in environmental factors on human health. Based on these criteria, the review authors identified the DPSEEA model (Driving Forces, Pressure, State, Exposure, Effect, Action), to be the more flexible and suitable for framing different areas of prevention and public health. [16,19]

By *Driving forces,* the model means the factors that motivate and promote the causal chain processes at the environmental and context level. *Driving forces* generate *Pressures*, phenomena acting on all the steps of the causal chain, thus modifying the *State*. Changes in the *State* may change the *Exposure*, that is, the relationship between the individual and protective or risk factors. Different levels of *Exposure* generate health effects, included in the framework under the heading *Effect*. To cope with these effects, it is necessary for the health care system to implement *Actions* which can act on different steps of the causal chain. [20]

The scientific board proposed the DPSEEA model to each subgroup as a starting point without any constraint. However, the subgroups had the task of repeating a review of the literature on conceptual models in their specific areas and possibly choosing a more suitable one.

Adapting DPSEEA model to cancer screening

The literature review did not identify any previous application of the DPSEEA model to cancer screening, although suggested by some authors. [21] The theoretical basis of the DPSEEA model are: the performance indicators identified by the European [2,4,5] and Italian [22-25] guidelines, the impact indicators developed for the assessment of screening effectiveness in real world, in particular from the Italian IMPATTO studies [26-30], and the epidemiological and social analysis coordinated by Agenas within a project on the implementation of screening programs. [31,32] The PNP and the PRPs were analysed to identify the proposed actions and the possible interconnections with actions aimed at other macro-objectives. The actions were then characterized by their intermediate target in the model, i.e. whether directed to modify a *Driving Forces*, *Pressure*, *State* or *Exposure*.

The indicators provided by the PNP, both for area specific objectives and for cross-sectional objectives, and the indicators reported by the Italian [22-25] and European [2,4,5] guidelines were analysed to identify those currently used.

The available indicators were included in the model based on the dimension they intend to measure (mostly *Exposure* and *Effect*, but also *State*).

The first version of the model developed by the subgroup (MZ, PGR, AF, SB) was submitted to the scientific board. The redefined model was also submitted to the advisory committee, firstly by mean of standard documents including the results of literature review, the PNP conceptual frameworks

relevant to the scope, the factors to be included in each dimension of the model, a list of proposed indicators. Then, the model was discussed in plenary session. The feedbacks and comments of the advisory committee were received, and the models revised accordingly. Revised models were resubmitted to the advisory committee and discussed in plenary meeting. The method adopted is similar to Delphi rounds. [33] The saturation of the process was reached with the second consultation. Finally, a survey was carried out on the local availability and quality of data for the proposed indicators.

RESULTS

PNP analysis: conceptual framework and objectives

The specific objectives of the PNP concerning cancer screening were found in macro-objective 1 according to a conceptual framework (Table 1) including specific indicators (Table 2).

The first two objectives, actual coverage (part of the target population regularly invited) and participation (part of the target population that carries out the screening test within the programs), were already included in the monitoring of the LEA and aim to increase the exposure of the population to invitation and screening test.

The third objective was the implementation of screening with HPV tests, to increase effectiveness in the prevention of invasive cancer [34], reducing overall costs and the number of tests in a woman's life. [3, 35]

The fourth objective was the definition of diagnostic-therapeutic pathways dedicated to women with an inherited risk for breast cancer. This action was aimed at improving the screening performance and efficacy through risk-based stratification of the population, enhancing the sensitivity of screening (introduction of magnetic resonance imaging and shortening surveillance intervals) but also expanding the target age group and proposing several options in addition to surveillance, such as prophylactic mastectomy. [7, 36] The intervention impacts on a limited population.

In macro-objective 9, objective 9.6 concerning vaccination registries was relevant for the implementation of cervical cancer screening, as the interoperability of vaccine registries is a prerequisite for managing the screening of women vaccinated against HPV in the next future. [37]

Macro-objective	Risk factors / determinants	Strategies	Central objectives	Central indicators
Reduce the preventable and avoidable burden of morbidity, mortality and disability of Chronic Non-Communicable Diseases	Initial precancerous and cancerous lesions for cancer of the uterine cervix, breast and colorectum.	Early identification with population-based programs of people at risk for cervical, breast and colorectal cancers by age.	Increase the actual coverage of screening programs target populations (for each of the 3 cancer sites).	Percentage of people who receive an invitation to participate in the screening program out of the target population.
			Increase the cancer screening uptake in people at risk.	Percentage of invited people undergoing screening test out of the target population.
			Redefine / implement cervical cancer screening programs by introducing HPV-DNA testing.	Adoption of regional planning guidelines for cervical cancer screening by introducing HPV-DNA testing (within one year of publishing the PRP) Starting HPV-based cervical cancer screening program by 2018
	Inherited risk for breast cancer.	Definition of therapeutic-diagnostic pathways, integrated with existing screening programs, for women at high risk for breast cancer, carrying BRCA1 and BRCA2 genetic mutations .	Early identification of subjects at inherited risk for breast cancer	

Table 1. Conceptual framework for cancer screenings in the National Prevention Plan 2014-2018. [13]

Acronyms: HPV Human Papillomavirus; PRP Regional Prevention Plan.

Central objective	Indicator code	Indicator name	Operational definition	Baseline value	Standard	Source of data
12. Increase the actual coverage of screening programs to the target population (for each of the 3 cancer sites)	1.12.1	Percentage of people who receive an invitation to participate in the cervical cancer screening program for out of the target population.	Proportion of people of target age invited.	2012: 77%	+30% (to reach the 100% set by the LEA)	National Centre for Screening Monitoring (ONS)
		Percentage of people who receive an invitation to participate in the breast cancer screening program out of the target population		2012: 73%	+37% (to reach the 100% set by the LEA)	
		Percentage of people who receive an invitation to participate in the colorectal cancer screening program out of the target population		2012: 57%	+75% (to reach the 100% set by the LEA)	
13. Increase the subjects at risk undergoing cancer screening	1.13.1	Percentage of people who participated to cervical cancer screening after active invitation out of the target population	Proportion of people of target age invited who take the first level test	2012: 41% of the invited women or 32% of the target population	+55% (to reach the 100% set by the LEA)	National Centre for Screening Monitoring (ONS)
		Percentage of people who participated to breast cancer screening after active invitation out of the target population		2012: 57.5% of the invited women or 42% of the target population	+45% (to reach the 100% set by the LEA)	
		Percentage of people who participated to colorectal cancer screening after active invitation out of the target population		2012: 46% of the guests equal to 26% of the target population	+95% (to reach the 100% set by the LEA)	
14. Redefine / implement HPV-based cervical cancer screening programs	1.14.1	Adoption of regional planning guidelines for cervical cancer screening by introducing the HPV-DNA test (within one year of the Regional Prevention Plan publication)	Proportion of Regions that have adopted regional planning recommendations for cervical cancer screening by introducing HPV-DNA testing	Not detected	100% before 2016 All Regions adopted the regional guidelines by 2016	Regions
	1.14.2	Start of cervical cancer screening program by introducing HPV-DNA testing (by 2018)	Proportion of Regions that implemented the HPV-DNA test as	Not detected	100% All Regions activated the HPV-	Regions

Central objective	Indicator code	Indicator name	Operational definition	Baseline value	Standard	Source of data
			exclusive first level test for cervical cancer screening		DNA test as exclusive test	
15. Early identification of subjects at inherited risk for breast cancer	1.15.1	Adoption of regional planning guidelines (within one year from the Regional Prevention Plan publication)	Proportion of Regions that adopted regional guidelines	Not detected	100% All Regions adopted the regional guidelines by 2016	Regions
	1.15.2	Care pathways implementation in all local health authorities (LHA) according to regional planning guidelines (by 2018)	Proportion of Regions that implemented care pathways in all LHA	Not detected	100% All the Regions adopted care pathways in all the LHA	Regions

Table 2. Indicators for cancer screenings included in the agreement between the Italian Ministry of Health and Regional Health Authorities. Acronyms: HPV Human Papillomavirus; LEA Essential Levels of Assistance set by the Italian Ministry of Health; LHA: Local Health Authorities

Figure 1 shows the adaptation of the DPSEEA model to cancer screening and the factors identified for the different dimensions of the model.

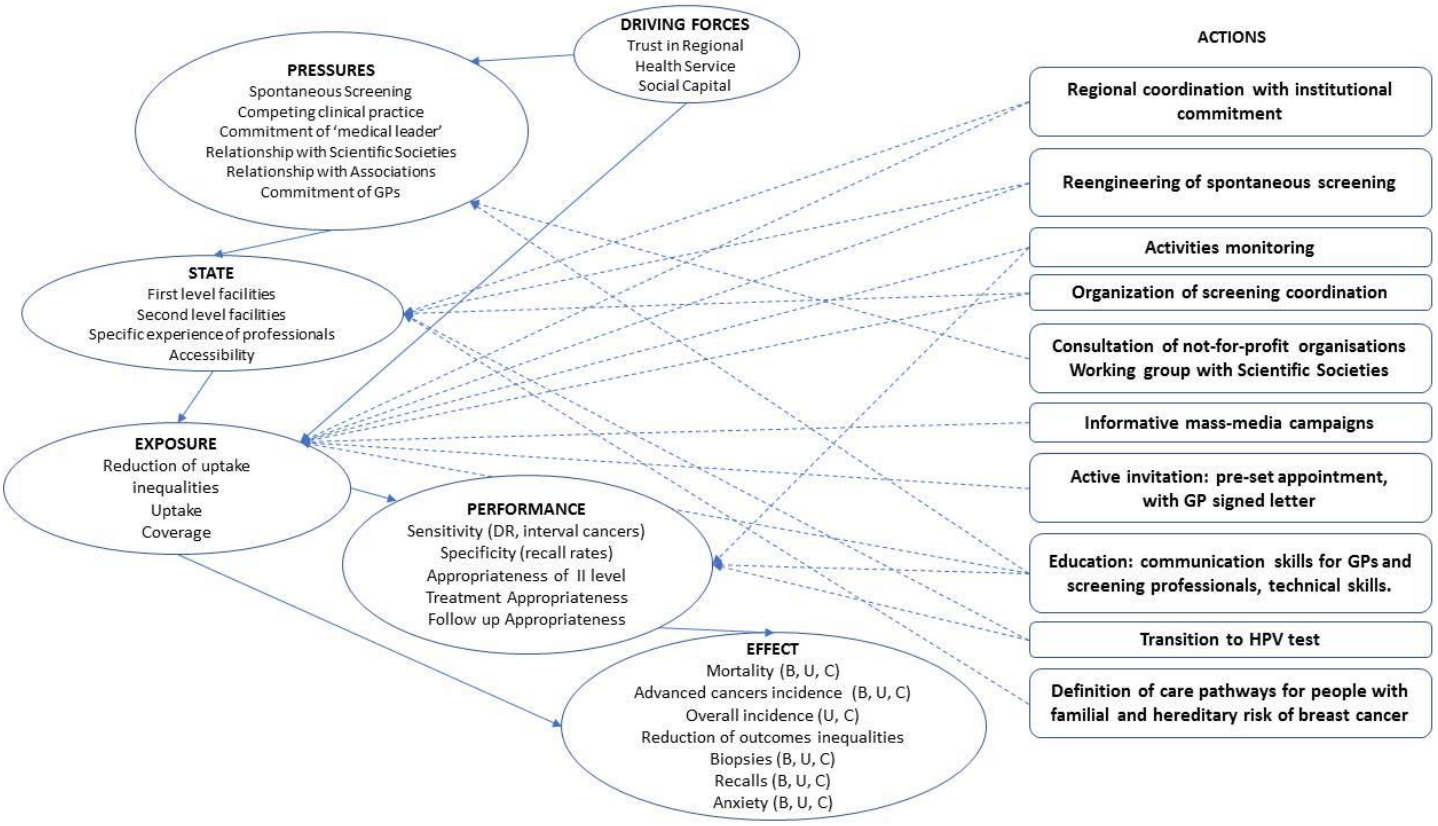


Figure 1. Adaptation of the DPSEEA framework to cancer screening programmes. Acronyms: B, Breast cancer; C, Colorectal cancer; DR, Detection Rate; GPs, General Practitioners, HPV Human Papillomavirus; U, Cervical cancer.

Table 3 summarizes the indicators suggested by the DPSEEA model. The survey on the analysis of local availability and quality of data was answered by 5 of 8 regions participating in the project. As an example, the indicator on advanced breast cancer incidence and on breast cancer incidence overall was available in Emilia-Romagna region and in the Autonomous Province of Trento. In Campania region, regional and Local Health Authorities (LHA) estimates are available based on data from Cancer Registers. In Lazio region, the Cancer Registry is being activated.

For each indicator a profile form was provided with a description of the indicator according to the model in Table 4.

Indicator ID	Indicator description
From the assessment plan document of the National Prevention Plan 2014-2018:	
1.12.1	Percentage of people who receive an invitation to participate in the cervical cancer screening program out of the target population
1.12.2	Percentage of people who receive an invitation to participate in the breast cancer screening program out of the target population
1.12.3	Percentage of people who receive an invitation to participate in the colorectal cancer screening program out of the target population
1.13.1	Percentage of people who participated to cervical cancer screening after active invitation out of the target population (CERVICAL SCREENING)
1.13.2	Percentage of people who participated to breast cancer screening after active invitation out of the target population (MAMMOGRAPH SCREENING)
1.13.3	Percentage of people who participated to colorectal cancer screening after active invitation out of the target population (COLORECTAL SCREENING)
1.14.1	Adoption of regional planning guidelines for cervical cancer screening by introducing the HPV-DNA test (within one year of the PRP publication)
1.14.2	Start HPV-based cervical cancer screening program introducing HPV-DNA testing by 2018
1.15.1	Adoption of regional planning guidelines (within one year from the Regional Prevention Plan publication)
1.15.2	Care pathways implementation in all Local Health Authorities (LHA) according to regional planning (by 2018)
9.6.1	Proportion of the regional population covered by computerized vaccination register
Other indicators proposed by the conceptual model (and available data sources):	
A1	Social capital (ISTAT)
A2	Spontaneous screening uptake (PASSI, ISTAT)
A3	Available facilities (mammographs; colonoscopy time; colposcopy time) (LHA, Regions)
A4	Differences in screening uptake by education level (PASSI, ISTAT)
A5	Differences in screening uptake by citizenship (PASSI, ISTAT)
A6	Detection rate (ONS)
A6bis	Detection rate of advanced adenomas (C) (ONS)
A7	Interval Cancers (B, C) (ONS, ad hoc studies)
A8	Recall rate (ONS)
A9	Appropriateness of second level (set of QA indicators ONS-scientific societies)
A10	Appropriateness of treatment (set of QA indicators ONS-scientific societies)
A11	Mortality (B, U, C) (ISTAT)
A12	Incidence of advanced cancers (B, U, C) (Cancer registries)
A13	Overall incidence (U, C) (Cancer registries)

Table 3. Indicators for cancer screenings included in the agreement between the Italian Ministry of Health and Regional Health Authorities and indicators defined by the DPSEEA framework for cancer screenings (with specific data sources). Indicator profiles are available from: http://www.ccm-network.it/documenti_Ccm/progetto_supporto_PNP/Screening_oncologici/Schede%20indicatori%20DPSEEA%20screening.pdf Acronyms: LHA, Local Health Authority; B Breast cancer; C Colorectal cancer; ISTAT Italian National Institute of Statistics; ONS, National Centre for Screening Monitoring; PASSI, "Progressi delle Aziende Sanitarie per la Salute in Italia" surveillance; PRP, Regional Prevention Plan; QA, Quality Assurance; U, Cervical cancer.

Definition	Brief description of the indicator and any context information to clarify what the indicator measures.
Purpose	Description of the rationale for choosing the indicator.
Priority level	Indication on the relevance of the indicator, based on the presence within the National Prevention Plan 2014-2018, its use for the assessment of the achievement of the LEA or the introduction through the DPSEEA conceptual model.
Required data	List of data necessary for the calculation of the indicator and available data sources.
Formula	Formula for calculating the indicator and multiplication factor.
Interpretation	Guide to the interpretation of the indicator, for example with indications on the timing necessary to measure a variation, on the factors contributing to the variation and on the possible meaning of the variation in relation to context factors and to the implementation of the National Prevention Plan 2014-2018.
Reference standard	Indication of the predefined target, if available, in quantitative terms and including timing to be achieved.

Table 4. Indicator profile form as defined within the project funded by the Centre for Diseases Control and Prevention of the Italian Ministry of Health, supporting the assessment of the implementation of the National Prevention Plan 2014-2018 strategies. Acronyms: LEA Essential Levels of Assistance set by the Italian Ministry of Health

DISCUSSION

Driving Forces:

Screening is a proactive medicine program based on a free choice of participation. Users' adherence is mainly influenced by the trust they place in the health service which, in turn, depends on the credibility of the system itself. Credibility can be measured but it is hard to change, at least in the short term, and must be considered as an effect modifier when assessing the performance of screening programs in Italy. Within the same region, however, this factor has much less impact and can hardly explain variations in screening uptake across different regional programs. The Social Capital, measurable but not modifiable in the short term [38–40], is understood as the set of resources of a community that could allow better interaction with the organization and screening proposals. Its association with the coverage and participation to screening has been observed in Italy. [31]

Pressures:

The uptake of opportunistic screening is easily measurable through the PASSI surveillance [32,41], ISTAT surveys [42] on health conditions and the use of health care services, and through the Information Technologies systems of specialist services. The active invitation to organized screening

programs could be perceived as an antagonist to opportunistic screening or, in any case, concurs with it. Actually, a recent uptake of a screening test, even outside the organized programs, is a reason for temporary exclusion from the active invitation if known by the screening coordination center when planning the invitations and, in any case, it is a reason not to take the test.

The commitment of General Practitioners (GPs) and medical leaders, the involvement of associations, and the agreement between the positions of the scientific societies and the recommendations adopted by the screening programs, are not measured but determine the factual indications of health professionals who deeply influence citizens' choices. It must be considered that such indications may be affected both by competing interests and by actual convictions of the inadequacy (of protocols, technical professional quality or organization) of organized screening services.

Four types of Actions can influence the *Pressures* (Figure 1):

1) strengthening of regional coordination;
2) active involvement of the GPs, medical leaders, scientific societies, voluntary associations, and other professionals in the field;
3) regulatory actions at the regional level which, for example, prevent or limit the use of spontaneous screening, also name actions for the re-engineering of spontaneous screening;
4) training and education of professionals internal and external to the organized screening programs, in particular GPs, gynecologists, gastroenterologists, and radiologists.

The direct impact of these strategies on intermediate factors is not easily measurable. Only the actions in the third point can be measured through the health services provided inside and outside the organized programs, before and after the policies' entry into force. The measurement of provided services is less feasible within the exclusive private sector.

State:

In analyzing the availability of resources, it is necessary to consider that a well-organized screening must aim at the capillary availability of the basic test across the country (to guarantee accessibility) and at a centralization of all the other phases (to guarantee technological and educational adequacy and an adequate volume of performance increasing expertise). This should involve the selection or change of the available offer (i.e. identifying the structures where to carry out the in-depth analysis with the technical-professional requirements provided for by the quality assurance guidelines) rather than adapting the screening program to the existing organization.[2,4,5] For factors such as human resources, convincing and validated indicators exists at national and European level (i.e. each screening radiologist must read at least 5,000 screening mammograms per year). [5]

The *Actions* can change the *state* by defining accreditation standards, information flows and monitoring criteria, but important structural limits require the re-engineering of spontaneous screening, ad hoc technical-professional training of the personnel and the acquisition of resources. A

clear institutional mandate is a necessary condition for achieving the objectives. Finally, the definition of pathways for breast cancer screening for inherited risk and the transition from Pap test to HPV test in cervical screening requires a *state* change in the resources of the SSR.

Exposure, Performances and *Effect:*

Exposure is defined as screening coverage and uptake. *Exposure* acts on the *effect* intended as diagnostic anticipation, improvement of prognosis and reduction of incidence (for the cervix and colorectal cancer). Changes in performance can be effect modifiers of the *Exposure*; adequate coverage and participation will have an effect on the population health (i.e. reduction of mortality) directly correlated with the performance of the program. The performance indicators measure both positive and undesirable effects (i.e. False positives, surgery on benign lesions in the breast, etc.), also providing direct assessment of the unwanted components of the effect.

The *Actions* that can directly influence on screening coverage and uptake are:

- coordination of screening across LHA with a strong commitment from the Strategic Management may have power to increase the coverage of organized programs;
- the monitoring and inclusion of coverage and participation in the LEA, which has often led to their inclusion in the objectives of the General Managers, can strengthen the commitment of Strategic Management;
- evidence-based actions to increase participation in programs (fixed appointments sent with active invitation, invitation letters signed by the GP, communication campaigns), [43] summarized in recommendations should be shared with Regional Councilors and General Managers to promote the implementation of coordinated screening programs, as done by Agenas; [31]
- training focused on operators internal and external to organized program, to promote active and appropriate participation.

Performance can be impacted by improving the sensitivity of the test, the technology used (i.e. shifting from Pap test to HPV and introducing magnetic resonance imaging for women with inherited risk and the technical-scientific capacity of staff through specific training. The indicators monitoring is therefore another strategic tool for continuous quality improvement.

Relationship between Actions, Exposure and Effect: the assessment of effectiveness

In assessing the relationship between *Actions*, *Exposure* and *Effect*, it is necessary to consider the factors that can bias their interpretation and make difficult to assess the causal links. The first is the time it takes for an *action* to have an *effect*. If the interaction between the natural history of the disease and screening tests is not considered, the *effects* could be misinterpreted. As an example, several studies show that the incidence begins to decrease after 6-8 years from the start of a

colorectal screening program, and before this timespan there is an increase in incidence in the screened population.

In addition, to assess the link between diagnostic anticipation and reduction of breast cancer mortality, it must be considered that mortality also depends on the incidence trend and the quality of treatment, which may change over the period considered. Surrogate indicators (i.e. incidence rate of advanced cancers) relating to intermediate stages of the causal chain between diagnostic anticipation and reduction of mortality may overcome the bias related to the quality of treatment and require shorter time to be observed.

Observational methods can be used to assess effectiveness outside an experimental context, mainly including:

- Before and after time comparisons; [44]
- Comparison of similar areas with different temporal implementation of screening programs; [29,45,46]
- Comparison between screened and non-screened people within the same population. [11.47]

These methods may be affected by comparability issues between groups and require the acquisition of additional information and adjustments in the analysis phase to make any measured link between changes in exposure to screening and variations in outcomes more robust. In general, the characteristics of the screening programs (active invitation of the population and a reliable collection of data related to process indicators) reduce the misclassification of exposure and the selection bias, due to inability of health professionals to select the target population based on health status. These points make the implementation or changes in screening program natural experiments where the assessment of effectiveness can be particularly convincing.

Selection of indicators

In Italy, the screening programs are monitored every year with an *ad hoc* questionnaire within a structured procedure, evaluated for formal and epidemiological consistency. This process is useful both for the evaluation of the Regional LEAs achievement, and for the continuous quality improvement of quality, including defined standards to be achieved and a benchmarking approach. The results are published and discussed annually at national, regional and single program level (www.Osservatorionazionalescreening.it). These indicators are an Italian contextualization of what is provided by the European guidelines. [48]

The indicators provided by the Document on the assessment of the PNP [14] for the objectives related to coverage and uptake were inserted in the DPSEEA model (Figure 1). Moreover, a summary of these indicators (i.e. the number of target people who perform a test following an invitation letter within organized programs) is currently used as an indicator for monitoring LEA on Screening.

For the objectives of shifting to HPV-based cervical cancer screening and of defining care pathways for inherited risk of breast cancer, the two process indicators identified are strictly connected to the *actions* and not to their *effects*. Nonetheless, screening monitoring and cancer registries may, in the future, show whether the introduction of the HPV test changed screening coverage and its *effects*.

It is more difficult to measure the *effects* of defining care pathways for women with inherited risk, acting on a small part of the target population and of the overall burden of disease. Process indicators are conceivable (use of magnetic resonance imaging or use of bilateral mastectomy) since their variations could have a plausible causal link with the implementation of the pathways themselves. However, the changes in these indicators are not directly correlated to an improvement in health status, therefore it was decided not to include them in the conceptual model.

CONCLUSIONS

Organized screening programs, in their implementation at European level, are born with their own set of performance and early outcome indicators, whose conceptual framework is strongly connected to the natural history of the disease and the theoretical basis of the mechanism of screening actions. [2–5] This may explain why, compared to other areas, there are few attempts to define a conceptual framework for identifying and interpreting screening indicators. To date, the assessment of screening programs followed a logic of monitoring the processes within the program itself and measuring the impact on population health. This was done without dealing with distal factors, external to the screening programs, which influence the implementation, coverage, and participation. For timing reasons, the approach proposed in this project has unfortunately not been able to inform the development of the PNP 2014-18 assessment document from the early stages. However, it represents an experiment that will be timely extended to all areas of the next PNP, to support the choice of central indicators. The collaboration with the Regions and the stakeholders represented in the advisory committee have shown a good acceptability of the model by final users, but its usefulness in current practice will be assessed.

Bibliography

1. The Council of the European Union. Council reccomendation of 2 December 2003 on cancer screening (2003/878/EC). Off J Eur Union. 2003;L 327/34:5.
2. Arbyn M, Anttila A, Jordan J, Ronco G, Schenck U, Segnan N, et al. European Guidelines for Quality Assurance in Cervical Cancer Screening. Second edition--summary document. Ann Oncol Off J Eur Soc Med Oncol. 2010 Mar;21(3):448–58.
3. Anttila A, Arbyn A, De Vuyst H, Dillner J, Dillner L, Franceschi S, et al. European guidelines for quality assurance in cervical cancer screening. Second edition, Supplements. Office for Official Publications of the European Union. Luxembourg. http://www.gisci.it/documenti/news/EW0115451ENN_002.pdf; 2015.
4. Segnan N, Patnick J, von Karsa L. European Commission. European guidelines for quality assurance in colorectal cancer screening and diagnosis. Luxembourg; 2011.
5. Perry N, Broeders M, de Wolf C, Tornberg S, Holland R, von Karsa L. European guidelines for quality assurance in breast cancer screening and diagnosis. Fourth edition--summary document. Ann Oncol. 2006 Oct 5;19(4):614–22.
6. Dimitrova N, Parkinson ZS, Bramesfeld A, Ulutürk A, Bocchi G. European Guidelines for Breast Cancer Screening and Diagnosis. Luxembourg; 2016.
7. NICE. Familial breast cancer: classification, care and managing breast cancer and related risks in people with a family history of breast cancer - Guidance and guidelines. UK; 2017.
8. Presidente consiglio dei ministri. D.P.C.M. 29-11-2001 - Definizione dei livelli essenziali di assistenza. Gazzetta Ufficiale, 33 2002 p. 5.
9. Zorzi M, Puliti D, Vettorazzi M, De Lisi V, Falcini F, Federico M, et al. Mastectomy rates are decreasing in the era of service screening: a population-based study in Italy (1997-2001). Br J Cancer. 2006 Nov 6;95(9):1265–8.
10. Foca F, Mancini S, Bucchi L, Puliti D, Zappa M, Naldoni C, et al. Decreasing incidence of late-stage breast cancer after the introduction of organized mammography screening in Italy. Cancer. 2013 Jun 1;119(11):2022–8.
11. Puliti D, Bucchi L, Mancini S, Paci E, Baracco S, Campari C, et al. Advanced breast cancer rates in the epoch of service screening: The 400,000 women cohort study from Italy. Eur J Cancer. 2017 Apr;75:109–16.
12. ONS. Osservatorio nazionale screening - Rapporto 2017. 2017.
13. Ministero della Salute. Piano Nazionale della Prevenzione 2014-2018. 2014.
14. Conferenza Stato Regioni. Accordo CSR n°56/2015 “Piano nazionale per la prevenzione per gli anni 2014-2018 - Documento per la valutazione.” Italy; 2015 p. 31.
15. Hambling T, Weinstein P, Slaney D. A review of frameworks for developing environmental health indicators for climate change and health. Int J Environ Res Public Health. 2011;8(7):2854–75.
16. Briggs D, World Health Organization Occupational and Environmental Health Team. Environmental Health Indicators: Framework and Methodologies. Geneva; 1999.
17. Green LW, Kreuter MW, Green LW. Health program planning : an educational and ecological approach. McGraw-Hill; 2005.
18. Federici A, Guarino A, Serantoni G. Adesione ai programmi di screening di prevenzione oncologica: proposta di una modellizzazione dei risultati di revisione della letteratura secondo il Modello PRECEDE-PROCEED. Epidemiol Prev. 2012;36(1):Appendice 3.
19. Waheed B, Khan F, Veitch B. Linkage-Based Frameworks for Sustainability Assessment: Making a Case for Driving Force-Pressure-State-Exposure-Effect-Action (DPSEEA) Frameworks. Sustainability. 2009 Aug 10;1(3):441–63.
20. Corvalán C, Nurminen M, Pastides H, World Health Organization Office of Global and Integrated Environmental Health. Linkage methods for environment and health analysis : technical guidelines : a report of the Health and Environment Analysis for Decision-making (HEADLAMP) project. Geneva; 1997.
21. Richter ED, Laster R. The Precautionary Principle, epidemiology and the ethics of delay. Int J Occup Med Environ Health. 2004;17(1):9–16.

22. Giordano L, Giorgi D, Frigerio A, Bravetti P, Paci E, Petrella M, et al. [Process indicators and standards for the evaluation of breast cancer screening programmes]. Epidemiol Prev. 2006;30(2 Suppl 1):5-9,11-47.
23. Ronco G, Zappa M, Naldoni C, Iossa A, Berrino F, Anghinoni E, et al. [Indicators and standards for evaluating the program process for cervical cancer screening. Operation manual. Italian Group for Cervical Carcinoma Screening]. Epidemiol Prev. 1999;23 Suppl:1–32.
24. Zorzi M, Giorgi Rossi P, Primario G di lavoro G sugli I dello screening con test HP V. Indicators for monitoring screening programs with primary HPV test. Indic per Monit dei Program di Screen con test HPV primario. 2017;41(1):1–32.
25. Zorzi M, De'Bianchi PS, Grazzini G, Senore C, GISCoR G di lavoro sugli indicatori del. Ouality indicators for the evaluation of colorectal cancer screening programmes. Epidemiol Prev. 2007;31(6 Suppl 1):6–56.
26. Paci E, Coviello E, Miccinesi G, Puliti D, Cortesi L, Lisi V De, et al. Evaluation of service mammography screening impact in Italy. The contribution of hazard analysis. Eur J Cancer. 2008 Apr;44(6):858–65.
27. Puliti D, Miccinesi G, Collina N, De Lisi V, Federico M, Ferretti S, et al. Effectiveness of service screening: a case–control study to assess breast cancer mortality reduction. Br J Cancer. 2008 Aug 29;99(3):423–7.
28. Zorzi M, Mangone L, Sassatelli R, Baracco S, Budroni M, Castaing M, et al. Screening for colorectal cancer in Italy: 2011-2012 survey. Epidemiol Prev. 2015;39(3 Suppl 1):115–25.
29. Zucchetto A, Ronco G, Giorgi Rossi P, Zappa M, Ferretti S, Franzo A, et al. Screening patterns within organized programs and survival of Italian women with invasive cervical cancer. Prev Med (Baltim). 2013 Sep;57(3):220–6.
30. Serraino D, Gini A, Taborelli M, Ronco G, Giorgi-Rossi P, Zappa M, et al. Changes in cervical cancer incidence following the introduction of organized screening in Italy. Prev Med (Baltim). 2015 Jun;75:56–63.
31. Sinopoli A, Saulle R, Guarino A, Cereda D, Giorgi Rossi P, Serantoni G, et al. [Experimenting an instrument for cancer screening programming in Italy]. Ig Sanita Pubbl. 2017;73(4):303–9.
32. Giorgi Rossi P, Carrozzi G, Federici A, Mancuso P, Sampaolo L, Zappa M. Invitation coverage and participation in Italian cervical, breast and colorectal cancer screening programmes. J Med Screen. 2018 Mar 14;25(1):17–23.
33. Dalkey N, Helmer O. An Experimental Application of the DELPHI Method to the Use of Experts. Manage Sci. 1963;9(3):458–67.
34. Ronco G, Dillner J, Elfström KM, Tunesi S, Snijders PJF, Arbyn M, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. Lancet (London, England). 2014 Feb;383(9916):524–32.
35. Ronco G, Biggeri A, Confortini M, Naldoni C, Segnan N, Sideri M, et al. [Health technology assessment report: HPV DNA based primary screening for cervical cancer precursors]. Epidemiol Prev. 2012 Aug;36((3-4 Suppl 1)):e1–72. PMID: 22828243.
36. Regione Emilia-Romagna. Contributi n. 91/2016: Protocollo assistenziale nelle donne a rischio ereditario di tumore della mammella e/o ovaio. Emilia-Romagna; 2016.
37. Giorgi Rossi P, Carozzi F, Federici A, Ronco G, Zappa M, Franceschi S. Cervical cancer screening in women vaccinated against human papillomavirus infection: Recommendations from a consensus conference. Prev Med (Baltim). 2017 May 25;98:21–30.
38. ISTAT. Il benessere equo e sostenibile in Italia - BES 2017. Roma; 2017.
39. Pitkin Derose K, Varda DM. Social Capital and Health Care Access. Med Care Res Rev. 2009 Jun 27;66(3):272–306.
40. Fiorillo D, Sabatini F. Structural social capital and health in Italy. Econ Hum Biol. 2015 Apr;17:129–42.
41. Venturelli F, Sampaolo L, Carrozzi G, Zappa M, Giorgi Rossi P, Rossi PG. Associations between cervical, breast and colorectal cancer screening uptake, chronic diseases and health-related behaviours: Data from the Italian PASSI nationwide surveillance. Prev Med (Baltim). 2019 Mar 16;120:60–70.
42. Istituto Nazionale di Statistica. Il sistema di indagini sociali multiscopo [Internet]. Metodi e Norme, n. 31. 2006 [cited 2017 Dec 6]. p. 279. Available from: https://www.istat.it/en/files/2014/06/met_-norme_06_31_il_sistema_di_indagini_multiscopo.pdf

43. Camilloni L, Ferroni E, Cendales BJ, Pezzarossi A, Furnari G, Borgia P, et al. Methods to increase participation in organised screening programs: a systematic review. BMC Public Health. 2013 May 13;13:464.
44. Giorgi Rossi P, Vicentini M, Sacchettini C, Di Felice E, Caroli S, Ferrari F, et al. Impact of Screening Program on Incidence of Colorectal Cancer: A Cohort Study in Italy. Am J Gastroenterol. 2015 Sep 25;110(9):1359–66.
45. Houssami N, Bernardi D, Caumo F, Brunelli S, Fantò C, Valentini M, et al. Interval breast cancers in the 'screening with tomosynthesis or standard mammography' (STORM) population-based trial. The Breast. 2018 Apr;38:150–3.
46. Giorgi Rossi P, Federici A, Farchi S, Chini F, Barca A, Guasticchi G, et al. The effect of screening programmes on the treatment of benign breast neoplasms: Observations from current practice in Italy. J Med Screen. 2006 Sep 22;13(3):123–8.
47. Ventura L, Mantellini P, Grazzini G, Castiglione G, Buzzoni C, Rubeca T, et al. The impact of immunochemical faecal occult blood testing on colorectal cancer incidence. Dig Liver Dis. 2014 Jan;46(1):82–6.
48. Anttila A, Lönnberg S, Ponti A, Suonio E, Villain P, Coebergh JW, et al. Towards better implementation of cancer screening in Europe through improved monitoring and evaluation and greater engagement of cancer registries. Eur J Cancer. 2015 Jan;51(2):241–51.

2.3. Research article #02: Associations between cervical, breast and colorectal cancer screening uptake, chronic diseases and health-related behaviours: data from the Italian PASSI nationwide surveillance.

The findings of this research article were published in *Preventive Medicine*.

Venturelli F, Sampaolo L, Carrozzi G; PASSI Working Group, Zappa M, Giorgi Rossi P. Associations between cervical, breast and colorectal cancer screening uptake, chronic diseases and health-related behaviours: Data from the Italian PASSI nationwide surveillance. Prev Med. 2019 Mar;120:60-70. doi: 10.1016/j.ypmed.2019.01.007. Epub 2019 Jan 16. Erratum in: Prev Med. 2019 Sep;126:105788. PMID: 30659908.

Abstract:

Screening programmes have been proposed as a privileged setting for health promotion interventions. We aim to assess the associations between behavioural risk factors, chronic conditions and diseases and cervical, breast and colorectal cancer screening uptake. Secondly, we aim to assess whether these associations are due to underlying differences in socioeconomic characteristics.

In Italy, a random sample was interviewed by the PASSI surveillance (106,000 interviews) in 2014-2016. Screening uptake adjusted for age and gender alone and for age, gender and socioeconomic characteristics (educational attainment and self-reported economic difficulties) were estimated using multivariate Poisson regression models.

Screening uptake was 79%, 73% and 45% for cervical (age 25-64), breast (women aged 50-69) and colorectal cancer (both sexes age 50-69), respectively. People with low consumption of vegetables and fruits and those with insufficient physical activity had lower uptake than people with healthy behaviours (20-22% and 8-15% lower, respectively), as did those obese and diabetic compared to healthier people (7-10% and 5-8% lower, respectively). Those with high-risk drinking behaviour, self-reported driving after drinking alcohol, and former smokers had higher screening uptake (3-7%, 3-6%, and 7-14% higher, respectively). Differences in uptake decreased after adjusting for socioeconomic characteristics, but trends were unvaried.

In conclusion, screening uptake is negatively associated with unfavourable behaviours and health conditions that are also risk factors for breast and colorectal cancer incidence. Socioeconomic characteristics do not fully explain these differences. Health promotion interventions targeting diet and physical activity nested in screening programmes might miss part of the at-risk population.

Abbreviations: FOBT faecal occult blood test; HPV human papillomavirus; PASSI The Italian Behavioral Risk Factor Surveillance System (Progressi delle Aziende Sanitarie per la Salute in Italia)

INTRODUCTION

Behavioural risk factors are related to almost one third of attributable disability-adjusted life years, representing the main risk category, followed by metabolic and environmental risk factors. [1] Moreover, tobacco smoke, alcohol use and diet-related risk factors have an important role in the cancerogenic process. [2, 3] Indeed, human behaviours are a complex issue whose determinants have been extensively debated in the literature. [4, 5] The complexity of these determinants makes any attempt to change unhealthy habits challenging, even when the detrimental consequences on individual health are well known. [6] Thus, evidence-based primary and secondary prevention strategies should be implemented to decrease the prevalence of behavioural risk factors and to identify related diseases early to reduce disability and mortality.

Organised cervical, breast and colorectal cancer screening programmes have been implemented in Italy since the beginning of the 2000s. [7] Despite the demonstrated efficacy of the recommended screening tests, the effectiveness of screening programmes depends largely on organizational issues and on behavioural issues (i.e. whether invited men and women participate). [8-10]As for behavioural risk factors, participation in screening programmes also has individual- and context-related determinants. [11] Indeed, non-participation is a particularly important issue when it involves people with risk factors. [12, 13]

Although determinants of uptake in prevention programmes and determinants of behavioural risk factors have been explored by numerous previous studies [4, 5, 14-16], only few have assessed the association between the two with inconsistent results. [17-21] In addition, the contribution of socioeconomic characteristics to these potential associations has not been clearly reported.

To plan health promotion and to increase screening participation in an equitable way and to address the right populations, it is important to measure associations between health behaviours. This is particularly crucial when trying to exploit the capillary contacts with the healthy population that screening programmes offer as a vehicle for health promotion interventions addressing different lifestyle and behavioural risk factors. [22-24]

Furthermore, measuring the association between screening participation and health behaviour and conditions allows quantifying the possible influence of self –selection bias on observational studies aimed at evaluating screening effectiveness.

The main objective of the present study was to describe the associations between behavioural risk factors (diet, physical activity, alcohol use, smoking, road safety behaviours and vaccine uptake), chronic conditions strictly related to these behaviours (obesity, hypertension and hypercholesterolemia, diabetes, cancer, depressive symptoms) and breast, cervical and colorectal cancer organised and spontaneous screening uptake. Secondly, this study aimed to assess whether these associations were linked to underlying differences in socioeconomic characteristics in both screening uptake and other health-related behaviours.

METHODS

Study design

This study is an analysis of data from repeated cross-sectional surveys.

Setting

In Italy, 21 Regional Health Services are responsible for implementing organised cancer screening programmes for cervical, breast and colorectal cancer with active invitation of the target population. In accordance with European recommendations. [7, 25] (**Table 1**). In 2016, organized programmes regularly invited 81%, 80% and 75% of the resident target population for cervical, breast and colorectal cancer screening, respectively; participation was 40%, 56% and 40%, respectively. [26]

Spontaneous screening uptake in Italy, particularly for cervical cancer and breast cancer, contributes appreciably to overall screening uptake, probably because of the low cost of the test and gynaecologists' habit of performing a Pap test during reproductive health visits. [27]

Screening programs	Invited population	Recommended test and intervals	References
Cervical cancer	Women aged 25-29	Pap test every 3 years	(Ministero della Salute and Direzione Generale della Prevenzione, 2005)[7]
	Women aged 30-64	HPV-DNA test every 5 years	(Ronco et al., 2012; von Karsa et al., 2015)[9, 28]
Breast cancer	Women aged 50-69	Mammogram every 2 years	(Ministero della Salute and Direzione Generale della Prevenzione, 2005)[7]
	Women aged 45-74	Mammogram every 1 year between 45-49 years; every 2 years between 50-74 years	In Emilia-Romagna and Piedmont regions only. (Distante et al., 2007)[29]
Colorectal cancer	Men and Women aged 50-69	FOBT every 2 years	(Ministero della Salute and Direzione Generale della Prevenzione, 2005)[7]
	Men and Women aged 58-60	Once-only Sigmoidoscopy (or FOBT)	In Piedmont region only. FOBT offered only to those not participating in sigmoidoscopy. (Ministero della Salute and Direzione Generale della Prevenzione, 2005)[7]

Table 1 Screening protocols: Screening protocols according to Italian guidelines. Italy, April 2018. FOBT= faecal occult blood test.

Data sources and population

In the present study, the analysed data were collected by PASSI between 2014 and 2016, with interviews of more than 106,000 people, a representative sample of the Italian population aged 18-69. PASSI collects information on behavioural risk factors and the implementation of preventive health care services. The survey and sampling methods have been described elsewhere. [30, 31]

In addition, the PASSI questionnaire gathers information about educational attainment (4 classes: elementary school, middle school, high school or higher education), occupational status (employed, employment seeker, retired or other unemployed), perceived economic difficulties (3 classes: many, some or no economic difficulties) and citizenship (2 classes: Italian plus foreign nationals from high-income countries and foreign nationals from middle or low-income countries, according to the World Bank classification). [32]

Outcomes definition

*Cancer screening uptake (**Figure 1**)*

PASSI questionnaire collects the date of the last test before the interview, provider of the last test (free or paid out of pocket, proxy of organised and spontaneous screening, respectively), reception of invitation letter mailed by organised programmes and screening promotion by health professionals.

For the assessment of cervical cancer screening uptake, we considered a Pap test in the last three years or an HPV-DNA test in the last 5 years for women aged 25 to 64.

For breast cancer screening uptake assessment, we took into account a mammogram in the last two years for women aged 50-69.

For colorectal cancer screening uptake assessment, we considered an FOBT in the last two years or a colonoscopy or flex sigmoidoscopy in the last five years for people aged 50-69. Interviews of residents in the Piedmont region were excluded for colorectal cancer screening due to the different screening protocol.

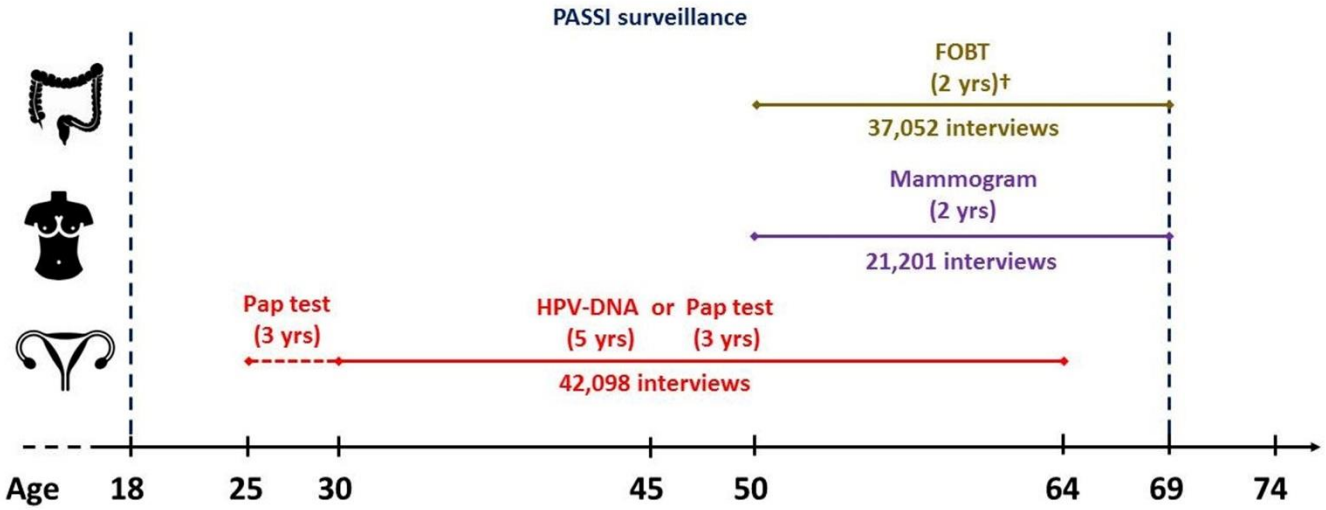


Figure 1 Study design. Screening tests (with recommended screening intervals) and age of target populations of the three organised cancer screening programmes implemented in Italy: colorectal cancer (for men and women), cervical cancer and breast cancer. For each screening, the number of people in the target population interviewed by PASSI surveillance in the period 2014-2016 is also reported. The vertical dashed lines show the target population of the PASSI surveillance. †For colorectal cancer screening, a colonoscopy or flex sigmoidoscopy in the last five years was also considered as screening uptake.

Exposure definition

Healthcare and lifestyle-related behaviours and chronic conditions

We included the following information: seasonal influenza vaccination uptake in people aged 65-69 or of any age but reporting at least one chronic disease, in accordance with current guidelines [33]; lifestyle-related behavioural risk factors, i.e. smoking habit (smoker, former smoker, non-smoker), daily fruit and vegetable intake (0, 1-2, 3-4, 5+ portions), high-risk drinking behaviour, physical activity level (active, partially active, sedentary) and road safety-related behaviours (i.e. frequency of front and back seat belt use, wearing a motorcycle helmet and driving after drinking alcohol); self-perceived health status (very good/good, normal, bad/very bad), nutritional status (underweight/normal, overweight, obese); diagnosis of hypertension, hypercholesterolemia, depressive symptoms and

diabetes. A positive anamnesis for cancer was also included among exposures, since several previous studies showed positive association with screening uptake. [17, 18, 34, 35]

The description of each variable and related categories is reported in **Supplementary material-Table A.1**.

Statistical Analysis

In this article, descriptive analyses and multivariate Poisson regression were performed with the Stata 11 software and were all appropriately weighed. In PASSI, each Local Health Authority extracts a proportionate stratified sampling for the sex and age class (18-34, 35-49, 50-69 years) of the resident population. Therefore, data analysis at regional and national level required data weighing to take into account stratification (geographic area of the Local Health Authority, age and sex) to be representative of the whole population. The weight used in the analysis is the inverse of the sample fraction.

In this work, screening uptake within the recommended time intervals are shown for socioeconomic variables as weighted percentages with 95% confidence interval (95%CI). The probabilities of performing screening tests within the recommended time intervals adjusted for age (five-year class) and gender (only for colon) or adjusted for age, gender, educational attainment and economic difficulties were calculated according to the presence/absence of behavioural risk factors, chronic conditions and/or chronic diseases. These probabilities were estimated using multivariate Poisson regression models. Age- and gender-adjusted results were compared with those adjusted for age, gender, educational attainment and economic difficulties.

Ethics and data sharing

In the PASSI surveillance system, personal data are processed in compliance with the privacy laws (Decree law 196/2003). PASSI was approved by the Ethics Committee of the National Institute of Public Health on January 23, 2007.

Although the anonymized dataset is not yet available, the National Institute of Public Health is working to make it available on request (http://www.epicentro.iss.it/passi/PresPolicy.asp).

Participation in the survey is free and voluntary. The interviewers receive specific training on the correct procedures to follow in the processing of personal data. The people selected for the interview are informed by letter and by the interviewer about the objectives of the investigation, its methods and the arrangements taken to ensure the confidentiality of the information collected. Individuals can also refuse to be interviewed or can interrupt the interview at any time.

Interviews are transferred anonymously to a national archive via a secure internet connection. Personal Identifiers on paper or computer are subsequently locally destroyed.

RESULTS

The overall self-reported screening uptake in our study was 79%, 73% and 45% for cervical, breast and colorectal cancer screening, respectively. There were strong associations with socioeconomic conditions, with lower uptake in more disadvantaged people, for all the screenings (**Table 2**).

Association with behaviours

All screening uptakes were associated with each other, i.e., uptake in one screening was associated with uptake in the other two, whether through organised screening programme or spontaneous uptake, while no association was observed between screening and influenza vaccination (**Table 3; Table 4**).

Non-smokers showed slightly higher test uptake than smokers in all screenings, while former smokers showed the highest uptake. High-risk drinking behaviour and driving after drinking alcohol were both associated with higher test uptake for all screenings. Instead, unhealthy behaviours concerning diet, physical activity and other road safety items were associated with lower screening uptake.

Association with chronic conditions

Hypertension was slightly inversely associated with screening uptake, while hypercholesterolemia was directly associated with screening uptake. Obese and overweight people had lower screening uptake, with a stronger trend for cervical and colorectal cancer screening than for breast; differences were smaller for the organised screening programme component. Depressive symptoms were slightly inversely associated with test uptake for all screenings.

Diabetes was negatively associated with uptake for all the screenings. Instead, previous cancer diagnosis increased screening uptake, particularly for colorectal cancer screening. Finally, feeling in good health was positively associated with test uptake for all screenings; the association was smaller in the organised screening component for cervical and breast cancer screening.

Model adjusted by socioeconomics

In general, adjusting for educational attainment and economic difficulties, differences in uptake became smaller than in the models only adjusted by age, but the trend was unvaried. There were only a few exceptions when the observed differences disappeared: diabetes and hypertension for all screenings, depressive symptoms for breast and colorectal cancer, high-risk drinking behaviour for breast cancer, and self-perceived health status in colorectal cancer (**Table 4**).

	Cervical cancer screening uptake according to screening protocols (n = 42098)						Breast cancer screening uptake according to screening protocols (n = 21201)						Colorectal cancer screening uptake according to screening protocols (n = 37052)					
	Organised programmes			Spontaneous			Organised programmes			Spontaneous			Organised programmes			Spontaneous		
	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI
Age classes																		
25-34	3716	**36.2**	35.0 - 37.4	2687	**36.4**	35.0 - 37.7												
35-49	8880	**43.9**	43.1 - 44.8	6004	**38.8**	37.9 - 39.7												
50-69[a]	8600	**51.0**	50.0 - 52.0	3644	**27.1**	26.2 - 28.1	12440	**53.7**	52.9 - 54.6	3558	**18.8**	18.1 - 19.5	15941	**37.2**	36.6 - 37.8	2606	**7.6**	7.2 – 7.9
Gender																		
Men													7582	**37.8**	37.0 – 38.6	1368	**8.2**	7.7 – 8.8
Women	21196	**44.9**	44.4 - 45.5	12335	**34.0**	33.4 – 34.6	12440	**53.7**	52.9 - 54.6	3558	**18.8**	18.1 - 19.5	8359	**36.7**	36.0 – 37.5	1238	**6.9**	6.4 – 7.5
Educational attainment																		
None/Elementary	1028	**40.8**	37.7 - 44.0	371	**20.5**	18.1 - 23.1	2199	**52.1**	49.8 - 54.4	345	**12.1**	10.4 - 14.0	2274	**34.1**	35.5 – 35.7	258	**5.3**	4.3 – 6.4
Middle school	6393	**48.6**	47.5 - 49.8	2831	**27.5**	26.4 - 28.6	4576	**54.7**	53.3 - 56.2	1086	**16.1**	15.0 - 17.3	5955	**37.3**	36.3 – 38.3	792	**6.1**	5.5 – 6.6
High school	9756	**45.5**	44.6 - 46.3	6007	**35.5**	34.6 - 36.4	4437	**54.4**	53.0 - 55.8	1547	**22.1**	20.9 - 23.3	5990	**38.6**	37.6 – 39.6	1070	**8.4**	7.8 – 9.1
Higher education	4006	**40.1**	38.9 - 41.4	3123	**43.2**	41.9 - 44.6	1224	**51.4**	48.9 - 53.9	578	**27.6**	25.4 - 30.0	1716	**36.8**	35.1 – 38.5	486	**12.3**	11.1 – 13.6
Economic difficulties																		
Many	2692	**41.3**	39.7 - 43.0	1432	**27.7**	26.2 - 29.3	1427	**42.2**	39.9 - 44.6	427	**15.2**	13.6 - 17.0	1543	**25.1**	23.6 – 26.6	276	**5.5**	4.7 – 6.4
Few	8765	**44.1**	43.1 - 45.0	5038	**33.4**	32.5 - 34.4	5065	**51.7**	50.3 - 53.0	1447	**19.1**	17.9 - 20.3	5338	**31.8**	30.9 – 32.7	1015	**7.6**	7.0 – 8.3
None	9642	**47.2**	46.3 - 48.1	5841	**37.2**	36.3 - 38.1	5889	**60.6**	59.4 - 61.8	1672	**19.8**	18.8 - 20.9	8694	**47.4**	46.5 – 48.2	1299	**8.3**	7.7 – 8.8
Citizenship[b] Italians + foreign nationals HIC	19692	**44.5**	43.9 - 45.1	11815	**34.8**	34.2 - 35.4	12030	**53.7**	52.8 - 54.5	3494	**19.0**	18.3 - 19.7	15577	**37.3**	36.7 – 37.8	2571	**7.6**	7.3 – 8.0
Foreign nationals MLIC	1494	**52.2**	49.9 - 54.5	507	**21.3**	19.4 - 23.4	403	**56.5**	52.1 - 60.8	60	**10.1**	7.7 - 13.0	358	**36.2**	32.7 – 39.9	34	**3.6**	2.5 – 5.2
Occupational status																		
Employed	13129	**46.3**	45.5 - 47.0	8187	**37.4**	36.6 - 38.1	4883	**55.1**	53.7 - 56.4	1728	**23.2**	22.0 - 24.4	7325	**36.0**	35.2 - 36.8	1467	**8.8**	8.3 - 9.4
Employment seeker	1746	**40.0**	38.1 - 42.0	996	**29.8**	27.9 - 31.8	353	**52.0**	46.8 - 57.1	108	**18.3**	14.6 - 22.6	504	**28.4**	25.7 - 31.3	66	**4.3**	3.2 - 5.9
Retired	1259	**54.7**	51.9 - 57.4	382	**21.0**	18.8 - 23.4	3502	**59.8**	58.1 - 61.4	586	**11.9**	10.9 - 13.0	5613	**46.1**	44.9 - 47.3	697	**7.1**	6.5 - 7.8
Other unemployed	4950	**41.9**	40.6 - 43.2	2707	**30.6**	29.4 - 31.9	3648	**48.2**	46.5 - 49.8	1110	**18.9**	17.6 - 20.3	2398	**28.6**	27.3 - 29.9	366	**6.0**	5.1 - 7.0
Living situation																		
Alone	1670	**41.3**	39.3 - 43.3	1004	**33.9**	31.8 - 35.9	1380	**53.4**	50.8 - 56.0	339	**15.3**	13.3 - 17.5	1647	**37.4**	35.5 - 39.3	242	**7.1**	5.9 - 8.5
With spouse/ partner	15226	**47.9**	47.1 - 48.6	8567	**34.7**	34.0 - 35.5	9196	**55.0**	54.0 - 56.0	2647	**19.4**	18.5 - 20.2	12411	**38.2**	37.6 - 38.8	2043	**7.8**	7.3 - 8.3
With other people except for partner	4293	**38.0**	36.8 - 39.3	2760	**32.1**	30.9 - 33.4	1860	**48.8**	46.6 - 51.0	570	**18.5**	16.9 - 20.3	1878	**32.0**	30.4 - 33.7	319	**6.6**	5.8 - 7.5

[a] Women aged 50-64 for cervical cancer screening; [b] Italian and foreign nationals from high-income countries (HIC); Foreign nationals from middle or low-income countries (MLIC).

Table 2. Spontaneous or within organised programme screening uptake (Number of people reporting screening uptake, weighted % and 95% Confidence Interval; 95%CI) for socio-demographic characteristics. PASSI 2014-2016, Italy. Cervical cancer screening: Pap test within three years before the interview or HPV-DNA test within five years before the interview; breast cancer screening: mammogram within two years before the interview; colorectal cancer screening: faecal occult blood test within two years before the interview or colonoscopy/ flex sigmoidoscopy within 5 years before the interview (residents in Piedmont were excluded due to the different colorectal cancer screening protocol).

	Cervical cancer screening uptake according to screening protocols (n = 42098)						Breast cancer screening uptake according to screening protocols (n = 21201)						Colorectal cancer screening uptake according to screening protocols (n = 37052)					
	Organised programmes			Spontaneous			Organised programmes			Spontaneous			Organised programmes			Spontaneous		
	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI
SCREENING TEST COVERAGE																		
CERVICAL CANCER SCREENING																		
Yes	-	-	-	-	-	-	7924	**59.9**	58.8-61.0	2572	**23.4**	22.4-24.4	5226	**39.8**	38.7-40.8	815	**7.8**	7.2-8.5
No	-	-	-	-	-	-	902	**28.5**	26.5-30.7	325	**12.0**	10.4-13.8	507	**17.1**	15.5-18.9	112	**4.1**	3.0-5.6
BREAST CANCER SCREENING																		
Yes	7395	**58.0**	56.9-59.1	3087	**30.1**	29.0-31.2	-	-	-	-	-	-	7456	**44.7**	43.8-45.7	1055	**8.1**	7.5-8.8
No	1148	**31.0**	29.0-33.1	530	**18.4**	16.6-20.3	-	-	-	-	-	-	840	**16.0**	14.7-17.5	177	**3.9**	3.3-4.7
COLORECTAL CANCER SCREENING																		
Yes	4464	**60.7**	59.1-62.2	1579	**27.9**	26.5-29.4	6970	**69.0**	67.7-70.3	1556	**18.2**	17.1-19.3	-	-	-	-	-	-
No	3105	**40.9**	39.5-42.4	1837	**28.6**	27.2-30.0	4134	**39.9**	38.6-41.1	1814	**20.8**	19.8-21.9	-	-	-	-	-	-
INFLUENZA VACCINATION UPTAKE*																		
Yes	307	**54.2**	48.8-59.5	112	**23.4**	19.3-28.1	477	**56.8**	52.6-61.0	86	**13.8**	10.9-17.2	725	**42.0**	39.0-45.1	102	**7.1**	5.7-8.8
No	1297	**49.2**	46.9-51.6	664	**29.9**	27.7-32.2	1127	**57.4**	54.5-60.2	247	**15.2**	13.3-17.3	1552	**41.3**	39.4-43.3	247	**7.8**	6.8-9.0
LIFESTYLE BEHAVIOURS																		
SMOKING HABIT																		
non smoker	13391	**44.7**	44.0-45.5	7664	**33.5**	32.7-34.2	7803	**53.3**	52.2-54.4	2269	**19.1**	18.2-20.0	8267	**36.3**	35.5-37.1	1306	**7.2**	6.7-7.7
former smoker	3383	**48.5**	46.9-50.1	1944	**36.4**	34.8-38.0	2344	**58.1**	56.0-60.1	658	**19.5**	17.9-21.2	4818	**44.4**	43.2-45.6	764	**8.7**	7.9-9.5
smoker	4379	**43.0**	41.7-44.3	2711	**34.1**	32.7-35.4	2273	**51.1**	49.0-53.2	630	**17.2**	15.7-18.9	2812	**30.6**	29.4-31.9	535	**7.3**	6.6-8.1
FRUIT AND VEGETABLE DAILY INTAKE																		
≥5 portions	2673	**49.3**	47.6-51.1	1498	**33.7**	32.1-35.4	1991	**58.4**	56.3-60.5	582	**19.4**	17.8-21.2	2353	**44.6**	42.8-46.3	384	**9.2**	8.1-10.3
3-4 portions	9754	**47.2**	46.3-48.1	5498	**34.3**	33.4-35.2	6206	**56.3**	55.0-57.5	1734	**19.1**	18.0-20.2	7552	**40.9**	40.0-41.9	1092	**7.3**	6.7-7.9
1-2 portions	8473	**42.0**	41.0-42.9	5125	**34.2**	33.3-35.2	4131	**49.1**	47.6-50.6	1200	**18.2**	17.1-19.4	5858	**31.8**	31.0-32.7	1093	**7.4**	6.9-8.0
0 portions	292	**35.2**	30.5-40.2	213	**25.6**	21.9-29.8	111	**39.8**	31.8-48.3	42	**15.5**	11.1-21.3	177	**27.2**	22.6-32.3	37	**5.9**	4.0-8.7
PHYSICAL ACTIVITY																		
Active	9980	**46.0**	45.1-46.9	5991	**35.4**	34.5-36.3	5630	**56.4**	55.2-57.7	1616	**19.2**	18.2-20.3	8199	**42.2**	41.3-43.0	1202	**7.4**	6.9-7.9
Partially active	5776	**48.3**	47.1-49.5	3179	**34.2**	33.0-35.4	3045	**58.6**	56.7-60.4	835	**18.8**	17.4-20.4	3913	**40.8**	39.5-42.1	635	**8.6**	7.7-9.5
Sedentary	5258	**40.1**	39.0-41.3	3095	**31.9**	30.7-33.1	3692	**47.6**	46.1-49.2	1085	**18.2**	16.9-19.5	3720	**28.2**	27.2-29.3	756	**7.2**	6.6-7.9
HIGH-RISK DRINKING BEHAVIOUR																		
No	18576	**44.9**	44.3-45.5	10792	**33.9**	33.3-34.5	11263	**53.8**	53.0-55.7	3211	**18.7**	18.0-19.5	13523	**36.7**	36.1-37.2	2230	**7.6**	7.2-8.0
Yes	2347	**45.9**	44.0-47.8	1386	**35.3**	33.4-37.3	1062	**56.9**	53.7-60.1	291	**18.0**	15.8-20.5	2235	**44.6**	42.7-46.4	324	**7.3**	6.4-8.3
ROAD SAFETY																		
DRIVING AFTER DRINKING																		
No	20930	**44.9**	44.3-45.5	12145	**34.0**	33.4-34.6	12360	**53.7**	52.9-54.6	3527	**18.7**	18.0-19.5	15264	**37.0**	36.5-37.6	2514	**7.6**	7.2-8.0
Yes	266	**45.1**	40.0-50.3	190	**38.3**	33.3-43.6	80	**55.6**	45.7-65.1	31	**20.9**	14.2-29.6	677	**43.3**	40.1-46.6	92	**7.2**	5.7-9.2

Research article #02: Associations between cervical, breast and colorectal cancer screening uptake, chronic diseases and health-related behaviours: data from the Italian PASSI nationwide surveillance.

	Cervical cancer screening uptake according to screening protocols (n = 42098)						Breast cancer screening uptake according to screening protocols (n = 21201)						Colorectal cancer screening uptake according to screening protocols (n = 37052)					
	Organised programmes			Spontaneous			Organised programmes			Spontaneous			Organised programmes			Spontaneous		
	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI
ANTERIOR SEAT BELT (ALWAYS USED)																		
Yes	19130	**46.3**	45.7-46.9	11045	**34.6**	34.0-35.2	11225	**55.6**	54.7-56.5	3178	**19.2**	18.4-19.9	14309	**39.7**	39.1-40.3	2249	**7.7**	7.3-8.1
No	1850	**36.4**	34.5-38.4	1205	**31.6**	29.6-33.6	1015	**43.6**	40.8-46.4	344	**16.9**	15.0-19.0	1455	**24.9**	23.4-26.5	334	**7.1**	6.2-8.1
POSTERIOR SEAT BELT (ALWAYS USED)																		
Yes	4720	**50.9**	49.5-52.2	8627	**32.8**	31.4-34.1	7910	**62.1**	60.2-63.9	680	**17.8**	16.4-19.3	3585	**49.8**	48.3-51.3	438	**6.9**	6.2-7.7
No	14280	**43.5**	42.8-44.2	2410	**34.6**	33.9-35.3	2760	**52.0**	51.0-53.1	2442	**19.7**	18.8-20.6	8742	**33.6**	32.9-34.3	1610	**8.1**	7.6-8.6
HELMET (ALWAYS USED)																		
Yes	2814	**40.9**	39.3-42.5	2239	**41.4**	39.8-43.2	985	**54.3**	51.1-57.5	353	**23.5**	20.8-26.4	2434	**41.7**	40.2-43.2	430	**8.8**	7.9-9.9
No	69	**42.6**	31.0-55.1	27	**15.9**	10.1-24.2	31	**69.2**	49.9-83.5	4	**6.2**	1.9-18.9	38	**26.6**	17.6-37.9	13	**8.8**	4.9-15.4
CHRONIC CONDITIONS AND NUTRITIONAL STATUS																		
Underweight/normal weight	14240	**44.8**	44.1-45.5	8857	**36.0**	35.3-36.7	6769	**54.8**	53.7-55.9	2141	**20.6**	19.7-21.6	7420	**39.1**	38.3-40.0	1221	**7.4**	7.0-7.9
Overweight	4878	**45.6**	44.2-46.9	2537	**31.8**	30.4-33.2	3805	**51.9**	50.3-53.5	1013	**17.8**	16.5-19.3	6171	**36.2**	35.3-37.2	1011	**7.9**	7.3-8.6
Obese	2004	**44.5**	42.3-46.6	904	**26.2**	24.3-28.2	1826	**54.2**	51.7-56.7	394	**14.2**	12.7-15.9	2308	**34.5**	33.0-36.1	371	**7.0**	6.1-8.0
HYPERCHOLESTEROLEMIA																		
No	14252	**45.2**	44.5-45.9	8812	**36.1**	35.4-36.9	7099	**52.8**	51.6-53.9	2161	**19.4**	18.5-20.4	9235	**36.3**	35.6-37.1	1471	**7.3**	6.8-7.8
Yes	4139	**49.9**	48.4-51.4	2103	**32.4**	31.0-33.9	4553	**58.1**	56.6-59.6	1188	**18.7**	17.5-19.9	5885	**43.3**	42.2-44.4	1000	**9.0**	8.3-9.7
HYPERTENSION																		
No	16582	**44.6**	44.0-45.3	10292	**36.4**	35.7-37.0	7812	**54.1**	53.0-55.2	2444	**20.3**	19.4-21.2	9778	**38.1**	37.4-38.8	1553	**7.2**	6.8-7.7
Yes	3320	**49.6**	47.9-51.3	1456	**26.7**	25.3-28.3	4192	**53.8**	52.2-55.4	976	**16.2**	15.0-17.5	5718	**37.3**	36.3-38.4	982	**8.5**	7.8-9.2
CHRONIC DISEASES																		
None	17661	**44.2**	43.6-44.9	10628	**34.9**	34.3-35.6	8941	**53.6**	52.6-54.6	2753	**19.8**	19.0-20.6	10898	**36.4**	35.7-37.1	1816	**7.2**	6.8-7.6
Cancer (current or past)	1069	**52.8**	49.7-55.9	500	**30.0**	27.3-32.9	1205	**60.0**	57.1-62.9	273	**17.2**	15.0-19.6	1429	**49.9**	47.4-52.3	193	**8.9**	7.6-10.5
Diabetes	644	**48.1**	44.0-52.2	266	**22.0**	19.0-25.4	896	**52.3**	48.5-56.0	160	**11.9**	9.2-15.3	1433	**33.8**	31.8-35.9	201	**7.3**	5.8-9.1
DEPRESSIVE SYMPHTOMS																		
No	19182	**45.2**	44.6-45.8	11286	**34.5**	33.9-35.1	11016	**54.7**	53.8-55.6	3126	**18.6**	17.9-19.4	14548	**38.2**	37.6-38.7	2346	**7.6**	7.2-7.9
Yes	1395	**44.1**	41.6-46.7	775	**30.9**	28.6-33.3	1054	**50.0**	46.8-53.2	287	**18.5**	15.9-21.5	1075	**34.6**	32.3-37.0	192	**8.5**	6.8-10.5
SELF PERCEIVED HEALTH STATUS																		
Good/very good	14280	**44.1**	43.4-44.8	8765	**35.5**	34.8-36.2	6173	**54.6**	53.4-55.8	1968	**20.7**	19.7-21.7	8491	**37.8**	37.0-38.6	1334	**7.0**	6.6-7.5
Normal	6228	**46.7**	45.5-47.9	3271	**31.6**	30.4-32.7	5529	**53.3**	52.0-54.6	1431	**17.4**	16.4-18.5	6572	**36.8**	35.9-37.7	1125	**8.1**	7.5-8.7
Bad/Very bad	682	**45.8**	42.1-49.6	296	**25.2**	22.1-28.5	733	**50.6**	46.6-54.6	157	**13.8**	11.0-17.1	873	**35.6**	32.8-38.4	144	**8.7**	6.7-11.2

Table 3: Raw uptake probabilities (number of people reporting screening uptake, % and 95% CI) of cervical, breast and colorectal cancer screening in target population, spontaneous or within organised programs, according to the uptake of other preventive interventions, lifestyle and road safety-related behaviours and chronic conditions and diseases. *Influenza vaccination uptake was assessed using as denominator people aged 65-69 and people with at least one chronic disease according to Italian guidelines. Cervical cancer screening: Pap test within three years before the interview or HPV-DNA test within five years before the interview; breast cancer screening: mammogram within two years before the interview; colorectal cancer screening: faecal occult blood test within two years before the interview or colonoscopy/flex sigmoidoscopy within 5 years before the interview (residents in Piedmont were excluded due to the different colorectal cancer screening protocol). PASSI 2014-2016, Italy.

	Cervical Cancer Screening				Breast Cancer Screening				Colorectal Cancer Screening			
	Probabilities % Adj. by Age		Probabilities % Adj. by Age and SES [a]		Probabilities % Adj. by Age		Probabilities % Adj. by Age and SES [a]		Probabilities % Adj. by Age and Gender		Probabilities % Adj. by Age, Gender, SES [a]	
	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI
Cervical cancer screening uptake												
Yes	-	-	-	-	**83.6**	82.7-84.5	**83.1**	82.2-84.1	**48.5**	47.4-49.6	**47.6**	46.5-48.8
No	-	-	-	-	**41.1**	38.7-43.4	**42.1**	39.7-44.5	**21.1**	19.1-23.1	**22.4**	20.3-24.5
Breast cancer screening uptake												
Yes	**88.2**	87.4-89.0	**87.7**	86.9-88.5	-	-	-	-	**53.5**	52.5-54.4	**52.6**	51.6-53.6
No	**51.0**	48.7-53.2	**51.9**	49.6-54.1	-	-	-	-	**20.5**	19.0-22.0	**21.4**	19.8-22.9
Colorectal cancer screening uptake												
Yes	**89.5**	88.4-90.7	**88.2**	87.0-89.4	**87.9**	86.9-88.8	**86.5**	85.5-87.5	-	-	-	-
No	**69.9**	68.5-71.3	**70.7**	69.3-72.0	**61.1**	59.8-62.4	**61.9**	60.6-63.1	-	-	-	-
Influenza vaccination uptake [b]												
Yes	**79.4**	74.9-83.8	**79.4**	75.0-83.8	**71.2**	67.8-74.5	**71.3**	68.0-74.6	**49.6**	46.8-52.4	**50.1**	47.3-52.9
No	**79.2**	77.0-81.4	**79.2**	77.0-81.3	**72.8**	70.7-74.9	**72.8**	7.7-74.8	**50.3**	48.6-51.9	**50.0**	48.4-51.6
Smoking habit												
Non-smoker	**78.7**	78.0-79.4	**78.6**	77.9-79.2	**73.0**	72.0-74.0	**73.1**	72.1-74.1	**44.2**	43.3-45.0	**44.0**	43.2-44.9
Former smoker	**85.0**	83.8-86.3	**83.9**	82.7-85.1	**77.9**	76.0-79.8	**76.5**	74.7-78.3	**52.7**	51.4-54.1	**51.6**	50.4-52.9
Smoker	**77.7**	76.5-78.9	**78.7**	77.6-79.9	**68.3**	66.3-70.3	**69.0**	67.1-71.0	**39.1**	37.8-40.5	**40.5**	39.1-41.9
Daily fruit and vegetable intake												
≥5 portions	**83.4**	82.0-84.7	**82.1**	80.8-83.4	**78.2**	76.4-80.1	**76.6**	74.8-78.4	**54.2**	52.4-56.0	**52.2**	50.4-53.9
3-4 portions	**81.8**	81.1-82.5	**81.2**	80.4-81.9	**75.8**	74.7-76.9	**75.2**	74.1-76.4	**48.9**	47.9-49.8	**48.1**	47.2-49.1
1-2 portions	**76.8**	75.9-77.6	**77.5**	76.7-78.3	**67.7**	66.3-69.2	**68.9**	67.5-70.4	**39.9**	38.9-40.8	**40.8**	39.8-41.8
0 portions	**62.6**	57.3-67.8	**65.6**	60.2-70.9	**55.8**	46.9-64.7	**58.6**	49.3-67.8	**34.3**	29.0-39.6	**37.3**	31.6-43.0
Physical activity												
Active	**81.8**	81.1-82.5	**81.3**	80.6-82.0	**76.0**	74.9-77.1	**75.1**	74.0-76.3	**50.3**	49.4-51.2	**49.0**	48.2-49.9
Partially active	**82.5**	81.5-83.5	**81.8**	80.8-82.8	**77.5**	75.8-79.2	**76.7**	75.1-78.4	**51.0**	49.6-52.4	**50.0**	48.6-51.4
Sedentary	**73.0**	71.9-74.1	**74.2**	73.1-75.3	**66.5**	64.9-68.0	**67.9**	66.4-69.4	**35.4**	34.3-36.6	**37.1**	36.0-38.3

	Cervical Cancer Screening				Breast Cancer Screening				Colorectal Cancer Screening			
	Probabilities % Adj. by Age		Probabilities % Adj. by Age and SES [a]		Probabilities % Adj. by Age		Probabilities % Adj. by Age and SES [a]		Probabilities % Adj. by Age and Gender		Probabilities % Adj. by Age, Gender, SES [a]	
	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI
High-risk drinking behaviour												
No	**79.1**	78.5-79.7	**79.2**	78.7-79.8	**73.0**	72.2-73.8	**73.1**	72.3-74.0	**44.9**	44.2-45.5	**45.0**	44.3-45.6
Yes	**83.2**	81.7-84.8	**82.1**	80.6-83.6	**75.5**	72.6-78.4	**74.2**	71.4-76.9	**52.3**	50.4-54.2	**51.1**	49.3-52.9
Driving after drinking alcohol												
No	**79.3**	78.8-79.9	**79.4**	78.9-79.9	**72.9**	72.1-73.7	**72.9**	72.2-73.7	**45.2**	44.6-45.8	**45.3**	44.7-45.9
Yes	**84.7**	80.3-89.0	**82.4**	78.2-86.6	**76.1**	67.5-84.7	**72.8**	64.5-81.1	**50.8**	47.4-54.1	**48.6**	45.5-51.7
Front seat belt (always used)												
Yes	**81.3**	80.8-81.8	**80.9**	80.4-81.5	**75.2**	74.3-76.0	**74.8**	74.0-75.6	**48.1**	47.4-48.7	**47.6**	46.9-48.2
No	**68.9**	67.0-70.8	**71.0**	69.1-72.9	**61.1**	58.3-63.8	**63.2**	60.4-66.1	**32.5**	30.9-34.2	**34.5**	32.7-36.2
Back seat belt (always used)												
Yes	**83.6**	82.5-84.7	**82.6**	81.5-83.7	**80.3**	78.8-81.9	**79.0**	77.5-80.6	**57.3**	55.8-58.8	**54.9**	53.5-56.4
No	**78.7**	78.1-79.3	**78.9**	78.3-79.5	**72.1**	71.1-73.1	**72.4**	71.4-73.4	**42.3**	41.5-43.0	**42.7**	41.9-43.5
Helmet (always used)												
Yes	**82.7**	81.4-84.0	**82.6**	81.3-84.0	**78.0**	74.9-81.1	**78.0**	75.0-80.9	**51.0**	49.4-52.6	**50.9**	49.3-52.5
No	**61.8**	48.7-74.9	**64.3**	51.7-76.9	**77.4**	61.4-93.3	**77.3**	62.0-92.6	**36.7**	25.5-48.0	**39.2**	27.6-50.9
Nutritional status												
Underweight/normal weight	**81.4**	80.8-82.0	**80.4**	79.8-81.0	**75.6**	74.6-76.7	**74.3**	73.3-75.3	**48.1**	47.1-49.0	**46.8**	45.9-47.8
Overweight	**77.5**	76.3-78.7	**78.7**	77.5-79.9	**70.4**	68.8-71.9	**71.2**	69.6-72.7	**44.2**	43.2-45.2	**44.6**	43.6-45.7
Obese	**71.0**	68.9-73.1	**74.2**	72.2-76.3	**69.1**	66.7-71.5	**72.1**	69.7-74.5	**41.4**	39.8-43.1	**43.4**	41.7-45.2
Hypercholesterolemia												
No	**81.5**	80.9-82.2	**81.5**	80.9-82.1	**72.4**	71.3-73.4	**72.3**	71.3-73.4	**44.5**	43.7-45.3	**44.5**	43.7-45.3
Yes	**83.6**	82.4-84.8	**83.7**	82.5-84.9	**77.8**	76.5-79.1	**77.8**	76.5-79.2	**52.2**	51.1-53.3	**52.1**	51.0-53.2
Hypertension												
No	**81.3**	80.7-81.9	**80.9**	80.4-81.5	**74.5**	73.5-75.5	**73.7**	72.7-74.7	**46.7**	45.9-47.5	**46.2**	45.4-47.0
Yes	**77.4**	75.9-79.0	**79.2**	77.6-80.8	**71.2**	69.6-72.7	**72.6**	71.0-74.1	**45.2**	44.2-46.3	**46.1**	45.0-47.2

	Cervical Cancer Screening				Breast Cancer Screening				Colorectal Cancer Screening			
	Probabilities % Adj. by Age		Probabilities % Adj. by Age and SES [a]		Probabilities % Adj. by Age		Probabilities % Adj. by Age and SES [a]		Probabilities % Adj. by Age and Gender		Probabilities % Adj. by Age, Gender, SES [a]	
	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI	Overall uptake	95%CI
Chronic diseases												
None	**79.6**	79.0-80.2	**79.3**	78.8-79.9	**73.6**	72.7-74.5	**72.9**	72.0-73.8	**44.9**	44.2-45.6	**44.0**	43.3-44.7
Cancer (current or past)	**83.1**	80.8-85.5	**83.3**	80.9-85.6	**78.1**	75.5-80.6	**78.5**	75.9-81.0	**57.9**	55.5-60.3	**58.3**	55.9-60.7
Diabetes	**72.0**	68.1-76.0	**75.8**	71.9-79.7	**65.8**	62.1-69.4	**69.3**	65.6-72.9	**39.7**	37.6-41.9	**42.7**	40.4-45.0
Depressive symptoms												
No	**80.2**	79.7-80.7	**80.0**	79.4-80.5	**73.7**	72.9-74.6	**73.4**	72.5-74.2	**46.4**	45.7-47.0	**46.0**	45.3-46.6
Yes	**75.5**	73.0-77.9	**78.3**	76.0-80.6	**69.2**	66.0-72.4	**72.7**	69.4-75.9	**43.6**	41.0-46.2	**48.3**	45.5-51.1
Self-perceived health status												
Good/very good	**80.3**	79.7-80.9	**79.5**	78.9-80.2	**75.3**	74.3-76.4	**73.8**	72.7-74.9	**46.0**	45.2-46.9	**44.2**	43.4-45.0
Normal	**78.2**	77.2-79.3	**79.5**	78.5-80.5	**71.4**	70.1-72.7	**72.3**	71.1-73.5	**45.0**	44.0-46.0	**46.4**	45.4-47.4
Bad/Very bad	**72.0**	68.7-75.3	**76.2**	72.8-79.6	**65.6**	61.7-69.4	**70.7**	66.7-74.7	**43.7**	40.8-46.6	**50.6**	47.3-53.9

[a] SES=educational attainment and economic difficulties
[b] Target population: People aged 50-69 or with at least one chronic disease.

Table 4. Overall screening uptake probabilities (reported as % with relative 95%CI) by the uptake of other preventive interventions (other screening and vaccination), lifestyle and road safety-related behaviours and chronic conditions. Left columns report probabilities calculated adjusting only for age (and gender for colorectal cancer screening); right columns report probabilities calculated adjusting for age (and gender for colorectal cancer screening) and SES (educational attainment and economic difficulties). PASSI 2014-2016, Italy

DISCUSSION

Our study shows that, with few exceptions, people with unhealthy behaviours or with poorer health conditions have lower test uptake for all the recommended screenings. In general, these differences are all smaller for participation in organized screening. These associations are not fully explained by underlying socioeconomic conditions since adjusting for educational attainment and economic difficulties only slightly reduced the observed differences in screening test uptake. In fact, differences in coverage are small, with higher participation among Italians, the highly educated, those with no economic difficulties, the employed and those living with partner, in accordance with most previous studies. [20, 36-38]Screening programmes are mitigating some of these inequalities, with higher participation by the most deprived groups, but not for economic difficulties, occupational status and living conditions.

Even though screening coverage in persons with low fruit and vegetable consumption or low physical activity is 10% to 20% lower, other associations are smaller: almost null for smoking and even positive for former smoking. This suggests that self-selection bias may go in the direction of overestimating the benefits of screening in observational studies evaluating the effect of test coverage both in organized screening programs and opportunistic screening. On the contrary, the net effect of self-selection bias is probably small or even in the direction of underestimating the screening benefit for studies considering only the impact of participation in organized screening. [39-41] This is consistent with the small differences observed in mortality for causes other than breast cancer between participants and non-participants in countries with population-based breast cancer screening programmes. [42]

We found a strong association between participation in the three screenings, which is clearly visible both in organized screening participation and in spontaneous uptake. This association cannot be explained by a contamination of the interventions because the intervals and the target populations are different for cervical cancer screening and, in Italy, there are no programmes that invite the population for colorectal and breast cancer screening on the same day. Similar findings were reported by previous studies, particularly for breast and cervical cancer. [17, 18, 43, 44] On the contrary, we found no association between screening uptake and influenza vaccination. Two previous studies showed a positive association with colorectal cancer screening uptake. [45, 46]

In our study, non-smokers had only a slightly higher uptake than did smokers, and we found a much higher uptake in former smokers, according to previous findings. [17, 19, 20, 44, 47]

Healthy diet and physical activity, two protective factors for breast and colorectal cancer incidence, were strongly associated with test uptake; the negative effects of unhealthy behaviours and non-participation in screening were therefore compounded. [48, 49] These results are consistent with previous studies on colorectal cancer screening uptake [45, 47], although no association with fruit and vegetable intake was suggested by a large recent study. [17] Furthermore, although screening test

uptake, unhealthy diet and reduced physical activity were each associated with socioeconomic deprivation, differences are still appreciable when we adjusted for educational attainment and economic difficulties. This therefore means not only that healthy behaviours are mediators of socioeconomic conditions, but that other common psychosocial characteristics act independently of the cultural and economic level.

A surprising finding was the positive associations between high-risk drinking behaviour and driving after drinking alcohol and test uptake. Although not widely explored in the literature [18], a slightly positive association between alcohol consumption and colorectal cancer screening uptake was already reported. [17] It is difficult to explain the positive association between screening uptake and driving after drinking alcohol because the association with all other road safety behaviours was inverse, consistent with a previous study. [47] It is noteworthy that adjusting for educational attainment and perceived economic difficulties, the differences in uptake for driving after drinking alcohol almost disappeared, especially for breast cancer screening, yet the associations with other road safety behaviours remained.

While test uptake was similar for people with or without hypertension, those with hypercholesterolemia had higher test uptake. This finding could be due to an association between cholesterol screening and cancer screening mediated by general practitioners' recommendations. The PASSI questionnaire does not distinguish between those with low cholesterol and those who have never been tested. Few previous studies explored these associations with inconsistent results. [20] Our findings on mental health are consistent with those of the few previous studies on this topic, which found an underuse of medical services among people suffering from depression [50], and in particular, a lower probability of being screened for women with a high depressive symptom burden. [51]

Overweight and obesity are negatively associated with test uptake, according to previous studies on breast and cervical cancer screening. [52] Some authors suggest that obesity may cause embarrassment, and thus may be a barrier to presenting to screening. [53] We found that the effect was smaller for organised screening, where active invitation is implemented, but probably privacy and embarrassment are no less an issue than in private clinics. We also observed this trend in colorectal cancer screening, which in Italy is based on FOBT, with the stool self-sampled at home. These counterintuitive results are consistent with some previous studies [17, 54, 55], while other studies found no or positive association between overweight and colorectal cancer screening uptake. [20] In summary, our findings do not support any role of embarrassment to justify lower participation of obese people.

People with a previous diagnosis of cancer had an only slightly higher test uptake rate than the average for breast and cervical cancer, while the effect was definitely stronger for colorectal cancer. Previous studies have found clear associations between having a history of cancer and breast [18] and colorectal cancer screening uptake. [17, 34, 35] Indeed, FOBT and endoscopic test uptake is much

lower than it is for mammography and Pap test; thus, there is much more room for improvement when an awareness-increasing factor is present. Furthermore, endoscopy screening is widely recommended in first-degree relatives of colorectal cancer patients. [56]

In general, all the other chronic diseases were negatively associated with screening uptake. This could be the consequence of an informed choice based on the fact that the balance of benefits and harms of screening goes in the direction of more harm and less benefit if life expectancy is shorter, but it could also be the consequence of lower awareness of the importance of screening. Unexpectedly, the lowest uptake rate was for those with diabetes, which does not have such a strong effect on life expectancy and because of which patients have repeated contact with health services. This suggests there are many missed opportunities to advise these patients on the importance of screening. [57] Finally, we observed lower uptake rates in those reporting a bad perceived health status, similar to previous studies. [17, 19] The difference was minimal for the organised screening component, at least for breast and cervical cancer screening. Differences for diabetes and self-perceived health almost disappeared when adjusting for educational attainment and economic concerns.

Implications for policy

Screening programmes offer a unique opportunity for contact between healthy individuals and health operators. Some researchers and public health decision makers have thus started exploring how to nest health promotion interventions within screening programmes, i.e. during history taking for cervical sample or mammography or when the kit for occult fecal test is delivered. [22, 23, 24, 58] As a general consideration, participants in organized screening programmes are almost evenly distributed in all the educational levels. Also, foreigners, often the most deprived part of the population in this historical period in Italy, participate only slightly less than do Italians. This implies that screening programmes may be an equitable vehicle for health promotion.

Brief advice interventions for smoking cessation [59] and screening for high-risk alcohol consumption followed by advice on possible motivational counselling or group programs [59] will probably reach the right target; interventions promoting healthy diet and physical activity, instead, are likely to miss a significant part of the at-risk population and might also increase inequalities because the most fragile part of the target population, i.e. those who are already overweight and obese, does not attend screening. Furthermore, organized programmes for cervical and colorectal cancer have low participation rates in Italy, particularly in southern regions that are the most deprived geographical areas. [26] Thus, the opportunity to contact young women and men is limited particularly in southern Italy.

Finally, given that on average people with diabetes have more contacts with their general practitioner and with the diabetes care clinics for drug prescription and checkup for possible complications, often within outreaching programs, it is surprising that they have lower test coverage for all the screening.

Our data suggest that check-ups for chronic conditions are not exploited to advise patients on the importance of screening, particularly for colorectal cancer.

Study limitations and strengths

The main limit of this study is that most of the observed associations are context- and time-specific. Thus, our conclusion about the impact on self-selection bias in observational studies cannot be generalised to other countries and other historical periods. Nevertheless, as some patterns are quite consistent among international studies, the implications of the selection of populations attending screening on possible health promotion interventions should be considered in all countries and specific assessment should be conducted locally.

The PASSI Surveillance System is based on self-reported data. Answers, therefore, can be affected by desirability bias and recall bias (particularly telescoping bias) and may be influenced by educational attainment and health conditions. Nevertheless, the entity of recall and telescoping bias was quantified in a similar Italian survey conducted on Pap test uptake just before the start of PASSI, finding minimal impact on test coverage estimates when present. [60]

The survey has, by definition, a cross-sectional design, which has associated limitations: we measure the exposure to risk factors and health conditions at the same time in which we measure the screening test uptake. In some cases, exposure (i.e. the risk factor or health condition onset) could occur after deciding whether to participate in screening. Actually, most of the putative determinants we considered are chronic conditions or behaviours that are very unlikely to change in as short a time span as that of screening testing (i.e. generally 2 or 3 years).
As the survey collects retrospective information on the timing and payment of the last exam, it can underestimate the use of the test within screening programmes due to the phenomenon of over-use, especially in cervical screening. Thus, screening participation in organised programmes is more accurately photographed by institutional screening information flows. [61]

CONCLUSIONS

Screening test uptake is negatively associated with some unfavourable behaviours and health conditions, in particular with obesity, unhealthy diet and low physical activity, which are also risk factors for breast and colorectal cancer incidence, thus exacerbating the existing risks for related morbidity. Socioeconomic characteristics do not fully explain these differences.

Bibliography

1. GBD 2015 Risk Factors Collaborators. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015 [published correction appears in Lancet. 2017 Jan 7;389(10064):e1]. Lancet. 2016;388(10053):1659-1724. doi:10.1016/S0140-6736(16)31679-8
2. Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Allen C, et al. Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study [published correction appears in JAMA Oncol. 2017 Mar 1;3(3):418]. JAMA Oncol. 2017;3(4):524-548. doi:10.1001/jamaoncol.2016.5688
3. O'Leary D, Suri G, Gross JJ. Reducing behavioural risk factors for cancer: An affect regulation perspective. Psychol Health. 2018;33(1):17-39. doi:10.1080/08870446.2017.1314480
4. Cortis C, Puggina A, Pesce C, et al. Psychological determinants of physical activity across the life course: A "DEterminants of DIet and Physical ACtivity" (DEDIPAC) umbrella systematic literature review. PLoS One. 2017;12(8):e0182709. Published 2017 Aug 17. doi:10.1371/journal.pone.0182709
5. Noll M, de Mendonça CR, de Souza Rosa LP, Silveira EA. Determinants of eating patterns and nutrient intake among adolescent athletes: a systematic review. Nutr J. 2017;16(1):46. Published 2017 Jul 28. doi:10.1186/s12937-017-0267-0
6. Macdonald G, Veen C, Tones K. Evidence for success in health promotion: suggestions for improvement. Health Educ Res. 1996;11(3):367-376. doi:10.1093/her/11.3.367
7. Ministero della Salute, Direzione Generale della Prevenzione, 2005. Raccomandazioni per la pianificazione e l'esecuzione degli screening di popolazione per la prevenzione del cancro della mammella, del cancro della cervice uterina e del cancro del colon retto. http://www.ccm-network.it/documenti_Ccm/pubblicazioni/Screening_oncologici.pdf (Accessed on December 15, 2017).
8. Perry N, Broeders M, de Wolf C, Törnberg S, Holland R, von Karsa L. European guidelines for quality assurance in breast cancer screening and diagnosis. Fourth edition--summary document. Ann Oncol. 2008;19(4):614-622. doi:10.1093/annonc/mdm481
9. von Karsa, L., Arbyn, A., De Vuyst, H., Dillner, J., Dillner, L., Franceschi, S., Patnick, J., Ronco, G., Segnan, N., Suonio, E., Törnberg, S., Anttila, A., 2015. European guidelines for quality assurance in cervical cancer screening. Second edition. Supplements. Luxembourg. doi:10.2875/859507
10. European Colorectal Cancer Screening Guidelines Working Group, von Karsa L, Patnick J, et al. European guidelines for quality assurance in colorectal cancer screening and diagnosis: overview and introduction to the full supplement publication. Endoscopy. 2013;45(1):51-59. doi:10.1055/s-0032-1325997
11. Mansfield C, Tangka FK, Ekwueme DU, et al. Stated Preference for Cancer Screening: A Systematic Review of the Literature, 1990-2013. Prev Chronic Dis. 2016;13:E27. Published 2016 Feb 25. doi:10.5888/pcd13.150433
12. Buron A, Auge JM, Sala M, et al. Association between socioeconomic deprivation and colorectal cancer screening outcomes: Low uptake rates among the most and least deprived people. PLoS One. 2017;12(6):e0179864. Published 2017 Jun 16. doi:10.1371/journal.pone.0179864
13. Douglas E, Waller J, Duffy SW, Wardle J. Socioeconomic inequalities in breast and cervical screening coverage in England: are we closing the gap?. J Med Screen. 2016;23(2):98-103. doi:10.1177/0969141315600192
14. Grosso G, Micek A, Godos J, et al. Health risk factors associated with meat, fruit and vegetable consumption in cohort studies: A comprehensive meta-analysis. PLoS One. 2017;12(8):e0183787. Published 2017 Aug 29. doi:10.1371/journal.pone.0183787
15. Pornet C, Dejardin O, Morlais F, Bouvier V, Launoy G. Socioeconomic determinants for compliance to colorectal cancer screening. A multilevel analysis. J Epidemiol Community Health. 2010;64(4):318-324. doi:10.1136/jech.2008.081117
16. Spencer AM, Roberts SA, Brabin L, Patnick J, Verma A. Sociodemographic factors predicting mother's cervical screening and daughter's HPV vaccination uptake. J Epidemiol Community Health. 2014;68(6):571-577. doi:10.1136/jech-2013-202629
17. He E, Lew JB, Egger S, et al. Factors associated with participation in colorectal cancer screening in Australia: Results from the 45 and Up Study cohort. Prev Med. 2018;106:185-193. doi:10.1016/j.ypmed.2017.10.032

18. Jepson R, Clegg A, Forbes C, Lewis R, Sowden A, Kleijnen J. The determinants of screening uptake and interventions for increasing uptake: a systematic review. Health Technol Assess. 2000;4(14):i-133.
19. Labeit A, Peinemann F, Baker R. Utilisation of preventative health check-ups in the UK: findings from individual-level repeated cross-sectional data from 1992 to 2008. BMJ Open. 2013;3(12):e003387. Published 2013 Dec 23. doi:10.1136/bmjopen-2013-003387
20. Wools A, Dapper EA, de Leeuw JR. Colorectal cancer screening participation: a systematic review. Eur J Public Health. 2016;26(1):158-168. doi:10.1093/eurpub/ckv148
21. Zappa M, Castiglione G, Giorgi D, Grazzini G, Paci E, Ciatto S. Re: Participation in colorectal cancer screening: a review. J Natl Cancer Inst. 1998;90(6):465. doi:10.1093/jnci/90.6.465
22. Anderson AS, Mackison D, Boath C, Steele R. Promoting changes in diet and physical activity in breast and colorectal cancer screening settings: an unexplored opportunity for endorsing healthy behaviors. Cancer Prev Res (Phila). 2013;6(3):165-172. doi:10.1158/1940-6207.CAPR-12-0385
23. Gorini G, Carreras G, Giordano L, et al. The Pap smear screening as an occasion for smoking cessation and physical activity counselling: effectiveness of the SPRINT randomized controlled trial. BMC Public Health. 2012;12:740. Published 2012 Sep 5. doi:10.1186/1471-2458-12-740
24. National Screening Unit, 2004. National Health Promotion Framework and Implementation Planning Guide for Screening Programmes. New Zealand. https://www.nsu.govt.nz/system/files/resources/hp-framework.pdf (Accessed on October 30, 2018)
25. The Council of the European Union, 2003. Council reccomendation of 2 December 2003 on cancer screening (2003/878/EC). Off. J. Eur. Union L 327/34, 5. https://ec.europa.eu/jrc/sites/jrcsh/files/2_December_2003 cancer screening.pdf (Accessed on October 30, 2018)
26. ONS, 2017. Osservatorio nazionale screening - Rapporto 2017. https://www.osservatorionazionalescreening.it/sites/default/files/allegati/ons rapporto 2017.pdf (Accessed on October 30, 2018)
27. Carrozzi G, Sampaolo L, Bolognesi L, et al. Cancer screening uptake: association with individual characteristics, geographic distribution, and time trends in Italy. Epidemiol Prev. 2015;39(3 Suppl 1):9-18.
28. Ronco G, Biggeri A, Confortini M, et al. Health technology assessment report: ricerca del DNA di papillomavirus umano (HPV) come test primario per lo screening dei precursori del cancro del collo dell'utero [Health technology assessment report: HPV DNA based primary screening for cervical cancer precursors]. Epidemiol Prev. 2012;36(3-4 Suppl 1):e1-e72.
29. Distante V, Ciatto S, Frigerio A, et al. Raccomandazioni di una conferenza di consenso italiana sull'opportunità di estendere lo screening mammografico organizzato alle donne di 40-49 e 70-74 anni di età [Recommendations of a national Italian consensus conference on the opportunity of extending screening service by mammography to 40-49 and 70-74 years of age women]. Epidemiol Prev. 2007;31(1):15-22.
30. Baldissera S, Campostrini S, Binkin N, et al. Features and initial assessment of the Italian Behavioral Risk Factor Surveillance System (PASSI), 2007-2008. Prev Chronic Dis. 2011;8(1):A24.
31. Baldissera S, Ferrante G, Quarchioni E, et al. Field substitution of nonresponders can maintain sample size and structure without altering survey estimates-the experience of the Italian behavioral risk factors surveillance system (PASSI). Ann Epidemiol. 2014;24(4):241-245. doi:10.1016/j.annepidem.2013.12.003
32. UNDP, 2007. Human Development Report 2007/2008. United Nations Development Programme. New York. http://hdr.undp.org/sites/default/files/reports/268/hdr_20072008_en_complete.pdf (Accessed on December 15, 2017).
33. European Centre for Disease Prevention and Control (ECDC), 2017. ECDC Technical Report: Seasonal influenza vaccination in Europe. doi:10.2900/153616
34. Giorgi Rossi P, Federici A, Bartolozzi F, Farchi S, Borgia P, Guasticchi G. Understanding non-compliance to colorectal cancer screening: a case control study, nested in a randomised trial [ISRCTN83029072]. BMC Public Health. 2005;5:139. Published 2005 Dec 22. doi:10.1186/1471-2458-5-139
35. So WK, Choi KC, Chan DN, et al. Colorectal cancer screening behaviour and associated factors among Chinese aged 50 and above in Hong Kong - a population-based survey. Eur J Oncol Nurs. 2012;16(4):413-418. doi:10.1016/j.ejon.2011.09.006

36. Broberg G, Wang J, Östberg AL, et al. Socio-economic and demographic determinants affecting participation in the Swedish cervical screening program: A population-based case-control study. PLoS One. 2018;13(1):e0190171. Published 2018 Jan 10. doi:10.1371/journal.pone.0190171
37. Deding U, Henig AS, Salling A, Torp-Pedersen C, Bøggild H. Sociodemographic predictors of participation in colorectal cancer screening. Int J Colorectal Dis. 2017;32(8):1117-1124. doi:10.1007/s00384-017-2832-6
38. Hanske J, Meyer CP, Sammon JD, et al. The influence of marital status on the use of breast, cervical, and colorectal cancer screening. Prev Med. 2016;89:140-145. doi:10.1016/j.ypmed.2016.05.017
39. Puliti D, Bucchi L, Mancini S, et al. Advanced breast cancer rates in the epoch of service screening: The 400,000 women cohort study from Italy [published correction appears in Eur J Cancer. 2017 Nov;85:160]. Eur J Cancer. 2017;75:109-116. doi:10.1016/j.ejca.2016.12.030
40. Puliti D, Miccinesi G, Collina N, et al. Effectiveness of service screening: a case-control study to assess breast cancer mortality reduction [published correction appears in Br J Cancer. 2008 Nov 18;99(10):1756]. Br J Cancer. 2008;99(3):423-427. doi:10.1038/sj.bjc.6604532
41. Ventura L, Mantellini P, Grazzini G, et al. The impact of immunochemical faecal occult blood testing on colorectal cancer incidence. Dig Liver Dis. 2014;46(1):82-86. doi:10.1016/j.dld.2013.07.017
42. Broeders M, Moss S, Nyström L, et al. The impact of mammographic screening on breast cancer mortality in Europe: a review of observational studies. J Med Screen. 2012;19 Suppl 1:14-25. doi:10.1258/jms.2012.012078
43. Bankhead CR, Brett J, Bukach C, et al. The impact of screening on future health-promoting behaviours and health beliefs: a systematic review. Health Technol Assess. 2003;7(42):1-92. doi:10.3310/hta7420
44. Labeit, A., Peinemann, F., 2015. Breast and cervical cancer screening in Great Britain: Dynamic interrelated processes. Health Econ. Rev. 5, 32. doi:10.1186/s13561-015-0065-3
45. Sewitch MJ, Fournier C, Ciampi A, Dyachenko A. Adherence to colorectal cancer screening guidelines in Canada. BMC Gastroenterol. 2007;7:39. Published 2007 Oct 2. doi:10.1186/1471-230X-7-39
46. Zimmerman RK, Nowalk MP, Tabbarah M, Grufferman S. Predictors of colorectal cancer screening in diverse primary care practices. BMC Health Serv Res. 2006;6:116. Published 2006 Sep 13. doi:10.1186/1472-6963-6-116
47. Shapiro JA, Seeff LC, Nadel MR. Colorectal cancer-screening tests and associated health behaviors. Am J Prev Med. 2001;21(2):132-137. doi:10.1016/s0749-3797(01)00329-4
48. Bradbury KE, Appleby PN, Key TJ. Fruit, vegetable, and fiber intake in relation to cancer risk: findings from the European Prospective Investigation into Cancer and Nutrition (EPIC). Am J Clin Nutr. 2014;100 Suppl 1:394S-8S. doi:10.3945/ajcn.113.071357
49. Namasivayam V, Lim S. Recent advances in the link between physical activity, sedentary behavior, physical fitness, and colorectal cancer. F1000Res. 2017;6:199. Published 2017 Mar 1. doi:10.12688/f1000research.9795.1
50. Zhang C, Brook JS, Leukefeld CG, Brook DW. Psychosocial Factors Related to Underuse of Medical Services. J Urban Health. 2016;93(4):652-665. doi:10.1007/s11524-016-0067-4
51. Pirraglia PA, Sanyal P, Singer DE, Ferris TG. Depressive symptom burden as a barrier to screening for breast and cervical cancers. J Womens Health (Larchmt). 2004;13(6):731-738. doi:10.1089/jwh.2004.13.731
52. Aldrich T, Hackley B. The impact of obesity on gynecologic cancer screening: an integrative literature review. J Midwifery Womens Health. 2010;55(4):344-356. doi:10.1016/j.jmwh.2009.10.001
53. William Flint, S., Lozano-Sufrategui, L., Trainer, S., Lee, J.A., Pausé CPause, C.J., Pausé, C.J., 2016. Stigma in Practice: Barriers to Health for Fat Women. Front. Psychol 7. doi:10.3389/fpsyg.2016.02063
54. Ferrante JM, Ohman-Strickland P, Hudson SV, Hahn KA, Scott JG, Crabtree BF. Colorectal cancer screening among obese versus non-obese patients in primary care practices. Cancer Detect Prev. 2006;30(5):459-465. doi:10.1016/j.cdp.2006.09.003
55. Rosen AB, Schneider EC. Colorectal cancer screening disparities related to obesity and gender. J Gen Intern Med. 2004;19(4):332-338. doi:10.1111/j.1525-1497.2004.30339.x
56. Ministero della Salute, Osservatorio Nazionale Screening, 2006. Raccomandazioni per la pianificazione e l'esecuzione degli screening di popolazione per la prevenzione del cancro della mammella, del cancro della cervice uterina e del cancro del colon retto. Roma. https://www.osservatorionazionalescreening.it/sites/default/files/allegati/screening_vers_completa.pdf (Accessed on December 15, 2017)

57. Rawshani A, Rawshani A, Franzén S, et al. Mortality and Cardiovascular Disease in Type 1 and Type 2 Diabetes. N Engl J Med. 2017;376(15):1407-1418. doi:10.1056/NEJMoa1608664
58. Ministero della Salute, 2014. Piano Nazionale della Prevenzione 2014-2018. http://www.salute.gov.it/imgs/C_17_pubblicazioni_2285_allegato.pdf (Accessed on October 30, 2018)
59. Prochaska, J., Norcross, J., Di Clemente, C.C., 1994. Changing for good: the revolutionary program that explains the six stages of change and teaches you how to free yourself from bad habits., 1st ed. New York.
60. Giorgi Rossi P, Esposito G, Brezzi S, Brachini A, Raggi P, Federici A. Estimation of Pap-test coverage in an area with an organised screening program: challenges for survey methods. BMC Health Serv Res. 2006;6:36. Published 2006 Mar 17. doi:10.1186/1472-6963-6-36
61. Zappa, M., 2017. Osservatorio Nazionale Screening - Rapporto 2016, Osservatorio Nazionale Screening. https://www.osservatorionazionalescreening.it/content/il-2014-2015-dello-screening-organizzato (Accessed on December 15, 2017).

2.4. PhD Candidate contribution to research articles in Chapter 2

Research article #01 Conceptual framework for interpretation of monitoring indicators of cancer screenings from the Italian National Prevention Plan.

The PhD Candidate contributed to the presentation of the conceptual framework for cancer screening monitoring, drafted all the versions of the paper, discussed revisions with the supervisor, and was responsible for correspondence.

Research article #02 Associations between cervical, breast and colorectal cancer screening uptake, chronic diseases and health-related behaviours: data from the Italian PASSI nationwide surveillance.

The PhD candidate conceptualized the study together with supervisor, defined the methodology, discussed results presentation, drafted all the paper versions, discussed revisions with the supervisor and was responsible for correspondence.

3. Chapter 3: Evidence generation on biomarkers in HPV based cervical cancer screening

3.1. Rationale

HPV-based cervical cancer screening needs a triage of HPV positive women to reduce colposcopy referral, overdiagnosis and overtreatment. Cytology triage is the only recommended by European guidelines. US guidelines also recommend managing HPV-positive women differently accordingly to HPV type, i.e. referring them directly to colposcopy if HPV16 or 18 positive and to surveillance if positive to other hrHPV. Other candidate biomarkers for HPV triaging are currently available on the market, but no guidelines recommend their use.

The third chapter provides evidence on how to evaluate a triage test and on the performance of two biomarkers in organised cervical cancer screening programs.

3.2. Research article #03: p16/ki67 and E6/E7 mRNA Accuracy and Prognostic Value in Triaging HPV DNA-Positive Women

The findings of this research article were published in *Journal of the National Cancer Institute*.

Giorgi Rossi P, Carozzi F, Ronco G, Allia E, Bisanzi S, Gillio-Tos A, Marco L, Rizzolo R, Gustinucci D, Del Mistro A, Frayle H, Confortini M, Iossa A, Cesarini E, Bulletti S, Passamonti B, Gori S, Toniolo L, Barca A, Bonvicini L, Mancuso P, Venturelli F, Benevolo M; the New Technology for Cervical Cancer 2 Working Group. p16/ki67 and E6/E7 mRNA Accuracy and Prognostic Value in Triaging HPV DNA-Positive Women. J Natl Cancer Inst. 2021 Mar 1;113(3):292-300. doi: 10.1093/jnci/djaa105. PMID: 32745170.

Abstract

Background. The study presents cross-sectional accuracy of E6/E7 mRNA detection and p16/ki67 dual staining, alone or in combination with cytology and HPV16/18 genotyping, as triage test in HPV DNA-positive women and their impact on CIN2+ overdiagnosis.

Methods. Women aged 25-64 were recruited. HPV DNA-positives were triaged with cytology and tested for E6/E7 mRNA and p16/ki67. Cytology positives were referred to colposcopy, while negatives were randomised to immediate colposcopy or to one-year HPV retesting. Lesions found within 24 months since recruitment were included.

Results: 40,509 women were recruited and 3147 (7.8%) tested HPV DNA-positive; 174 CIN2+ were found: sensitivity was 61.0% (95% CI 53.6-68.0), 94.4% (95% CI 89.1, 97.3), and 75.2% (95% CI 68.1, 81.6) for cytology, E6/E7 mRNA, and p16/ki67, respectively. Immediate referral was 25.6%, 66.8%, and 28.3%, respectively. Overall referral was 65.3%, 78.3%, and 63.3%. Cytology or p16/ki67 when combined with HPV16/18 typing reached higher sensitivity with a small impact on referral.

Among the 2306 HPV DNA-positive/cytology-negative women, relative CIN2+ detection in those randomized at 1-year retesting vs. immediate colposcopy suggests a -28% CIN2+ regression (95% CI -57%, +20%); regression was higher in E6/E7 mRNA-negatives (p=0.29). HPV clearance at 1 year in E6/E7 mRNA and in p16/ki67 negatives was about 2 times higher than in positive women ($P < 0.001$ for both).

Conclusions: p16/ki67 showed good performance as triage test. E6/E7 mRNA showed the highest sensitivity, at the price of too high positivity rate to be efficient for triage but, when negative, it showed a good prognostic value for clearance and CIN2+ regression.

Introduction.

HPV DNA-based screening has been shown to be more effective than cytology-based screening [1] and its implementation is fundamental for cervical cancer elimination. [2] It is replacing cytology-based screening in most industrialized and is now also the recommended strategy for low-income countries. [3]

Positivity to HPV DNA is too high and prevalence of lesions in HPV DNA-positive women too low to refer all HPV DNA-positive women to colposcopy. [4] It is therefore necessary to apply a triage test to identify those women who need immediate colposcopy and those who should undergo surveillance. To date, cytology, alone or in combination with partial genotyping, is the only triage strategy recommended by European [4] and US guidelines. [5, 6] Several biomarkers have been proposed as triage tests, [7] including those targeting molecular changes due to overexpression of the viral oncogenes E6 and E7, which are technologically well developed and mature for implementation, if proven to have better performance. [8-17]

HPV DNA-based screening can also potentially increase false positive histology results [18] and especially unnecessary treatments of regressive lesions. High-grade cervical intraepithelial neoplasia (CIN), particularly CIN2, are highly regressive and their treatment is associated with negative pregnancy outcomes. [19] Therefore, the ability of biomarkers to predict the persistence of lesions is important. Very little evidence is, however, available about the association between biomarkers' positivity and the probability of CIN2+ regression or virus clearance.

The clinical performance of a triage test depends on its accuracy but also on the algorithm of which it is part, e.g. the sequence of tests, the interval for surveillance, and the test to be used in surveillance. In some of the most widespread algorithms, including that adopted by Italian screening programs (supplementary figure S1), HPV DNA-positive/cytology-negative women are recalled after 12 months to be retested for HPV DNA; [4,6,20,21] and return to routine screening if negative whereas, if positive, they are referred to colposcopy. With such algorithms the association between being negative to the triage test and clearance of HPV DNA is a strong determinant of overall colposcopy referral. [22] However, direct information on how biomarkers predict clearance is lacking.

In the NTCC2 (New Technologies for Cervical Cancer screening 2) study, all the HPV DNA-positive women were tested for cytology and putative biomarkers (E6/E7 mRNA and p16/ki67). Furthermore, HPV DNA-positive/cytology-negative women were randomly assigned to immediate colposcopy or to one-year HPV DNA retesting. This design permits a comparison of the performance of these biomarkers, alone or in combination with cytology and partial genotyping, as triage test and of their ability to predict CIN2+ regression and virus clearance.

Methods

Setting and recruitment

Women were recruited from five Italian HPV DNA-based organised cervical cancer screening programmes using active call/recall (Table 1). Colposcopies and follow-up visits were managed with scheduled appointments at the screening programme facilities.

Centre	Age range	Recruited women	HPV DNA assay	Date start	Date stop	HPV DNA positive	% HPV DNA positive
Umbria	35-64	15145	Cobas	04/2013	07/2016	898	5.9
Veneto	25-59	7478	Cobas	05/2015	07/2016	447	6.0
	25-59	2596	HC2	09/2014	05/2015	198	7.6
Florence	34-59	1049	Cobas	06/2016	10/2016	101	9.6
	34-59	7136	HC2	06/2015	06/2016	710	9.9
Turin	30-59	7105	HC2	02/2016	01/2017	793	11.2
Total included in the present analyses	25-64	40509		04/2013	01/2017	3147	7.8
Total Cobas	25-64	23672		04/2013	10/2016	1446	6.1
of which HPV16 and/or 18						391	1.7
Total HC2	25-59	16837		09/2014	01/2017	1701	10.1
Trento*	35-60	618	HC2	07/2016	10/2016	33	5.3
Total recruited	25-64	41127		04/2013	01/2017	3180	7.7
Total Cobas	25-64	23672		04/2013	10/2016	1446	6.1
Total HC2	25-60	17455		09/2014	01/2017	1734	9.9

Table 1. Accrual timing, number of women, type of HPV DNA test, and HPV positivity rate, by NTCC2 study recruiting centre

Study design

The NTCC2 trial aimed at evaluating different biomarkers (E6/E7 mRNA (Aptima, Hologic); p16/ki67 expression (Roche)) as putative triage tests for HPV DNA-positive women. Specific objectives were:

1) to compare the cross-sectional accuracy of cytology, HPV E6/E7 mRNA, and p16/ki67 as triage tests;

2) to measure the value of these tests to predict CIN2 regression and HPV clearance in one year;

3) to measure the 5-year CIN2+ and CIN3+ cumulative detection in HPV DNA-positive/ triage-negative women.

To assess point 2, HPV DNA-positive/cytology-negative women were randomized to immediate colposcopy or one-year HPV DNA retesting. Here we report on points 1 and 2. Data include all the

CIN2+ found up to 24 months from recruitment, so that the assessment of HPV DNA-positive women is complete and all the information necessary for the purpose is presented. Analyses include women recruited in four of the five centres (Table 1).

Women aged 25-59 years, resident in programme areas and undergoing a new screening episode were eligible for the study (Figure 1). In Umbria, women aged 60-64 years were also recruited and excluded from long-term follow-up but not from the present analyses. Exclusion criteria were pregnancy and treatment for a CIN2+ lesion in the previous 5 years. All women meeting the inclusion criteria were asked to provide written informed consent to participate in the trial. Refusers were screened by HPV DNA according to routine practice. In these analyses original histological diagnoses are considered. In all centres p16/ki67 staining for histology was used only for equivocal cases.

Sample size

The planned 60,000 women provided a 95% confidence interval of +/- 0.5/1000 of the CIN2+ cumulative incidence at 5 years in HPV+ E6/E7 mRNA-negative women when assuming: a) cumulative incidence of 1/1000 in all the E6/E7 mRNA-negative women; b) 50% of the E6/E7 mRNA-negative women who developed a lesion in the following five years were HPV DNA+ at recruitment; c) 70% completed follow up. This sample size would give an estimate of more than 400 CIN2+ lesion at baseline, in the hypothesis of a detection of 7/1000; the corresponding power to observe as significantly different (alpha 0.05) two biomarkers with sensitivity 70% and 80%, respectively, would be over 90% (McNemar's two tail test, under the hypothesis of correlation ≥ 0.01). This sample size would have given 62% power to detect as significant ($P < 0.05$) an 80% regression of the HPV DNA-positive E6/E7 mRNA-negative CIN2+ in the one-year control arm vs the immediate colposcopy arm when assuming that 7% of the CIN2+ found in HPV DNA+ women are negative to E6/E7 mRNA and that the total detection rate with HPV is 6/1000. Unfortunately, the actual detection was much lower (see below).

Primary testing

All women were tested for HPV DNA. Cervical samples were collected in PreservCyt solution (Thin Prep, Hologic) and tested locally. Two different tests were used, based on the collection period and centre (Table 1): 1) COBAS 4800 HPV test (Roche), which separately reports positivity for HPV 16, 18 and for ≥1 of the remaining genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68); 2) Hybrid Capture 2 (HC2, Qiagen, Hilden, Germany) for the pooled detection of 13 high-risk types (same as COBAS except HPV66). DNA was extracted using the QIAsymphony DSP HPV Media Kit (Qiagen, Hilden, Germany) and hybridization was done by the Rapid Capture System (RCS) (Qiagen) according to manufacturer's instructions.

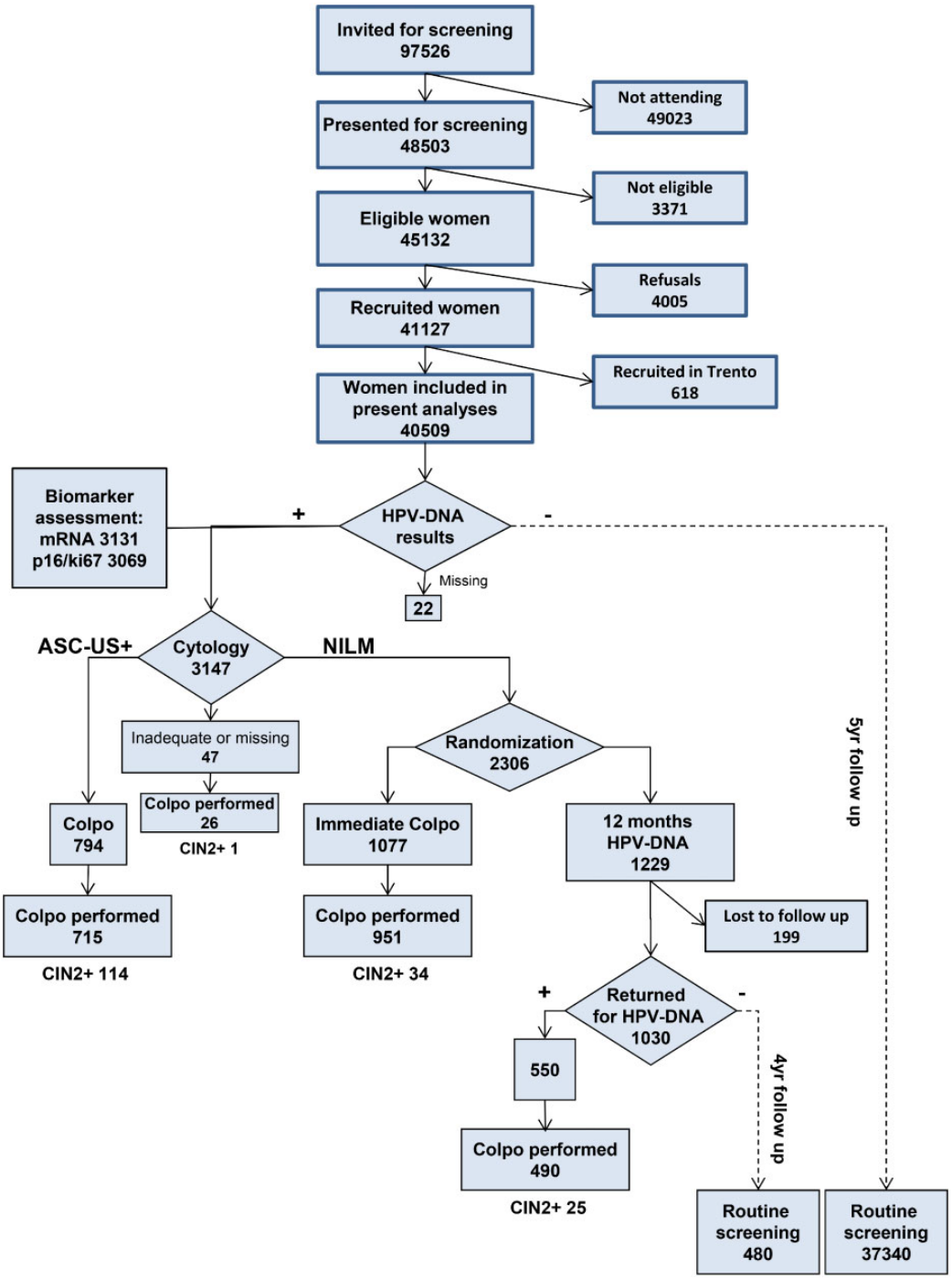


Figure 1. Study flowchart reporting recruitment process, randomization, and primary endpoint results. Women were invited for screening by the standard program management system. Participating women were assessed for eligibility and, if eligible, were asked to participate; those willing signed informed consent. All women underwent screening with HPV DNA test with reflex cytology if positive; those included in the study and positive for HPV DNA were also tested for biomarkers (E6/E7 mRNA, p16/ki67). Women with ASC-US or more severe cytology were referred to colposcopy, those negative for intraepithelial lesion or malignancy were randomized to immediate colposcopy or to 1-year retesting (the routine protocol in Italy, Supplementary figure S1). If HPV DNA-positive at retesting, women were referred to colposcopy.

Management of HPV DNA-positive women

All HPV DNA-positive women had reflex cytology. Liquid-based slides (ThinPrep, Hologic) were prepared and interpreted in local screening pathology laboratories using the Bethesda 2001 classification. [24] All biomarker analyses were performed on the same cervico-vaginal sample.

Cytology-positive women were referred to colposcopy, as in routine practice (Supplementary figure S1). HPV DNA-positive/cytology-negative women were randomly assigned, with 1:1 ratio, to immediate colposcopy or to repeating HPV DNA test after 12 months. Women were referred to colposcopy if still positive or returned to a new screening round if negative. [20] Gynaecologists performing colposcopies and colposcopy-guided biopsies were blind to biomarkers' results. Women with CIN2+ were treated almost exclusively with loop electrosurgical excision procedure (LEEP).

Randomization procedures

Women were randomized using locally implemented systems nested in the screening management software. Randomization was automatically activated when the result of cytology was uploaded to/ registered in the screening database. Arm allocation automatically determined the next steps. Unfortunately, the randomization procedure failed to work for a few short periods in two centres because of routine software updates and in these periods all women were allocated to one-year retesting (Supplementary Table S1).

Biomarkers

Biomarkers were assessed only in HPV DNA-positive samples.

The Aptima HPV Assay test (Hologic) detects E6-E7 viral mRNA from 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, detected as a pool). The assay was performed by the Panther system, according to manufacturer's instructions, as previously described. [24] Signal-to-cut off (S/CO) ratios >=0.5 were considered as positive.

Slides for P16/Ki67 immunostaining were prepared from the same liquid-based cytological sample, after having obtained a slide for cytology, using a ThinPrep 2000 or 5000 processor (Hologic) and were immunostained using the CINtec® PLUS kit (Roche Diagnostics, Basel, Switzerland), according to manufacturer's instructions, in four laboratories (Florence, Umbria, Turin, which also stained the slides from Trento, and Regina Elena Institute, Rome, for samples collected in Veneto).

A plan for harmonising interpretation criteria was implemented before the start of the trial. [24] All slides were read by three different centres, blind to cytology and histology results, and classified as positive, negative, or inadequate. Samples were scored as positive when double immunoreaction was revealed within at least one cell. Slides with <5000 squamous epithelial cells were considered as inadequate but recorded as positive if showing p16/ki-67 immunopositive cells. Here we used the

majority diagnosis. If only two readings were informative, we considered the interpretation made in the first reading.

Statistical analyses

For stand-alone cytology, E6/E7 mRNA, and p16/ki67, we estimated, among HPV DNA-positive women, the proportion of test-positives, sensitivity for histology-proven CIN2+ and CIN3+, specificity for < CIN2, immediate and 1-year referral to colposcopy when referring women positive for each putative triage test immediately and the remaining after 1-year if still HPV DNA positive (Supplementary figure S1) and its positive predictive value (PPV). Both raw values and values adjusted for unequal arm size are reported (see Supplementary methods sections 1-5 for details).

We estimated the same parameters also for the combined use (colposcopy referral of women positive to either test, see Supplementary methods section 2) of HPV16/18 partial genotyping (obtained from the Cobas test, thus available only for about half of study subjects) and cytology and of p16/ki67. Other combinations with weaker rationale are reported in Supplementary Table S2.

Missing/invalid samples were excluded from analyses. In the case of test combinations, women with at least one valid positive test were always kept because they would in any case be referred to colposcopy (see Supplementary methods section 2).

We computed sensitivity and specificity of E6/E7 mRNA, p16/ki67, and biomarker combinations including only women randomized to immediate colposcopy (see Supplementary methods section 3-5).

All 95% confidence intervals (95% CI) of proportions were obtained from the exact binomial distribution.

To study the regression of CIN2+ we compared their detection in the one-year repeat arm (p1) to that in the immediate colposcopy arm (p0) and used (p1-p0)/p0 as estimate (see Supplementary methods section 6). Thus, the estimate is based only on cytology-negative women. For each of the two studied biomarkers we report estimates for biomarker-positive and -negative women at baseline. In addition, we computed the *P* value for the arm-biomarker interaction in a logistic model as estimate of the probability that similar or larger differences in the relative risk occur under the null hypothesis of equal regression in biomarker positives and negatives; given the low power of the study for this comparison, we did not set a significance threshold and no formal statistical test was performed. Non-compliers to colposcopy in both arms and non-compliers to test repeat in the retesting arm were excluded. To evaluate whether biomarker positivity was a predictor of HPV DNA persistence we computed the relative probability of clearance with 95% CI.

Ethics

The study protocol was approved by the S. Giovanni Battista University Hospital, Turin, Italy, on 20 June 2012 (N. CEI513) and by the local committees of all recruiting centers.

Results

The four centers included in this analysis recruited 40,509 women (Figure 1), including 600 over age 60. Of the 40,487 women with a valid HPV DNA test, 3147 were positive (Table 1). Cytology was available for 3137, positive in 794 (25.3%) and unsatisfactory in 37 (1.2%); positivity for type 16-18 was 27.0% (391/1446 tested with Cobas); E6/E7 mRNA was available for 3131 and positive in 2092 (66.8 %); p16/ki67 was available for 3069 of whom 822 (26.8%) were positive and 164 (5.3%) unsatisfactory.

Among the 2306 HPV DNA-positive/cytology-negative women, 1077 (46.7%) were randomly assigned to immediate colposcopy and 1229 (53.3%) to 12-month HPV DNA retesting (figures 1 and 2); their characteristics are reported in Supplementary Table S1. Delay in colposcopy was longer in the cytology-negative women randomized to immediate colposcopy than in those cytology-positive (median time from HPV DNA testing to colposcopy 93 vs. 70 days, respectively). Overall, 174 CIN2+ were diagnosed (including 95 CIN3 and 1 AIS).

The sensitivity of ASC-US+ cytology was just 61.0% for CIN2+ and 68.2% for CIN3+, with 76.6% specificity for <CIN2. E6/E7 mRNA had very high sensitivity (94.4% for CIN2+ and 96.9% for CIN3+) but just 34.4% specificity for <CIN2. With p16/ki67 dual staining sensitivity (75.2% and 80.6% for CIN2+ and CIN3+, respectively) was lower than with E6/E7 mRNA but higher than with cytology, while specificity (74.8%) was much higher than with E6/E7 mRNA and similar to that of cytology (Table 2).

When referring to immediate colposcopy all women who were either cytology- or HPV16/18-positive, sensitivity reached 93.8% for CIN2+ and 100 for CIN3+, but specificity was only 57.4%. Results were similar for p16/ki-67 combined with HPV16/18 genotyping (Table 2, supplementary table S2).

The estimated overall proportion of HPV DNA-positive women who would have been referred to colposcopy either immediately or after one year with the assumed algorithm was slightly lower with p16/ki67 (63.3%) than with cytology at ASC-US threshold (65.3%). Overall referral was highest for E6/E7 mRNA assay (78.3%) despite very limited (11.4%) referral after one-year retesting. The overall PPV was similar for all biomarkers: 9.5% for cytology, 8.3% for E6/E7 mRNA, and 10.1% for p16/ki67 (Table 3, supplementary table S3).

	Tested*	Test positive	TP	FP	TN	FN	Sensitivity for CIN2+ (95% CI)		Specificity for <CIN2+ (95% CI)		Sensitivity for CIN3+ (95% CI)	
							Raw	Adj‡	Raw	Adj‡	Raw	Adj‡
Cytology ASC-US+†	2636	715	114	601	1862	59	65.9% (58.3% - 72.9%)	61.0% (53.6% - 68.0%)	75.6% (73.9% - 77.3%)	76.6% (74.5% - 78.5%)	71.9% (61.8% - 80.6%)	68.2% (60.6% - 75.2%)
E6/E7 mRNA	2650	1784	167	1617	859	7	96.0% (91.9% - 98.4%)	94.4% (89.1% - 97.3%)	34.7% (32.8% - 36.6%)	34.4% (31.9% - 37.0%)	96.9% (91.1% - 99.4%)	96.9% (90.0% - 99.3%)
p16/ki67†	2471	714	131	583	1720	37	78.0% (70.9% - 84.0%)	75.2% (68.1% - 81.6%)	74.7% (72.9% - 76.5%)	74.8% (72.4% - 77.1%)	85.1% (76.3% - 91.6%)	80.6% (70.9% - 88.3%)
Cytology ASC-US+ or HPV 16/18 typing	1203	548	47	501	650	5	90.4% (79.0% - 96.8%)	93.8% (82.8% - 98.7%)	56.5% (53.6% - 59.4%)	57.4% (53.2% - 61.4%)	96.2% (80.4% - 99.9%)	91.9% (74.0% - 99.0%)
p16/ki67 or HPV 16/18 typing	1121	521	44	477	595	5	88.8% (77.8% - 96.6%)	90.1% (76.9% - 96.5%)	55.5% (52.5% - 58.5%)	53.7% (49.9% - 57.5%)	100.0% (86.8% - 100%)	100.0% (85.8% - 100%)

*All women with valid test and complete assessment

†Only valid test included

‡ adjusted for completeness of follow up. Formulas used for raw and adjusted estimates are in Supplementary methods sections 1-4 and 6

TP, true positive; FP, false positive; TN, true negative; FN, false negative.

Table 2. Accuracy of different triage tests. Sensitivity for CIN2+ and CIN3 and specificity for CIN2+, with relative 95% confidence interval (95% CI) of cytology, E6/E7 mRNA assay, and p16/ki67 dual staining.

Immediate colposcopy referral	Tested	Test positive	Referral (%)	PPV (%)	Referral (%)	PPV (%)	Referral (%)	PPV (%)
Cytology ASC-US+*	3100	794	25.6	16.2	39.7	5.1	65.3	9.5
E6/E7 mRNA	3131	2092	66.8	9.5	11.4	0.8	78.3	8.3
p16/ki67*	2905	822	28.3	18.5	35.0	2.9	63.3	10.1
Cytology ASC-US+ or HPV 16/18 typing	1446	638	44.1	8.3	31.4	1.8	75.5	5.6
p16/ki67 or HPV 16/18 typing	1446	614	42.5	8.5	30.4	1.7	72.8	5.7

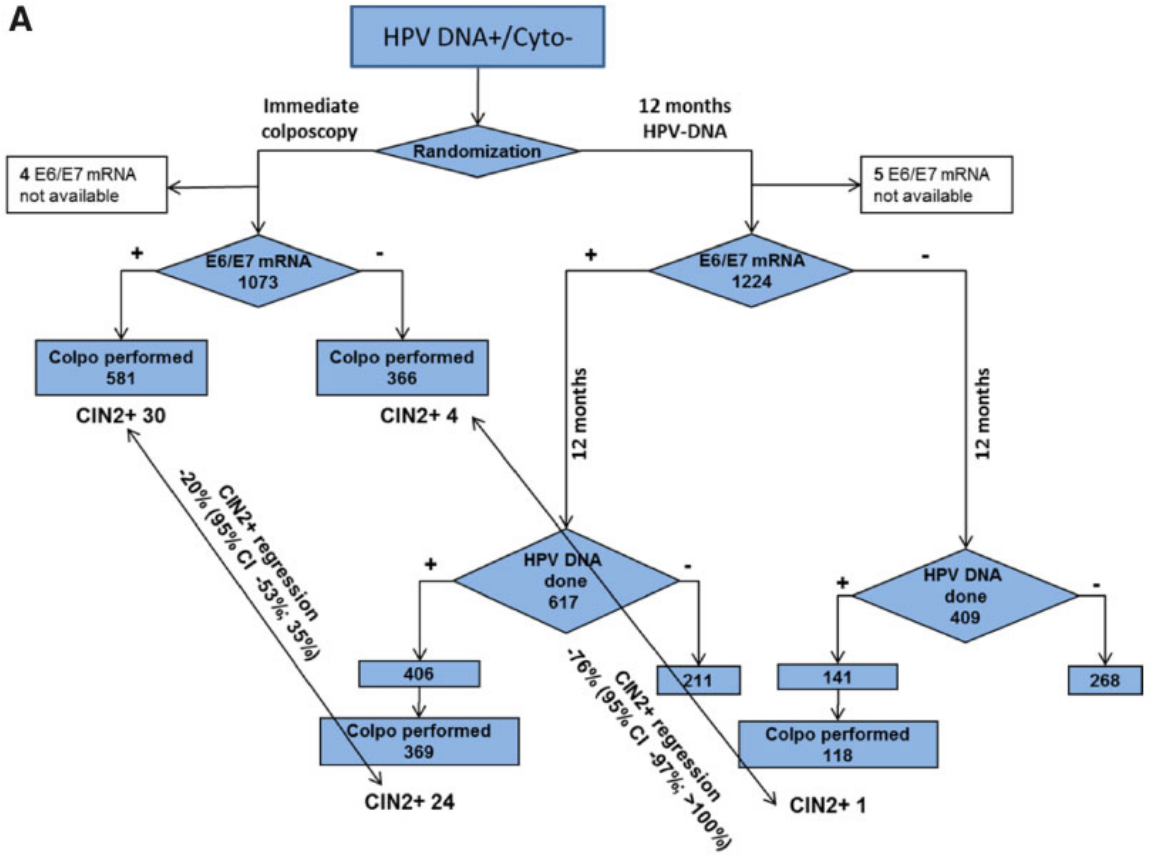
***Only valid test included

Table 3. Immediate, at one-year retesting, and overall estimated referral rate and positive predictive value (PPV) for cytology, E6/E7 mRNA assay, p16/ki67 dual staining, and combinations of biomarkers. Values are estimated assuming that HPV DNA-positive women are referred to immediate colposcopy according to test or combination of tests specified in table lines and the remaining to colposcopy after 12 months if still HPV DNA positive.

Regression of CIN2+ and clearance of HPV DNA.

Among all HPV DNA+/cytology- women the CIN2+ detection was 2.6% (25/971) and 3.6% (34/951) in the 1-year retesting and immediate-colposcopy arm respectively (Figure 1). Thus, the estimated overall 1-year CIN2+ variation was -28% (ie that 28% of them regressed) with 95 % CI from a -57% regression to a +20% increase). Point estimates of regression were smaller in women who were biomarker-positive at baseline [-20% (95% CI -53% to +35%) and -22% (95% CI -61% to +56%) for E6/E7 mRNA and p16/ki67, respectively] than in biomarker-negatives [-76% (95% CI -97% to +110%) and -39% (95% CI -72% to +33%) for E6/E7 mRNA and p16/ki67, respectively] but confidence intervals were very wide and the test for interaction was 0.29 and 0.67 for E6/E7 mRNA and p16/ki67, respectively) (figure 2).

Moreover, among HPV DNA-positive/cytology-negative women, the clearance of HPV DNA after 12 months in mRNA-negative women (268/409 = 65.5%) was 1.9 times (95% CI 1.7 to 2.2) that in mRNA-positive women (211/617 = 34.2%) and clearance in p16/ki67-negative women (393/768 = 51.2%) was 1.9 times (95% CI 1.5 to2.5) that in p16/ki67-positive women (48/182 = 26.4%) (figure 2).


A
HPV DNA+/Cyto-
Immediate colposcopy
Randomization
12 months HPV-DNA
4 E6/E7 mRNA not available
5 E6/E7 mRNA not available
+
E6/E7 mRNA 1073
-
+
E6/E7 mRNA 1224
-
Colpo performed 581
CIN2+ 30
Colpo performed 366
CIN2+ 4
12 months
12 months
-20% (95% CI -53%; 35%) CIN2+ regression
HPV DNA done 617
HPV DNA done 409
406
211
141
268
Colpo performed 369
CIN2+ 24
-76% (95% CI -97%; >100%) CIN2+ regression
Colpo performed 118
CIN2+ 1

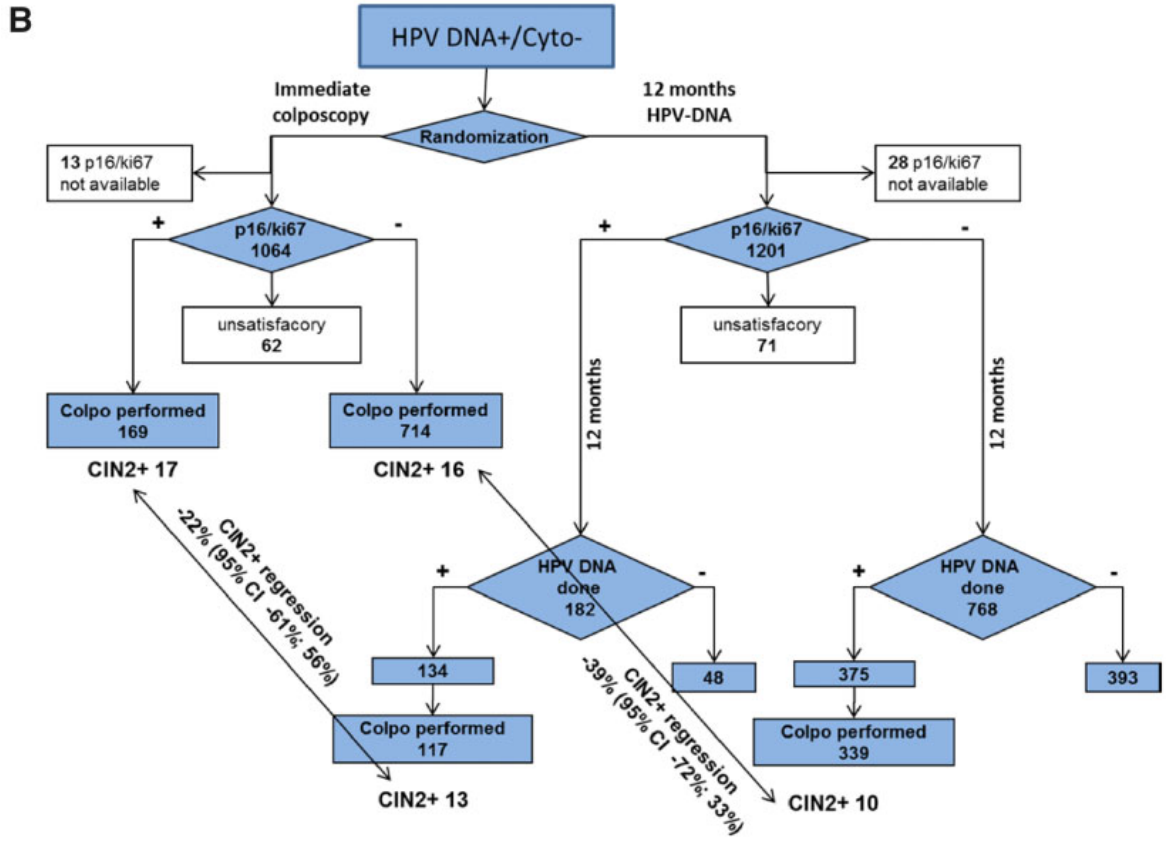

B
HPV DNA+/Cyto-
Immediate colposcopy
Randomization
12 months HPV-DNA
13 p16/ki67 not available
28 p16/ki67 not available
+
p16/ki67 1064
-
+
p16/ki67 1201
-
unsatisfacory 62
unsatisfacory 71
Colpo performed 169
CIN2+ 17
Colpo performed 714
CIN2+ 16
12 months
12 months
-22% (95% CI -61%; 56%) CIN2+ regression
HPV DNA done 182
HPV DNA done 768
134
48
375
393
Colpo performed 117
CIN2+ 13
-39% (95% CI -72%; 33%) CIN2+ regression
Colpo performed 339
CIN2+ 10

Figure 2. Flowchart of HPV DNA-positive/cytology-negative women randomized to immediate colposcopy or one-year HPV retesting, distinguishing between E6/E7 mRNA-positive and E6/E7 mRNA-negative women (a) distinguishing between p16/ki67-positive and p16/ki67-negative women (b). Baseline and up to 24 months' results are reported. Among the 2306 HPV-positive/cytology-negative women randomized, 2297 had a valid E6/E7 mRNA test and 2132 had a valid p16/ki67 test. Dotted arrows show the comparison between CIN2+ detection in the two arms; for the two biomarkers, 1 - ratio between detection in the 1-year referral arm vs. that in the immediate colposcopy arm represents an unbiased estimate of lesion regression in one year. Here it is reported as reduction (negative percentage) of the CIN2+ proportion found in the immediate colposcopy arm. In panel a, overall CIN2+ detection was 3.6% in the immediate colposcopy referral arm (34/951) and 2.6% in the 1-year referral arm (25/971), resulting in a 28% reduction (95 % CI from a 57% reduction to a 20% increase).

Discussion

Consistent with available data, we observed that the sensitivity of p16/ki67 was slightly higher than that of cytology [10-12] and that E6/E7 mRNA sensitivity was close to that of HPV DNA testing, [13, 15, 17] but that over 60% of HPV DNA positives was also E6/E7 mRNA positive. Combined use of cytology or p16/ki67 and HPV 16-18 typing increases sensitivity with a modest loss of specificity. [11, 25]

Based on the relative detection of CIN2+ in the two study arms (figure 2), our data suggest that, overall, less than one of four cytology-negative CIN2+ regress in one year. Similar estimates were obtained from the ALTS study [26] and in a systematic review. [27] In principle, some of the lesions detected in women randomised to 12-month retesting might not yet have been detectable at baseline, leading to a possible underestimate of regression. We estimated almost no regression in women positive for p16/ki67 or E6/E7 mRNA, while regression was estimated to be 70% and 40% in women E6/E7 mRNA and p16/ki67 negative, respectively. These data suggest that the two biomarkers under study may predict CIN2+ clearance, which is also plausible given what we know about the molecular pathogenesis of the disease. The association was, however, far from being statistically significant, likely because of the small number of CIN2+ found.

Conversely, our data clearly show that HPV infections in women negative to the p16/ki67 test or the E6/E7 mRNA assay have strongly increased probability of clearing within 12-months. Low persistence of HPV infection in tissues not overexpressing these two viral oncogenes is consistent with their functional suppression activity on p53 and RB and suggests that such tissues have little or no malignant potential, thus logically supporting high regression of only apparently abnormal histological findings.

HPV DNA persistence after a negative p16/ki67 or E6/E7 mRNA test was also lower than after a normal cytology. This reduces colposcopy referral if screening algorithms based on HPV DNA retesting of women negative to the triage test are applied. With retesting after 12 months, as currently in Italy, the total referral (immediate + at 12 months) would be similar with p16/ki67 or cytology as triage

test, despite higher immediate referral with the former. Conversely, total referral with E6/E7 mRNA would be more than 10% higher due to very high initial referral.

Strengths and limitations

We acknowledge that, given the study design, we estimate HPV DNA persistence after negative p16/ki67 or E6/E7 mRNA just from cytology-negative women. Actually, cytology positivity occurred together with p16/ki67 negativity in only 11.6 % of tested women and only in 3.7 % with E6/E7 mRNA-negativity. Thus, even if clearance were lower in these women, the underestimation of overall referral would be minimal.

In two centres, more women were assigned to the conventional arm due to software malfunctioning. Because the staff could not manipulate randomization, the resulting bias could at most be unequal centre and season distributions in the study arms. Excluding all the women recruited during periods of possible malfunctioning had virtually no effect on results.

Some women randomized to immediate colposcopy were assessed >6 months after HPV DNA testing. This led to underestimating the overall regression of triage- cytology-negative CIN2+ and to a dilution of its association with p16/ki67 and E6/E7 mRNA negativity.

In this study, two liquid-based slides, one for cytology and one for p16/ki67, were prepared for each HPV DNA-positive woman. The slide for cytology was prepared first because used for actual management. This may explain the relatively high proportion of unsatisfactory dual-stained slides.

NTCC2 was implemented in population-based organised screening programmes. This, along with the high proportion of eligible women who accepted being enrolled, guarantees high applicability of results to routine activity. Computerised management, centralisation of procedures in dedicated facilities, availability of fail-safe systems, and systematic computerized registration of results enabled high adherence to protocols and completeness of follow up (including very high participation to retesting and colposcopy), thus assuring high-quality data.

Finally, the main limit was the much lower prevalence of CIN2+ than that assumed in power calculations. Indeed, although 30% fewer women than planned were recruited, the low CIN2+ prevalence was the main reason for low power. It was mainly due to an unexpectedly high proportion of women previously screened by HPV testing. Detection was particularly low at 1-year follow up, because of higher sensitivity of cytology than that observed in previous trials. [28]

Considering practical conclusions, p16/ki67 is more sensitive than cytology and entails similar colposcopy referral even when keeping the same interval to retesting, thus leading to increased efficiency. High sensitivity would plausibly guarantee the safety of prolonged intervals to retesting, which would further increase efficiency.

The E6/E7 mRNA assay has very high sensitivity but its immediate colposcopy referral was higher than the total (immediate + delayed) referral with cytology. On the other hand, its sensitivity was so high that only seven lesions were missed, and we estimate that most of these may regress in one year. This makes E6/E7 mRNA a good candidate as primary screening test. Thus, if over-diagnosis is an issue, the relevance of the biomarker really depends on what being positive implies. Results from the long-term follow up of this study and of other ongoing cohorts [16, 29-33] are necessary to determine the longest safe interval after E6/E7 mRNA-negative primary testing.

Registration: Clinicaltrials.gov registration number: NCT01837693, New Technology in Cervical Cancer 2 (NTCC2) study. [34]

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Bibliography

1. Ronco G, Meijer CJL, Segnan N, et al. Invasive cervical cancer after HPV-based screening. *Lancet*. 2014; 383:1295. doi: 10.1016/S0140-6736(13)62218-7.
2. Bosch FX, Robles C, Díaz M, et al. HPV-FASTER: broadening the scope for prevention of HPV-related cancer. *Nat Rev Clin Oncol*. 2016; 13:119-32. doi: 10.1038/nrclinonc.2015.146.
3. WHO guidelines for screening and treatment of precancerous lesions for cervical cancer prevention. Geneva: World Health Organization; 2013. Available at https://www.who.int/reproductivehealth/publications/cancers/screening_and_treatment_of_precancerous_lesions/en/, accessed June 27, 2019
4. Ronco G, Arbyn M, Meijer CJLM, et al. Screening for cervical cancer with primary testing for human papillomavirus. In: European guidelines for quality assurance in cervical cancer screening. Second edition, Supplements. Anttila A, Arbyn A, De Vuyst H, Dillner J, Dillner L, Franceschi S, Patnick J, Ronco G, Segnan N, Suonio E, Törnberg S & von Karsa L (eds.). Office for Official Publications of the European Union, Luxembourg, 2015 pp:1-68
5. US Preventive Services Task Force, Curry SJ, Krist AH, et al. Screening for Cervical Cancer: US Preventive Services Task Force Recommendation Statement. *JAMA*. 2018; 320:674-686. doi: 10.1001/jama.2018.10897.
6. Saslow D, Solomon D, Lawson HW, et al. ACS-ASCCP-ASCP Cervical Cancer Guideline Committee. 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. doi: 10.3322/caac.21139.
7. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive women in cervical cancer screening. *J Clin Virol*. 2016; 76 Suppl 1:S49-S55. doi: 10.1016/j.jcv.2015.11.015.
8. Carozzi F, Gillio-Tos A, Confortini M, et al. Risk of high grade CIN on follow up in HPV positive women according to baseline p16-INK4A results.follow up p16 NTCC. *Lancet Oncol*. 2013; 14:168-176. doi: 10.1016/S1470-2045(12)70529-6.
9. Carozzi F, Confortini M, Dalla Palma P, et al. Use of p16 overexpression to increase the specificity of human papillomavirus testing: a study nested in the NTCC randomised controlled trial. *Lancet Oncol*. 2008, 9:937-945. doi: 10.1016/S1470-2045(08)70208-0.
10. Clarke MA, Cheung LC, Castle PE, et al. Five-Year Risk of Cervical Precancer Following p16/Ki-67 Dual-Stain Triage of HPV-Positive Women. *JAMA Oncol*. 2019; 5:181-186. doi: 10.1001/jamaoncol.2018.4270.
11. Wentzensen N, Clarke MA, Bremer R, et al. Clinical Evaluation of Human Papillomavirus Screening With p16/Ki-67 Dual Stain Triage in a Large Organized Cervical Cancer Screening Program. *JAMA Intern Med*. 2019; 179:881-888. doi: 10.1001/jamainternmed.2019.0306.
12. Gustinucci D, Giorgi Rossi P, Cesarini E, et al. Use of Cytology, E6/E7 mRNA, and p16INK4a-Ki-67 to Define the Management of Human Papillomavirus (HPV)-Positive Women in Cervical Cancer Screening. *Am J Clin Pathol*. 2016; 145:35-45. doi: 10.1093/ajcp/aqv019.
13. Monsonego J, Hudgens MG, Zerat L, et al. Evaluation of oncogenic human papillomavirus RNA and DNA tests with liquid-based cytology in primary cervical cancer screening: the FASE study. *Int J Cancer.* 2011; 129:691-701. doi: 10.1002/ijc.25726.
14. Monsonego J, Hudgens MG, Zerat L, Zerat JC, Syrjänen K, Smith JS. Risk assessment and clinical impact of liquid-based cytology, oncogenic human papillomavirus (HPV) DNA and mRNA testing in primary cervical cancer screening (the FASE study). *Gynecol Oncol* 2012;125:175-80. doi: 10.1016/j.ygyno.2012.01.002.
15. Heideman DA, Hesselink AT, van Kemenade FJ, et al. The Aptima HPV assay fulfills the cross-sectional clinical and reproducibility criteria of international guidelines for human papillomavirus test requirements for cervical screening. *J Clin Microbiol.* 2013; 51:3653-57. doi: 10.1128/JCM.01517-13.
16. Iftner T, Neis KJ, Castanon A, et al. Longitudinal Clinical Performance of the RNA-Based Aptima Human Papillomavirus (AHPV) Assay in Comparison to the DNA-Based Hybrid Capture 2 HPV Test in Two Consecutive Screening Rounds with a 6-Year Interval in Germany. *J Clin Microbiol.* 2019; 57(1). pii: e01177-18. doi:10.1128/JCM.01177-18.
17. Cuzick J, Cadman L, Mesher D, et al. Comparing the performance of six human papillomavirus tests in a screening population. *Br J Cancer*. 2013; 108:908-13. doi: 10.1038/bjc.2013.22.

18. Dalla Palma P, Giorgi Rossi P, Collina G, et al. The risk of false-positive histology according to the reason for colposcopy referral in cervical cancer screening: a blind revision of all histological lesions found in the NTCC trial. *Am J Clin Pathol*. 2008; 129:75-80. doi: 10.1309/EWYGWFRRM8798U5P.
19. Kyrgiou M, Athanasiou A, Paraskevaidi M, et al. Adverse obstetric outcomes after local treatment for cervical preinvasive and early invasive disease according to cone depth: systematic review and meta-analysis. *BMJ*. 2016; 354:i3633. doi: 10.1136/bmj.i3633.
20. Ronco G, Accetta G, Angeloni C, et al. HTA report: Ricerca del DNA di papillomavirus umano (HPV) come test primario per lo screening dei precursori del cancro del collo uterino. *Epidemiol Prev*. 2012; 36 (3/4 suppl 1):e1-e72.
21. Moyer VA, U.S. Preventive Services Task Force. Screening for cervical cancer: U.S. Preventive Services Task Force recommendation statement. *Ann Intern Med*. 2012; 156(12):880-91. doi: 10.7326/0003-4819-156-12-201206190-00424.
22. Ronco G, Zappa M, Franceschi S, Tunesia S, Caprioglio A, Confortini M, Del Mistro A, Carozzi F, Segnan N, Zorzi M, Giorgi-RossiP, and the Italian HPV Survey Working Group. Effect of the accuracy of tests for triaging HPV positive women on the overall screening performance. *Eur J Cancer.* 2016 ;68:148-155.
23. Solomon D, Davey D, Kurman R, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. *JAMA.* 2002; 287:2114-99. doi: 10.1001/jama.287.16.2114.
24. Giorgi Rossi P, Bisanzi S, Allia E, et al. Determinants of Viral Oncogene E6-E7 mRNA Overexpression in a Population-Based Large Sample of Women Infected by High-Risk Human Papillomavirus Types. *J Clin Microbiol.* 2017; 55:1056-65. doi: 10.1128/JCM.01794-16.
25. Cuzick J, Adcock R, Carozzi F, Gillio-Tos A, De Marco L, Del Mistro A, Frayle H, Girlando S, Sani C, Confortini M, Zorzi M, Giorgi-Rossi P, Rizzolo R, Ronco G; New Technologies for Cervical Cancer Screening Working Group. Combined Use Of Cytology, P16 Immunostaining And Genotyping For Triage Of Women Positive For High Risk Human Papillomavirus At Primary Screening. Int J Cancer. 2020 Mar 14. doi: 10.1002/ijc.32973.
26. Castle PE, Schiffman M, Wheeler CM, Solomon D. Evidence for frequent regression of cervical intraepithelial neoplasia-grade 2. *Obstet Gynecol*. 2009; 113:18-25. doi: 10.1097/AOG.0b013e31818f5008.
27. Tainio K, Athanasiou A, Tikkinen KAO, et al. Clinical course of untreated cervical intraepithelial neoplasia grade 2 under active surveillance: systematic review and meta-analysis. *BMJ*. 2018; 360:k499. doi: 10.1136/bmj.k499.
28. Ronco G, Segnan N, Giorgi-Rossi P, et al. Human papillomavirus testing and liquid-based cytology: results at recruitment from the new technologies for cervical cancer randomized controlled trial. *J Natl Cancer Inst*. 2006; 98:765-74. doi: 10.1093/jnci/djj209.
29. Reid JL, Wright TC Jr, Stoler MH, et al. Human Papillomavirus Oncogenic mRNA Testing for Cervical Cancer Screening: Baseline and Longitudinal Results From the CLEAR Study. *Am J Clin Pathol* 2015; 144:473-83. doi: 10.1309/AJCPHVD7MIP3FYVV.
30. Cook DA, Smith LW, Law JH, et al. Comparative performance of human papillomavirus messenger RNA versus DNA screening tests at baseline and 48 months in the HPV FOCAL trial. *J Clin Virol* 2018; 108:32-7. doi: 10.1016/j.jcv.2018.09.004.
31. Iftner T, Becker S, Neis KJ, et al. Head-to-Head Comparison of the RNA-Based Aptima Human Papillomavirus (HPV) Assay and the DNA-Based Hybrid Capture 2 HPV Test in a Routine Screening Population of Women Aged 30 to 60 Years in Germany. *J Clin Microbiol* 2015; 53:2509-16. doi: 10.1128/JCM.01013-15.
32. Maggino T, Sciarrone R, Murer B, et al. Screening women for cervical cancer carcinoma with a HPV mRNA test: first results from the Venice pilot program. *Br J Cancer*. 2016; 115:525-32. doi: 10.1038/bjc.2016.216.
33. Zorzi M, Del Mistro A, Giorgi Rossi P, et al. Risk of CIN2 or more severe lesions after negative HPV-mRNA E6/E7 overexpression assay and after negative HPV-DNA test: concurrent cohorts with a 5-year follow up. *Int J Cancer*. 2019 Oct 3. doi: 10.1002/ijc.32695.
34. Clinicaltrial.gov. Cervical Cancer Prevention: From DNA to mRNA? - New Technologies for Cervical Cancer Screening 2 (NTCC2). Study protocol registration number NCT01837693 Available at: https://clinicaltrials.gov/ct2/show/NCT01837693

3.3. Research article #04: Interlaboratory Concordance of p16/Ki-67 Dual-Staining Interpretation in HPV-Positive Women in a Screening Population

The findings of this research article were published in *Cancer Cytopathology*.

Benevolo M, Mancuso P, Allia E, Gustinucci D, Bulletti S, Cesarini E, Carozzi FM, Confortini M, Bisanzi S, Carlinfante G, Rubino T, Rollo F, Marchi N, Farruggio A, Pusiol T, Venturelli F, Giorgi Rossi P; New Technologies for Cervical Cancer 2 Working Group. Interlaboratory concordance of p16/Ki-67 dual-staining interpretation in HPV-positive women in a screening population. Cancer Cytopathol. 2020 May;128(5):323-332. doi: 10.1002/cncy.22248. Epub 2020 Feb 12. PMID: 32168431.

ABSTRACT

Background: p16/ki67 dual staining is a candidate biomarker for triaging HPV-positive women. Reproducibility is needed for adopting a test in the screening. We assessed inter-laboratory reproducibility in HPV-positive women.

Methods: An unselected screening HPV-positive population was enrolled within the Italian NTCC2 study. ThinPrep slides were immunostained for p16/ki67 in four laboratories and interpreted in seven laboratories. Each slide had three reports from different labs. Slides were classified as valuable or inadequate, and, among valuable, as positive (at least one double-stained cell) or negative. Inter-laboratory reproducibility was evaluated by kappa values.

Results: Overall, we obtained 9300 reports for 3100 cases; 905 reports were inadequate (9.7%). The overall adequacy concordance was poor (K=0.224, 95% CI: 0.183-0.263). The overall positivity concordance was moderate (K=0.583, 95% CI 0.556-0.610). Of the 176 CIN2+ lesions found in HPV DNA-positive women, 158 had a valid result: 107 were positive in all 3 reports (sensitivity for CIN2+: 67.7%, 95% CI: 59.8%-74.9%), 23 in two (sensitivity of majority report: 82.3%, 95% CI: 75.4%-87.9%) and 15 in one (sensitivity of at least one positive result: 91.8%, 95% CI: 86.3%-95.5%). Thirteen CIN2+ cases were negative in all 3 reports. The overall positivity concordance in CIN2+ samples was K=0.487 (95% CI: 0.429-0.534), while in the non-CIN2+ samples it was K=0.558 (95% CI: 0.528-0.588).

Conclusions: The p16/ki67 assay showed poor reproducibility for adequacy and good reproducibility for positivity, comparable to that of cervical cytology. Nevertheless, the low reproducibility does not impact on sensitivity for CIN2+.

Trial registration: Clinicaltrials.gov registration number: NCT01837693, New Technology in Cervical Cancer 2 (NTCC2) study

Funding support: Funded by the Italian Ministry of Health, data owner; grant number: RF-2009-1536040

INTRODUCTION

Nowadays, great effort is being made in cervical cancer screening programs to assess the best method to triage HPV DNA-positive women to increase specificity so as to decrease colposcopy referral rate. The ideal triage biomarker should also have high sensitivity for baseline CIN2+ cases among HPV-positive women, thus ensuring sufficient time to allow virus clearance. Finally, it should also be able to distinguish between infections with high and with low probability of progression.

So far, cervicovaginal cytology has been the most diffuse triage test worldwide. [1-6] Before the HPV test introduction, cervical cytology was the most used screening primary test, and is still the primary tool in many low-income countries. [7] However, cytology presents some limitations. Although it is inexpensive and easy to perform, experienced and trained readers are needed to obtain a quality result. [8, 9] Although interpretation can be partially automated, it still remains highly subjective. Stoler has shown that the interobserver reproducibility of liquid-based cytology was only moderate (overall k= 0.46; at ASCUS+ cut-off k=0.56) and that the greatest source of disagreement involved ASCUS and H-SIL categories.[10] An Italian study showed a similar overall concordance (K= 0.44). [11] In the Bethesda Interobserver Reproducibility Study -2 (BIRST-2), the mean agreement on the Pap test results between participants and the reference panel interpretation was 62.8%, with a variability among the different diagnostic groups; in particular, the L-SIL showed the highest value and the H-SIL and ASC-H had the lowest. [12] Moreover, the Pap test reports status at the time of sampling but cannot provide information on the potential of progression. Finally, with the introduction of HPV testing, and especially of the HPV vaccination, a decrease in the number of CIN2+ lesions is expected, with a consequent decrease in the positive predictive value (PPV) of cytology, which is linked to CIN2+ prevalence. For these reasons, the scientific community is looking for new biomarkers that can overcome these limitations. Many biomarkers have been proposed but only few have been investigated on large screening populations; one of the most promising is p16/ki67 double staining. [13-15] In a recent publication, the p16/ki67 dual stain was used as the triage test of HPV positivity in a screening population; the results showed its better sensitivity and specificity and lower referral to colposcopy compared to cytology. [16] Also, preliminary findings from the NTCC2 study confirm that in HPV triage, the accuracy for CIN2+ detection of p16/ki67 dual stain is better than that of cytology. [17] However, very few studies have been published on dual stain interpretation concordance [18-22] and none has investigated p16/ki67 interpretation reproducibility in the screening context. Data from pragmatic studies set in the context of routine screening are of utmost importance to consider this new biomarker as a triage test of HPV-positivity in public population health programs.

New Technologies for Cervical Cancer 2 (NTCC2) is a large randomized clinical trial within organized cervical screening programs in Italy using HPV DNA as primary screening test. The aim of NTCC2 is to evaluate new biomarkers as triage test of HPV DNA positivity in comparison to cytology. In particular, we are evaluating the Aptima HPV assay for HPV E6-E7 mRNA and the CINtec® PLUS assay for the immunocytochemical dual staining of p16 and ki67 proteins. In the NTCC2 study, liquid-based

cytology slides immunostained using the CINtec PLUS assay were analyzed and scored independently by three different labs. In a previous study by our group, p16/ki67 dual staining on selected immunostained slides showed a good reproducibility between readers from nine different laboratories. [21] Here, we assessed the inter-laboratory reproducibility of the test interpretation and described the determinants of agreement in a large unselected population of HPV-positive women from five Italian screening centers.

MATERIALS AND METHODS

Study design

The study protocol was approved by the S. Giovanni Battista University Hospital, Turin, Italy, on 20 June 2012 (N. CEI513) and then by the local ethic committees of all the recruiting centers.

Women 25-64 years old attending a new screening episode were asked to participate and to sign informed consent. Participating women were tested for HPV DNA; if positive, cytology and p16/ki67 immunostaining were performed. All the NTCC2 HPV-positive cases were included in the present study (n=3180 out of 41127 women enrolled in the study). After the first interpretation in the lab which performed the immunostaining, the slides circulated among centers between October 2016 and May 2019, since every slide needed three reports from three different labs. The circulation among centers was balanced so that the workload was similar among labs and included all the possible combinations of readers (Table 1). Seven Italian centers involved in cervical cancer screening and/or cervical cancer research took part in the study. For the purpose of the study, they were identified as Lab1 – Lab7.

Table 1. Frequency of the p16/ki67 interpretation reports and order of readings according to reading labs

	Total	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**	**Lab 7**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Reports	**9300**	**1299 (14.0)**	**1397 (15.0)**	**1297 (13.9)**	**1197 (12.9)**	**1341 (14.4)**	**1352 (14.5)**	**1417 (15.2)**
Evaluable	8395 (90.3)	1241 (95.5)	1250 (89.5)	1251 (96.5)	1108 (92.6)	1136 (84.7)	1215 (89.9)	1194 (84.3)
Negative	*5763 (68.6)*	*891 (68.6)*	*861 (61.6)*	*850 (65.5)*	*888 (74.2)*	*862 (64.3)*	*794 (58.7)*	*617 (43.5)*
Positive	*2632 (31.4)*	*350 (26.9)*	*389 (27.8)*	*401 (30.9)*	*220 (18.4)*	*274 (20.4)*	*421 (31.1)*	*577 (40.7)*
Inadequate	905 (9.7)	58 (4.5)	147 (10.5)	46 (3.5)	89 (7.4)	205 (15.3)	137 (10.1)	223 (15.7)
1st report	3100	593 (45.7)	889 (63.6)	-	-	-	801 (59.2)	817 (57.7)
2nd report	3100	606 (46.7)	402 (28.8)	401 (30.9)	795 (66.4)	896 (66.8)	-	-
3rd report	3100	100 (7.7)	106 (7.6)	896 (69.1)	402 (33.6)	445 (33.2)	551 (40.8)	600 (42.3)

Immunostaining

Slides were prepared from liquid-based cytological samples using the ThinPrep 2000 or the ThinPrep 5000 processor (Hologic, Bedford, Mass, USA), fixed in 99% ethanol for 10 minutes/1 hour, air-dried for 20 minutes/16 hours, and then immunostained using the CINtec® PLUS kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions in a Ventana autostainer (Benchmark XT or Ultra). Four of the seven participating centers performed immunocytochemistry (Lab1, Lab2, Lab6, and Lab7). Each slide was analyzed and scored independently by 3 labs in the 7 participating centers. All the readers in the 7 participating labs have been trained in the interpretation of CINtec PLUS. Reading was blinded to the cytologic, histologic, and mRNA results, although all readers were aware that only HPV DNA-positive cases were included and that the study was population-based, i.e., samples were unbiased consecutive series of HPV-positive women participating in screening. Results were reported in three categories: positive, negative, or inadequate. The criterion for positivity was the presence of at least one double-stained cell (cytoplasmic and/or nuclear brown staining for p16, together with nuclear red staining for ki67), as recommended by the manufacturer. Moreover, we recorded an additional arbitrary cut-off for positivity when at least 5 double-positive cells were counted. Slides were scored as negative when immunoreactivity was evident for neither, only one, or both of the markers but on different cells. Criteria for an inadequate report were scant cellularity according to the 2001 Bethesda System [23], excessive p16 background, and/or too weak counterstaining. However, specimens with insufficient nucleate squamous cells or faint staining but showing at least one p16/ki67 immunostained cell were reported as satisfactory for evaluation and recorded as positive. Readers also detailed both the presence of p16 and ki67 as a single staining and an evaluation of the cellularity of the slide.

Covariates

We evaluated the median interval between the sampling and the immunostaining (192 days) and between the immunostaining and the microscopic evaluation (418 days), and defined the reports as recent, when the time lag was shorter, and old, when it was longer than the median interval. Based on the interval between the sampling and the immunostaining, we divided the reports into two groups: recent vs old; based on the median interval between the immunostaining and the microscopic evaluation, we divided the reports into three groups: first and second reports both recent; first report recent and second old; both the interpretations old.

Women in the study who were positive for CIN2+ were those who had a histology of ≥CIN2. Women in the study who were negative for CIN2+ were those who had a histology of <CIN2, those who did not have a biopsy taken under colposcopy, or those who resulted negative at HPV re-testing at 1 year follow up. All diagnoses obtained within 24 months of recruitment were included.

Data analysis

We defined concordance for adequacy (evaluable/not evaluable) considering all cases, and for positivity (positive/negative) considering only the cases with all evaluable reports. For all the analyses we considered the presence of one double-stained cell as the cut-off, except when evaluating the overall concordance and the concordance in cases with or without CIN2+, for which we reported both the 1-cell positivity and the 5-cell positivity cut-off.

Concordance was assessed by the kappa (K) statistics for multiple readers. [24] Kappa values > 0.75 are regarded as excellent agreement beyond chance, values <0.40 as poor agreement beyond chance, and values between 0.40 and 0.75 as fair to good agreement beyond chance. [25] Ninety-five percent confidence intervals (95% CI) of the kappa values were calculated, using the bootstrap method with bias correction and 1000 simulations. Multiple-rater kappa was computed also by stratifying by the presence or absence of CIN2+, by time from sampling to slide preparation and immunostaining (recent vs old), and by lab preparing and staining the slides. Two-rater kappa statistic was computed considering each pair of reports, stratified by the following variables: pair of readers (independently of the order of interpretation); order of reading (1^{st} vs 2^{nd}; 2^{nd} vs 3^{rd}; and 1^{st} vs 3^{rd}); time elapsed between immunostaining and reading (first recent vs second recent; first recent vs. second old; both old). We also built logistic models to identify the determinants of agreement between reports for cases with all evaluable reports. We calculated odds ratios (OR) and 95% CI for the odds of each pair of reports being in agreement or not in the groups defined by the following variables: time from sampling to slide immunostaining, time from immunostaining to interpretation of the slide, order of reading, lab preparing and staining the slide, and presence or absence of CIN2+ lesions. All the analyses were performed using the STATA 13.0 statistical package.

RESULTS

Of the 3180 HPV-positive cases, 80 cases were not available for staining. Thus, a total of 3100 liquid-based cervicovaginal slides were immunostained. Overall, we obtained 9300 reports equally distributed among the 7 participating centers (range 12.9%-15.2% of the total reports). Of the 9300 reports 905 were inadequate (9.7%): 325 first, 269 second, and 311 third reports. When we took into consideration only the 8395 evaluable reports, we observed 2632 positive (31.4%) and 5763 negative readings (68.6%) (Table1).

Among the 21 pairs of readers, we observed fair concordance for adequacy in 5 pairs (Lab1-Lab2, Lab1-Lab6, Lab2-Lab3, Lab2-Lab4, Lab2-Lab6), and disagreement in two pairs (Lab3-Lab4, Lab3-Lab6). The concordance for positivity was good for 18 pairs of readers, whereas it was poor for three pairs and rather lower than the overall concordance (Lab1-Lab7, Lab2-Lab7, Lab4-Lab7) (Table 2).

Table 2. Interobserver K values for p16/ki67 positivity and for adequacy by combinations of readers

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
Lab 1							
Lab 2	0.490 (0.385; 0.594) (n=1008) **0.722 (0.674; 0.770) (n=906)**						
Lab 3	0.137 (-0.130; 0.405) (n=291) **0.757 (0.674; 0.840) (n=279)**	0.480 (0.297; 0.663) (n=189) **0.569 (0.430; 0.708) (n=154)**					
Lab 4	0.044 (-0.077; 0.164) (n=302) **0.749 (0.659; 0.839) (n=238)**	0.461 (0.349; 0.574) (n=502) **0.829 (0.764; 0.893) (n=403)**	0.000 (0.000; 1.000) (n=100) **0.657 (0.473; 0.840) (n=91)**				
Lab 5	0.114 (-0.000; 0.229) (n=290) **0.581 (0.470; 0.692) (n=234)**	0.376 (0.224; 0.528) (n=189) **0.474 (0.312; 0.636) (n=129)**	0.233 (0.151; 0.314) (n=896) **0.575 (0.513; 0.638) (n=741)**	0.167 (0.044; 0.291) (n=445) **0.544 (0.435; 0.652) (n=379)**			
Lab 6	0.420 (0.247; 0.593) (n=456) **0.554 (0.476; 0.633) (n=415)**	0.427 (0.289; 0.565) (n=456) **0.561 (0.479; 0.644) (n=392)**	-0.024 (-0.046; -0.002) (n=301) **0.664 (0.572; 0.756) (n=284)**	0.195 (0.098; 0.293) (n=595) **0.561 (0.478; 0.643) (n=497)**	0.188 (0.076; 0.299) (n=445) **0.529 (0.431; 0.628) (n=330)**		
Lab 7	0.016 (-0.033; 0.065) (n=250) **0.374 (0.272; 0.475) (n=187)**	0.067 (-0.011; 0.145) (n=450) **0.299 (0.231; 0.367) (n=315)**	0.195 (0.093; 0.297) (n=817) **0.628 (0.570; 0.687) (n=718)**	0.101 (0.007; 0.195) (n=450) **0.363 (0.284; 0.442) (n=343)**	0.109 (-0.010; 0.228) (n=416) **0.570 (0.478; 0.661) (n=331)**	0.222 (0.074; 0.371) (n=451) **0.623 (0.547; 0.699) (n=395)**	

In bolt K for positivity

The overall concordance for adequacy was poor (multi-reader-K=0.224, 95% CI: 0.183 - 0.263). However, considering only the consecutive first and second interpretations, the kappa value increased to 0.345 (95% CI 0.292 – 0.398). Taking into account only the evaluable reports, the overall concordance for positivity was moderate (K=0.583; 95% CI 0.556 - 0.610). When we considered >5 positive cells as the cut-off for positivity, we obtained a slightly lower value (K=0.506, 95% CI 0.468 - 0.538). Considering only the first and the second reports, the kappa value for positivity rose to 0.662 (95% CI 0.631 - 0.692). When the time interval between the sampling and the immunostaining was shorter, the concordance for adequacy was no better (K=0.190, 95% CI: 0.135 - 0.247), while for concordance for positivity, it was slightly better (K=0.609, 95% CI: 0.574 – 0.643).

Concordance for both adequacy and positivity were higher when both reports were in the same period, i.e., both recent or both old, than when the first reading was recent and the second old (Table 3).

Table 3. Overall concordance for adequacy and for positivity according to different parameters

	Concordance for adequacy			Concordance for positivity		
	N slides	% evaluable	Kappa (95% CI)	N slides	% positive	Kappa (95% CI)
Overall* (at least 1 cell +)	3100	77.8	0.224 (0.183 - 0.263)	2413	22.0	0.583 (0.556 - 0.610)
Overall* (at least 5 cells +)				2409	6.5	0.506 (0.468 - 0.538)
Order of reading**						
1st vs 2nd	3100	84.7	0.345 (0.292 - 0.398)	2627	23.8	0.662 (0.631 - 0.692)
1st vs 3rd	3100	82.2	0.184 (0.135 - 0.232)	2549	25.8	0.564 (0.530 - 0.597)
2nd vs 3rd	3100	83.4	0.145 (0.096 - 0.193)	2585	22.8	0.547 (0.514 - 0.581)
Laboratory preparing the slide*						
Lab 1	593	77.2	0.163 (0.091 - 0.251)	458	24.5	0.702 (0.654 - 0.761)
Lab 2	889	73.2	0.312 (0.237 - 0.379)	651	23.8	0.479 (0.423 - 0.530)
Lab 6	801	78.7	0.212 (0.138 - 0.286)	630	17.3	0.559 (0.502 - 0.608)
Lab 7	817	82.5	0.124 (0.056 - 0.208)	674	22.8	0.613 (0.566 - 0.663)
Time from sampling to immunostaining*,^						
Recent	1552	82.1	0.190 (0.135 - 0.247)	1274	23.2	0.609 (0.574 - 0.643)
Old	1548	73.6	0.477 (0.406 - 0.548)	1139	20.6	0.551 (0.513 - 0.590)
Time from immunostaining to interpretation of the slide,°**						
First report recent vs second report recent	2880	85.2	0.258 (0.202 - 0.314)	2455	23.9	0.699 (0.668 - 0.730)
First report recent vs second report old	3562	80.0	0.196 (0.152 - 0.240)	2849	24.9	0. 498 (0.465 - 0.531)
First report old vs second report old	2858	86.0	0.227 (0.170 - 0.283)	2457	23.4	0.594 (0.560 - 0.628)
Presence of CIN2+* (at least 1 cell+)						
CIN2+				158	67.7	0.487 (0.429 - 0.534)
Non-CIN2+				1902	18.8	0.558 (0.528 - 0.588)
Presence of CIN2+* (at least 5 cells+)						
CIN2+				156	27.6	0.427 (0.327 - 0.537)
Non-CIN2+				1900	4.8	0.461 (0.419 - 0.506)

(*) multiple rater (three readers); (**) two readers; (^) recent when the time lag was shorter than the median interval between the sampling and the immunostaining (192 days); old when it was longer than 192 days; (°) recent when the time lag was shorter than the median interval between the immunostaining and the interpretation (418 days); old when it was longer than 418 days

All the 176 cases in which a CIN2+ lesion was found in the HPV DNA-positive women were immunostained. Of these, 158 had an evaluable p16/ki67 result: 107 were positive in all three reports (sensitivity for detection of CIN2+: 67.7%, 95% CI: 59.8% - 74.9%), 23 in two out of the three reports (sensitivity of majority report: 82.3%, 95% CI: 75.4% - 87.9%), and 15 in one out of the three reports (sensitivity of at least one positive result: 91.8%, 95% CI: 86.3% - 95.5%). Thirteen CIN2+ cases were negative in all the three reports (Figure 1).

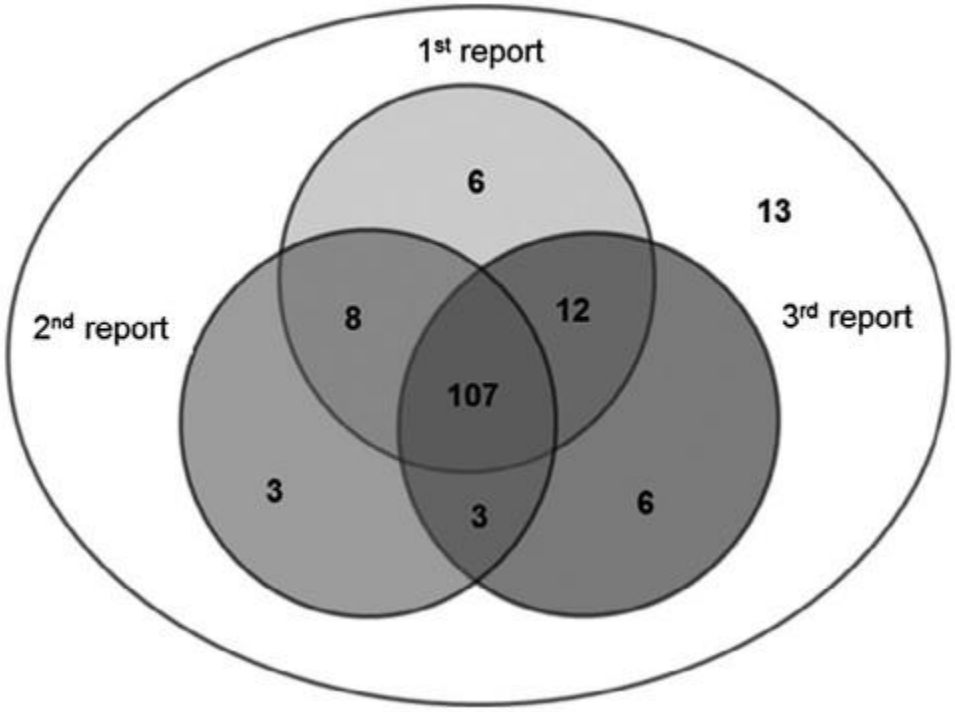

Figure 1. Venn diagram of the p16/ki67 positivity distribution in CIN2+ cases

The overall concordance for positivity in CIN2+ samples was K=0.487 (95% CI: 0.429 - 0.534), while in the non-CIN2+ samples it was K=0.558 (95% CI: 0.528 - 0.588). When we evaluated the overall concordance for positivity considering >5 positive cells as the cut-off, we estimated the K=0.427 (95% CI: 0.327 - 0.537) in CIN2+ samples and the K=0.461 (95% CI: 0.419 - 0.506) in the non-CIN2+ samples (Table 3).

Multivariate analysis (Table 4) confirmed the effect on agreement of most of the variables observed in the kappa analysis except for the time between sampling and slide immunostaining, for which we observed higher agreement for more recent slides (OR=1.128, 95% CI: 0.977 - 1.303). Differences in agreement according to the laboratory which performed immunocytochemistry remain in the multivariate analysis. We observed a higher agreement in cases CIN2+ than in cases without CIN2+ (OR=1.236, 95% CI: 0.959 - 1.594), although kappa for positivity was lower. This effect is simply due to the very high proportion of p16/ki67-positive reports among CIN2+ cases, regardless of the lab reader, and this imbalance in the judgements increases the agreement due to chance. Therefore, the kappa value and the proportion of agreement may have opposite behavior.

Table 4. Multivariate logistic model: determinants of agreement between readers

	OR	P-value	95% CI
Time from sampling to immunostaining^			
Recent	1		
Old	1.128	0.101	0.977-1.303
Time from immunostaining to interpretation of the slide°			
First report recent vs second report recent	1		
First report recent vs second report old	0.594	0.000	0.475-0.743
First report old vs second report old	0.983	0.902	0.742-1.300
Order of reading			
1st vs 2nd	1		
1st vs 3rd	0.750	0.000	0.643-0.875
2nd vs 3rd	0.621	0.000	0.526-0.734
Laboratory preparing the slide			
Lab 2	1		
Lab 1	1.597	0.001	1.206-2.113
Lab 6	1.195	0.054	0.997-1.431
Lab 7	1.572	0.000	1.314-1.880
Presence of CIN2+			
No	1		
yes	1.236	0.102	0.959-1.594
No colpo*	0.974	0.756	0.826-1.149

(^) recent when the time lag was shorter than the median interval between the sampling and the immunostaining (192 days); old when it was longer than 192 days;
(°) recent when the time lag was shorter than the median interval between the immunostaining and the interpretation (418 days); old when it was longer than 418 days;
(*) women not compliant to colposcopy referral
Pair of reports are the primary statistical unit; variance estimate takes into account non-independence of different pairs on the same slide
Only slides with all valid reports are included (2587 slides, 7761 pairs of reports)
OR>1 indicates higher probability of agreement than in the reference group

DISCUSSION

To date, guidelines recommend cytology as the triage test for HPV DNA-positive women. [1-6] Nonetheless, several new biomarkers have been developed which are able to suggest the active transcription of the viral E6/E7 oncogenes instead of the mere presence of viral DNA. Among them, the dual staining for p16 and ki67 proteins is one of the most promising and it has been largely validated in selected as well as in screening populations. [16, 26-28] However, limited data have been published on the reproducibility of dual staining interpretation on selected series [21, 22], and some articles have considered only positivity agreement. [19, 20] To the best of our knowledge, NTCC2 is the first study in which the reproducibility of the test interpretation has been investigated on a large unselected screening population, taking into consideration agreement both for adequacy and for positivity, to better investigate the role of this biomarker as a triage test of HPV positivity in real screening practice. In fact, besides the accuracy for CIN2+ lesions, to be applied to the screening as a triage test, a putative biomarker should also have been evaluated in other respects, for example the reproducibility of its interpretation. In particular, for a potentially useful biomarker in prevention, the standardization of the interpretation of positivity is of the utmost importance. In cytological samples, the interpretation of p16 immunostaining alone lacks standardization [29], thus making the evaluation of this biomarker somewhat difficult. Instead, the criteria and the cut-off for dual staining are well established. However, as the test requires a microscopic evaluation, it is affected by subjectivity. Despite the very few criteria for positivity, which should reduce subjectivity of interpretation compared to Pap test, reproducibility obtained in our study was surprisingly comparable to that observed for Pap test. [10-12] As a matter of fact, p16/ki67 double staining is not always easy to interpret and many factors may cause misinterpretation. For example, the interpretation of staining in cell groups and/or the detection of dual staining in cells with scant cytoplasm or in samples with a weak p16 staining background may lead to interpretation problems. [18, 22] Nevertheless, low reproducibility does not impact on sensitivity. Indeed, the majority of the CIN2+ cases were positive in all the three reports. Moreover, when we considered cases with two out of three positive reports, sensitivity for CIN2+ was high and in line with values observed in other studies on screening populations [16, 26, 27], and even higher considering the cases with at least one positive report. The kappa value for positivity was similar but slightly lower than those calculated in the previous studies on dual staining reproducibility. [19-21] However, several differences among studies have to be taken into consideration, making it difficult to compare our findings with those of previous studies. In the study by Wentzensen et al., only 320 selected HPV-positive cases were analyzed by more than 2 readers and the reproducibility analysis was carried out considering a reference interpretation for comparisons. Conversely, in the present study a nearly 10 times bigger series of consecutive and unselected HPV-positive samples was analyzed by 3 readers and the agreement was assessed by the kappa statistic for multiple readers. Furthermore, Wentzensen's study was conducted on SurePath-based liquid cytology, which might have introduced a further source of variability. Allia's study included 500 selected cases, each interpreted by 7 readers from the

same center. This characteristic of the study may have increased the agreement found among readers as they may have a similar approach to immunocytochemistry interpretation. In a previous study by our group, we investigated p16/ki67 reproducibility on a small number of cases because our primary aim was to generate agreement on the criteria to be used in the NTCC2 trial. [21] To this end, we included a large proportion of difficult and equivocal cases, and readers were allowed to classify ambiguous cases as inconclusive. This classification is obviously not acceptable in clinical practice, in which these cases would give rise to test repetition or inadequate reports. Therefore, it did not represent a real population of HPV-positive women. Nevertheless, in our previous study, in which many of the readers were the same as those included in the present study, we obtained similar or slightly better kappa values. This suggests that there is no short-term learning curve leading to higher agreement.

Limits

The present study presents some limitations. First of all, the reports were not always obtained in conditions reflecting a routine triage setting, due both to the time elapsed between sample collection and slide immunostaining, and to the time between immunostaining and interpretation. The time between sample collection and immunostaining showed a modest effect on kappa. Instead, the time between immunostaining and interpretation showed a larger effect, particularly when we compared the interpretation made when the slide was recently prepared with the interpretation made when the slide was old. Moreover, the slide for immunocytochemistry was obtained after that for Pap test. As reported by McMenamin et al., performing CINtec PLUS cytology post cervical cytology may increase the number of paucicellular samples. [22] However, this will not happen in real clinical practice if dual staining is adopted instead of cytology as triage test. Moreover, regarding inadequacy for staining problems, it is possible in a screening setting to repeat uncertain cases. In this study, the above-mentioned technical issues could have increased the proportion of inadequate reports and decreased the reproducibility of the judgement. In addition, even if a large number of immunostained slides were interpreted in a pragmatic trial involving many laboratories in both research institutes and routine screening centers, readers were aware that the report was only to be used for research purposes. Thus, readers did not have the responsibility of clinical management of the women. However, we included a large sample size and we drew up a well-balanced study design, which allowed the investigation of many possible determinants of the concordance.

Conclusion and implication for practice

In conclusion, in a large, consecutive, unselected HPV-positive screening population, the dual-staining for p16/ki67 showed poor agreement for adequacy and good reproducibility for positivity, comparable to that of cervical cytology. The reproducibility of triage for HPV positivity was therefore not improved, and a crucial issue in cervical cancer screening was not resolved. Implementation efforts and training may be needed for a better standardization in the interpretation of the double staining, but no evident improvement of reproducibility was obtained in this second study of our

group compared to the previous one, at least in the medium-short time frame of this study. If low reproducibility does not impact too much on sensitivity, it does impact on specificity, thus if a better standardization will be achieved, there is room for an improvement of test accuracy. Further investigations on accuracy, as well as analyses of the feasibility and the costs, are needed for adoption of this biomarker as a triage test in cervical cancer screening programs with HPV DNA as primary test.

Bibliography

1. Ronco G, Biggeri A, Confortini M, et al. [Health technology assessment report: HPV DNA based primary screening for cervical cancer precursors]. *Epidemiol Prev*. 2012;36: e1-72.
2. Ronco G, Arbyn M, Meijer CJLM, Snijders PJF, Cuzick J. Screening for cervical cancer with primary testing for human papillomavirus. S1. In: Anttila A, Arbyn M, De Vuyst H, et al. editors. European guidelines for quality assurance in cervical cancer screening. Second edition, Supplements. Luxembourg: Office for Official Publications of the European Union. 2015; p. 1–68.
3. Anttila A, Arbyn M, de Vuyst H, et al, editors. European guidelines for quality assurance in cervical cancer screening. Second edition, Supplements. Luxembourg: Office for Official Publications of the European Communities. 2015.
4. 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. *Am J Clin Pathol*. 2012;137(4): 516-42.
5. Huh WK, Ault KA, Chelmow D, .et al. Use of primary high-risk human papillomavirus testing for cervical cancer screening: interim clinical guidance. *Obstet Gynecol*. 2015;125: 330–337.
6. US Preventive Services Task Force Screening for Cervical Cancer US Preventive Services Task Force Recommendation Statement. *JAMA*. 2018;320(7): 674-686.
7. Bruni L, Albero G, Serrano B, et al. ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the World. Summary Report 17 June 2019, [Internet], [cited 30 September 2019]. Available from https://www.hpvcentre.net/statistics/reports/XWX.pdf
8. International Agency for Research on Cancer. Cervix Cancer Screening. Lyon: IARC Handbooks of Cancer Prevention, Volume 10 , 2005.
9. World Health Organization. Guidelines for screening and treatment of precancerous lesions for cervical cancer prevention. Geneva: WHO guidelines, 2013.
10. Stoler MH, Schiffman M. Interobserver reproducibility of Cervical Cytologic and Histologic Interpretations: realistic estimates from the ASCUS-LSIL triage study. *JAMA.* 2001;285(11): 1500-1505.
11. Confortini M, Bondi A, Cariaggi MP, et al. Interlaboratory reproducibility of Liquid-Based Equivocal Cervical Cytology Within a Randomized Controlled Trial Framework. *Diagn Cytopathol.* 2007; 35(9): 541-44.
12. Kurtycz DFI, Staats PN, Chute DJ, et al. Bethesda Interobserver Reproducibility Study-2(BIRST-2): Bethesda System 2014. *J Am Soc Cytopathol*. 2017; 6: 131e144.
13. Clarke MA, Cheung LC, Castle PE, et al. Five-Year Risk of Cervical Precancer Following p16/Ki-67 Dual-Stain Triage of HPV-Positive Women. *JAMA Oncol.* 2019;5: 181–186.
14. Wentzensen N, Schiffman M, Palmer T, Wentzensen, Arbyn M. Triage of HPV positive women in cervical cancer screening. *J Clin Virol.* 2016;76 Suppl 1:S49–S55.
15. Gustinucci D, Rossi PG, Cesarini E, et al. Use of Cytology, E6/E7 mRNA, and p16INK4a-Ki-67 to Define the Management of Human Papillomavirus (HPV)-Positive Women in Cervical Cancer Screening. *Am J Clin Pathol.* 2016;145: 35–45.
16. Wentzensen N, Clarke MA, Bremer R, et al. Clinical Evaluation of Human Papillomavirus Screening With p16/Ki-67 Dual Stain Triage in a Large Organized Cervical Cancer Screening Program. *JAMA Intern Med*. 2019;179: 881–888.
17. Carozzi F. Triage markers for HPV-positive women: p16 vs p16/ki67. Paper presented at:

EUROGIN 2018 - International Multidisciplinary HPV Congress; December 02-05, 2018; Lisbon, Portugal.

18. Waldstrom M, Christensen RK, Ornskov D. Evaluation of p16(INK4a)/Ki-67 dual stain in comparison with an mRNA human papillomavirus test on liquid-based cytology samples with low-grade squamous intraepithelial lesion. *Cancer Cytopathol.* 2013;121: 136–145.
19. Wentzensen N, Fetterman B, Tokugawa D, et al. Interobserver reproducibility and accuracy of p16/Ki-67 dual-stain cytology in cervical cancer screening. *Cancer Cytopathol*. 2014;122: 914–920.
20. Allia E, Ronco G, Coccia A, et al. Interpretation of p16(INK4a) /Ki-67 dual immunostaining for the triage of human papillomavirus-positive women by experts and nonexperts in cervical cytology. *Cancer Cytopathol.* 2015;123: 212–218.

21. Benevolo M, Allia E, Gustinucci D, et al. Interobserver reproducibility of cytologic p16(INK4a) /Ki-67 dual immunostaining in human papillomavirus-positive women. *Cancer Cytopathol.* 2017;125: 212–220.
22. McMenamin M, McKenna M, McDowell A, Dawson C, McKenna R. Intra- and inter-observer reproducibility of CINtec((R)) PLUS in ThinPrep((R)) cytology preparations. *Cytopathology*. 2017;28: 284–290.
23. Solomon D, Davey D, Kurman R, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. *JAMA.* 2002;287: 2114–2119.
24. Fleiss JL. measuring nominal scale agreement among many raters. *Psychol Bullettin.* 1971;76:378–382.
25. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics*. 1977;33: 159–174.
26. Stanczuk GA, Baxter GJ, Currie H, et al. Defining Optimal Triage Strategies for hrHPV Screen-Positive Women-An Evaluation of HPV 16/18 Genotyping, Cytology, and p16/Ki-67 Cytoimmunochemistry. *Cancer Epidemiol Biomarkers Prev*. 2017;26: 1629–1635.
27. Wright TC Jr, Behrens CM, Ranger-Moore J, et al. Triaging HPV-positive women with p16/Ki-67 dual-stained cytology: Results from a sub-study nested into the ATHENA trial. *Gynecol Oncol.* 2017;144: 51–56.
28. Ebisch RM, van der Horst J, Hermsen M, et al. Evaluation of p16/Ki-67 dual-stained cytology as triage test for high-risk human papillomavirus-positive women. *Mod Pathol.* 2017;30: 1021–1031.
29. Tsoumpou I, Arbyn M, Kyrgiou M, et al. p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. *Cancer Treat Rev.* 2009;35:210–220.

3.4. Research article #05: Determinants of p16/Ki-67 Adequacy and Positivity in HPV-Positive Women From a Screening Population

The findings of this research article were published in *Cancer Cytopathology*.

Benevolo M, Mancuso P, Allia E, Gustinucci D, Bulletti S, Cesarini E, Carozzi FM, Confortini M, Bisanzi S, Rubino T, Rollo F, Marchi N, Farruggio A, Pusiol T, Venturelli F, Giorgi Rossi P; New Technologies for Cervical Cancer 2 (NTCC2) Working Group. Determinants of p16/Ki-67 adequacy and positivity in HPV-positive women from a screening population. Cancer Cytopathol. 2020 Nov 3. doi: 10.1002/cncy.22385. Epub ahead of print. PMID: 33142029.

Abstract

Background: The aim of this study was to describe the determinants of adequacy and positivity of the p16/ki67 assay in an HPV-positive screening population enrolled within the NTCC2 study.

Methods: ThinPrep slides were immunostained for p16/ki67; each slide had three reports from different labs. We included population-related, sampling/staining-related, and interpretation-related variables in the analyses. Adequacy and positivity proportions were stratified by variables of interest. Univariate and multivariate logistic models identified determinants of adequacy and positivity.

Results: We analyzed 3100 consecutive HPV-positive cases. Since every slide was interpreted by 3 centers, we obtained 9300 reports: 905 (9.7%) were inadequate and 2632 (28.3%) were positive. The percentage of cases with all 3 reports inadequate increased with increasing age of the women and with inadequate cytology. The highest percentage of adequacy in all 3 reports as well as of cases with all 3 reports positive was observed in specimens from women with a CIN2+, with ASC-US+ cytology, or with mRNA positivity. The number of inadequate reports was significantly associated with increasing age, inadequate cytology, mRNA negativity, and scant cellularity. A positive p16/ki67 report was associated with an ASC-US+ result and with a positive mRNA result, both in cases with and without CIN2+, but with an HPV16/18 infection only in CIN2+ cases. The presence of CIN2+ was strongly associated with dual staining positivity.

Conclusions: p16/ki67 interpretation may be influenced by several different variables which are part of all the steps of the procedure, and by the characteristics of the screened population.

Trial registration: Clinicaltrials.gov registration number: NCT01837693, New Technology in Cervical Cancer 2 (NTCC2) study

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INTRODUCTION

Cervical cytology is the most used triage test for HPV-positive women in cervical cancer screening and is recommended by national and international guidelines. [1, 2] Dual staining for the simultaneous detection of $p16^{INK4a}$ and ki67 represents one of the most promising tests which could substitute cytology in triaging HPV-positive women. The concomitant expression of p16 and ki67 within the same cell is suggestive of a transforming infection and may serve as an indicator of a deregulated cell cycle caused by HPV oncoproteins. In fact, under physiological conditions, the co-expression of these proteins does not occur since they typically induce opposite effects; while p16 has an anti-proliferative activity, triggering cell cycle arrest, ki67 is a proliferation-associated protein detectable only in cycling cells. Dual staining for p16 and ki67 proteins has been largely validated on selected as well as on screening populations. [3-6] It has been shown to be very accurate in referral populations, with very high sensitivity and acceptable specificity compared to cytology. However, the test currently involves microscopic evaluation and is thus affected by subjective interpretation. Even if the positivity criteria and the settlement cut-off for positivity are well established, the interpretation reproducibility for positivity is not as high as expected and is comparable to that of the Pap test (Allia E. 2015: k=0.70; Benevolo M. 2017: k=0.61; Wentzensen N. 2014: k=0.71; McMenamin M. 2017: k=0.83-0.88; Benevolo M. 2020: k=0.58). [7-11] In addition, many studies have observed a variable but not negligible percentage of inadequate cases (Ebish RM. 2017: 46%; Clarke MA. 2019: 8.2%; Wright TC Jr 2017: 3.7%; Stanczuk GA.2017: 7-8%; Wentzensen N. 2012: 7.1%; Wentzensen N. 2015: 6.3%; Wentzensen N. 2019: 2.5%; Stoler MH. 2020: 8%). [3-6, 12-15] Agreement for adequacy was far less investigated because most of the previous studies excluded the inadequate cases from the analyses. In a recent study from our group, it was poor (k=0.224). [11]

Many factors can introduce variability in dual staining interpretation and may influence its adequacy and positivity. Even using the same assay and reporting the results according to the same criteria, many variables linked to the characteristics of the population under study, the laboratory which performs the immunostaining, and/or which interprets the slides should be taken into consideration. Very few studies have investigated the determinants of p16/ki67 positivity in slide interpretation and the determinants of adequacy of both specimens and slides in real screening practice. [10, 11]

We conducted this study within the New Technologies for Cervical Cancer 2 (NTCC2) project, a large randomized clinical trial conducted in organized cervical screening programs in Italy using HPV DNA as primary test, which aimed at evaluating new biomarkers as triage test of HPV DNA positivity in comparison to cytology. [16] Among others, dual staining was used as triage test of HPV DNA positivity.

The aim of the present study is to investigate the determinants of adequacy and positivity of the p16/ki67 assay, taking into account several variables related to the individual women, to the cell samples, and to the immunostaining process and interpretation.

MATERIALS AND METHODS

Setting and study design

As described elsewhere, cervicovaginal specimens were collected in 5 Italian centers (Turin, Florence, Perugia, Trento, and Veneto) and stored in ThinPrep vials (Hologic) between April 2014 and February 2017. [16] The HPV DNA test was performed by Cobas 4800 (Roche Diagnostics) or Hybrid Capture 2 (HC2, Qiagen). HPV DNA-positive women were triaged with cytology and were tested first with mRNA assay and the residual material with p16/ki67 dual staining. From all the HPV DNA-positive women, 2 slides (the first for cytology, the second for immunostaining) were prepared from PreservCyt liquid material using a ThinPrep 2000 or ThinPrep 5000 Processor (Hologic). Morphological assessment was carried out in the local screening pathology laboratory, according to the Bethesda 2001 system classification. [17]

p16/ki67 immunostaining

The second liquid-based slide was fixed in 99% ethanol for 10 minutes/1 hour and then air-dried.

Dual staining was performed in four participating centers (Turin, Perugia, Florence, and Rome) using the CINtec® PLUS Cytology kit (Roche Diagnostics), according to the manufacturer's instructions, on Benchmark XT or ULTRA VENTANA automated systems (Roche Diagnostics).

Each slide was interpreted independently and blinded to cytology, histology, and E6/E7 mRNA results. All readers have been trained in the interpretation of CINtec® PLUS. Slides were considered positive when at least one cervical cell showed a brown cytoplasmic and a red nuclear staining simultaneously. Slides without any evident double-immunoreaction within the same cell, i.e., showing immunoreactivity for none or only one of the two markers, were referred as negative.

As for pap test interpretation, according to the 2001 Bethesda System criteria, immunostained slides with fewer than 5,000 cells were considered unsatisfactory for dual staining evaluation unless they contained double-stained cells. In addition, immunostained slides were also deemed inadequate for interpretation in cases of immunostaining decay or too weak counterstaining. Thus, regarding p16/ki67 dual staining, insufficient cellularity and staining problems were considered together as a source of inadequate interpretation.

Each reader reported positive and negative results and adequacy for each slide. The presence of either p16 or ki67 as a single staining was also recorded. The immunostained slides circulated among seven centers in order to ensure three interpretations for each slide and so that all the possible couples of readers were guaranteed. The design of the concordance study and the scheme of reader triplets have been described elsewhere. [11]

To assign each slide to a different category (inadequate, positive, or negative), the first report was considered. Otherwise, the criteria for assignment were based on the 3 available interpretations. When all three reports were adequate, we considered the majority diagnosis. If only two reports were informative, we considered the report of the first interpretation.

HPV mRNA Aptima test

The Aptima HPV assay (Hologic) detects E6/E7 viral mRNA from 14 HR HPV types detected as a pool, as described elsewhere. [16] The assay was performed in two participating centers on the Panther system (Hologic) according to the manufacturer's instructions.

Endpoints and Variables of interest

p16/ki67 adequacy and positivity were considered as the endpoints. We included in our analyses woman-related variables, i.e., age, presence of grade 2 or higher cervical intraepithelial neoplasia (CIN2+), cytology, and mRNA result. Women were positive for CIN2+ when they had a confirming histological diagnosis. Women were considered negative for CIN2+ if they had a histology of CIN1 or less, if they had negative colposcopy with no biopsy performed, or if they had negative cytology and HPV testing at 12 month-retesting. Partial genotyping was taken into consideration as a variable only for HPV-positive women tested by Cobas.

Among the sampling/staining-related variables, we investigated the role of specimen cellularity, of the time interval between sampling and immunostaining, of the recruiting center, and of the lab that prepared the slide and performed the immunostaining.

Finally, among the interpretation-related variables, we considered the center which interpreted the immunostaining and the time between immunostaining and interpretation of the slide. We evaluated the median time interval between sampling and immunostaining (192 days) and between immunostaining and microscopic evaluation (418 days), and we defined the report as recent when the time lag was shorter than the median interval and as old when it was longer than the median interval.

For the purpose of the study, the seven Italian centers involved in at least one step (recruitment, immunostaining, or interpretation) were identified as Lab1 to Lab7.

Analyses

We summarized reading results for each slide overall and stratified by variables of interest. For p16/ki67 adequacy we considered all cases; for positivity we considered only cases with three adequate reports. Adequacy and positivity proportions are reported stratified by variables of interest.

We also performed univariate and multivariate logistic models to identify determinants of inadequate reports and of positivity, adjusted for age, recruiting center, reading center, and time between

immunostaining and interpretation and between sampling and immunostaining. Models for positive reports were split for women with or without a histologically confirmed CIN2+.
Statistical analyses were performed with STATA 13.

RESULTS

Overall, we analyzed 3100 HPV-positive cases. The mean age was 45.5 years. Considering only the first interpretation, 35.9% were inadequate cases and 34.1% were positive cases; when considering the majority diagnosis, we found 5.3% (165/3100) and 26.9% (833/3100) of inadequate and positive cases, respectively. Table 1 shows the distribution of inadequate slides according to the variables under study. As each slide received three interpretations, we divided the cases into 4 groups: cases with all 3 reports evaluable, those with 2 of 3 reports evaluable, those with only 1 report adequate, and those with all 3 reports inadequate. Overall, we found 2413 cases with all 3 reports adequate (77.8%) and only 53 cases with all 3 reports inadequate (1.7%). We observed that the percentage of cases with all 3 reports inadequate increased with the increasing age of the women and was the highest over age 55. Moreover, we found a strong association with inadequate cytology. On the other hand, cases from women with a CIN2+ lesions, with ASC-US+ cytology, or with mRNA positivity showed the highest percentage of adequacy in all 3 reports. HPV typing, center that recruited, or that which performed immunocytochemistry had no effect on adequacy.

Table 2 shows the distribution of cases with all 3 reports evaluable (n=2413, mean age= 45.4 years), stratified by variables under study. In this case as well, we divided the cases into four groups according to p16/ki67 results: all three reports positive, 2 positive and 1 negative, 2 negative and 1 positive, all 3 reports negative. Overall, we observed that 530 cases had all 3 reports positive (22%), whereas 1195 (49.5%) had all 3 reports negative. Neither age, recruiting center, nor lab that immunostained the slides had any effect on the distribution of p16/ki67-positive interpretations. On the other hand, a higher proportion of cases with all 3 reports positive was observed among women with a CIN2+ lesion, with ASC-US+ cytology, with positive mRNA result, or with HPV16/18 infection.

Table 1. Distribution of cases according to p16/ki67 adequacy stratified by variables under study

	All adequate n (%)	Discordant 2:1* n (%)	Discordant 1:2** n (%)	All inadequate n (%)	Total n
Overall	**2413 (77.8)**	**522 (16.8)**	**112 (3.6)**	**53 (1.7)**	**3100**
Age (mean)	45.4	45.6	44.9	49.9	45.5
Age					
25-34	237 (80.9)	43 (14.7)	12 (4.1)	1 (0.3)	293
35-44	964 (77.6)	211 (17.0)	55 (4.4)	13 (1.0)	1243
45-54	872 (78.5)	193 (17.4)	24 (2.2)	22 (2.0)	1111
55+	340 (75.1)	75 (16.6)	21 (4.6)	17 (3.8)	453
Presence of CIN2+					
CIN2+	158 (89.8)	12 (6.8)	5 (2.8)	1 (0.5)	176
Not CIN2+	1902 (77.5)	420 (17.1)	91 (3.7)	42 (1.7)	2455
No assessment***	353 (75.3)	90 (19.1)	16 (3.4)	10 (2.1)	469
Cytology result					
Negative	1719 (75.2)	432 (18.9)	89 (3.8)	45 (1.9)	2285
ASC-US+[§]	673 (87.4)	77 (10)	16 (2)	4 (0.5)	770
Inadequate	15 (41.7)	11 (30.5)	6 (16.6)	4 (11.1)	36
Missing	6 (66.7)	2 (22.2)	1 (11.1)	0 (0)	9
mRNA result					
Negative	729 (71.5)	223 (21.8)	43 (4.2)	25 (2.4)	1020
Positive	1679 (81)	296 (14.2)	69 (3.3)	28 (1.3)	2072
Missing	5 (62.5)	3 (37.5)	0 (0)	0 (0)	8
HPV typing					
16 and/or 18 (with or without other HR)	287 (77.8)	58 (15.7)	13 (3.5)	11 (2.9)	369
Other HR types	765 (75.2)	187 (18.3)	37 (3.6)	28 (2.7)	1017
Cellularity					
Good in all 3 reports	2232 (95.2)	100 (4.2)	12 (0.5)	0 (0)	2344
Scant in 1 or more reports	181 (23.9)	422 (55.8)	100 (13.2)	53 (7)	756
Recruiting center					
Lab 6	630 (78.7)	129 (16.1)	31 (3.8)	11 (1.3)	801
Lab 2	651 (73.2)	167 (18.7)	38 (4.2)	33 (3.7)	889
Lab 3	18 (64.3)	9 (32.1)	0 (0)	1 (3.5)	28
Lab 7	656 (83.1)	111 (14)	20 (2.5)	2 (0.2)	789
Lab 5	458 (77.2)	106 (17.8)	23 (3.8)	6 (1)	593
Lab which immunostained the slides					
Lab 6	630 (78.7)	129 (16.1)	31 (3.8)	11 (1.3)	801
Lab 1	458 (77.2)	106 (17.8)	23 (3.8)	6 (1)	593
Lab 2	651 (73.2)	167 (18.7)	38 (4.2)	33 (3.7)	889
Lab 7	674 (82.5)	120 (14.6)	20 (2.4)	3 (0.3)	817

*2 adequate/1 inadequate reports

**1 adequate/2 inadequate reports

***women who did not undergo colposcopy or 1-year retest

[§]ASCUS+ : ASCUS, L-SIL, H-SIL, ASC-H, AIS, CA

Table 2. Distribution of cases according to p16/ki67 positivity stratified by variables under study

VARIABLES	All positive n (%)	Discordant 2:1* n (%)	Discordant 1:2** n (%)	All negative n (%)	Total[#] n
Overall	**530 (22.0)**	**262 (10.9)**	**426 (17.7)**	**1195 (49.5)**	**2413**
Age (mean)	44.0	46.1	46.3	47.1	45.4
Age					
25-34	60 (25.3)	23 (9.7)	33 (13.9)	121 (51.1)	237
35-44	245 (25.4)	94 (9.8)	167 (17.3)	458 (47.5)	964
45-54	177 (20.3)	103 (11.8)	147 (16.9)	445 (51.0)	872
55+	48 (14.1)	42 (12.4)	79 (23.2)	171 (50.3)	340
Presence of CIN2+					
CIN2+	107 (67.7)	23 (14.6)	15 (9.5)	13 (8.2)	158
Non-CIN2+	358 (18.8)	202 (10.6)	346 (18.2)	996 (52.4)	1902
No assessment***	65 (18.4)	37 (10.5)	65 (18.4)	186 (52.7)	353
Cytology result					
Negative	230 (13.4)	152 (8.8)	324 (18.8)	1013 (58.9)	1719
ASC-US+[§]	295 (43.8)	106 (15.8)	98 (14.6)	174 (25.9)	673
Inadequate	4 (26.7)	3 (20.0)	3 (20.0)	5 (33.3)	15
Missing	1 (16.7)	1 (16.7)	1 (16.7)	3 (50.0)	6
mRNA result					
Negative	27 (3.7)	34 (4.7)	105 (14.4)	563 (77.2)	729
Positive	502 (29.9)	227 (13.5)	319 (19.0)	631 (37.6)	1679
Missing	1 (20.0)	1 (20.0)	2 (40.0)	1 (20.0)	5
HPV type					
16 and/or 18 (with or without other HR)	107 (37.3)	24 (8.4)	62 (21.6)	94 (32.8)	287
Other HR types	147 (19.2)	66 (8.6)	177 (23.1)	375 (49.0)	765
Cellularity					
Good in all 3 reports	463 (20.7)	234 (10.5)	355 (15.9)	1180 (52.9)	2232
Scant in 1 or more reports	67 (37.0)	28 (15.5)	71 (39.2)	15 (8.3)	181
Recruiting center					
Lab 6	109 (17.3)	79 (12.5)	99 (15.7)	343 (54.4)	630
Lab 2	155 (23.8)	56 (8.6)	186 (28.6)	254 (39.0)	651
Lab 3	4 (22.2)	5 (27.8)	2 (11.1)	7 (38.9)	18
Turin	150 (22.9)	83 (12.7)	86 (13.1)	337 (51.4)	656
Lab 5	112 (24.5)	39 (8.5)	53 (11.6)	254 (55.5)	458
Lab which immunostained the slides					
Lab 6	109 (17.3)	79 (12.5)	99 (15.7)	343 (54.4)	630
Lab 1	112 (24.5)	39 (8.5)	53 (11.6)	254 (55.5)	458
Lab 2	155 (23.8)	56 (8.6)	186 (28.6)	254 (39.0)	651
Lab 7	154 (22.8)	88 (13.1)	88 (13.1)	344 (51.0)	674

[#]Only cases with all 3 reports evaluable
* 2 positive/1 negative reports
** 1 positive/2 negative reports
***women who did not undergo colposcopy or 1-year retest
[§]ASCUS+ : ASCUS, L-SIL, H-SIL, ASC-H, AIS, CA

Since every slide was interpreted by 3 centers, we obtained 9300 reports overall: 905 (9.7%) were inadequate, 2632 (28.3%) were positive, and 5763 (62%) were negative. We stratified the inadequate and the positive reports according to the determinants under study (Table 3). Regarding the number of inadequate reports, we observed that it significantly increased with increasing age and was associated with inadequate cytologic results, negative mRNA results, and with scanty cellularity, whereas it was not affected by HPV typing or by both time between sampling and immunocytochemistry and time between slide immunostaining and interpretation. Considering the positive reports, they were directly associated with the presence of CIN2+, ASC-US+ cytology, positive mRNA result, and HPV16/18 infection. In addition, we observed a significant variability in the proportion of both adequate and positive reports among the centers which enrolled the women, the centers which immunostained, and those which interpreted the slides.

The univariate and multivariate analyses were performed in order to assess the association between the covariates and the adequacy of the interpretation (Table 4). We observed that the only variable strongly associated with adequacy in both the analyses was cytologic inadequacy. Age weakly increased the risk of an inadequate p16/ki67 report. As shown in Supplementary Table, the results were substantially similar when excluding from the analysis all the readings from slides that were classified as unsatisfactory for cytology (36 slides) or had a missing cytologic result (9 slides).

Table 5 reports the univariate and multivariate models for a positive interpretation, taking into consideration only the adequate reports of the women who underwent colposcopy (N=7140). A cytologic ASC-US+ result and a positive mRNA result were associated with a positive p16/ki67 report both in cases with and without CIN2+, while an HPV16/18 infection was associated only in CIN2+ cases. Women's age and the different centers that recruited, immunostained, or interpreted did not affect the probability of a positive report. Overall, the presence of a CIN2+ lesion was strongly associated with dual staining positivity.

Table 3. Proportion of inadequate and of positive reports according to variables under study

	Total reports (n)	Inadequate reports n(%)	*P*	Positive reports n(%)	*P*
Overall	**9300**	**905 (9.7)**		**2632 (28.3)**	
Age (mean)	45.5	45.5		46.2	
Age					
25-34	879	70 (8.0)		265 (30.1)	
35-44	3.729	360 (9.7)	0.002	1136 (30.5)	0.000
45-54	3.333	307 (9.2)		912 (27.4)	
55+	1.359	168 (12.4)		319 (23.5)	
Presence of CIN2+					
CIN2+	528	25 (4.7)		388 (73.4)	
Not CIN2+	7365	728 (9.8)	0.000	1897 (25.7)	0.000
No assessment*	1407	152 (10.8)		347 (24.6)	
Cytology result					
Negative	6855	745 (10.8)		1385 (20.2)	
ASC-US+	2310	121 (5.2)	0.000	1218 (52.7)	0.000
Inadequate	108	35 (32.4)		22 (20.3)	
Missing	27	4 (14.8)		7 (25.9)	
mRNA result					
Negative	3060	384 (12.5)		266 (8.6)	
Positive	6216	518 (8.3)	0.000	2357 (37.9)	0.000
Missing	24	3 (12.5)		9 (37.5)	
HPV type					
16 and/or 18 (with or without other HR)	1107	117 (10.5)	0.503	447 (40.3)	0.000
Other HR types	3051	345 (11.3)		776 (25.4)	
Cellularity					
Good	8103	144 (1.7)		2271 (28)	
Scant	939	692 (73.6)		172 (18.3)	
Missing	258	69 (26.7)	0.000	189 (73.2)	0.000
Recruiting center					
Lab 6	2403	224 (9.3)		600 (24.9)	
Lab 2	2667	342 (12.8)		792 (29.6)	
Lab 3	84	12 (14.2)	0.000	25 (29.7)	0.000
Lab 7	2367	157 (6.6)		733 (30.9)	
Lab 5	1779	170 (9.5)		482 (27.0)	
Lab which prepared the slides					
Lab 6	2403	224 (9.3)		600 (24.9)	
Lab 1	1779	170 (9.5)	0.000	482 (27.0)	0.000
Lab 2	2667	342 (12.8)		792 (29.6)	
Lab 7	2451	169 (6.8)		758 (30.9)	

	Total reports (n)	Inadequate reports n(%)	*P*	Positive reports n(%)	*P*
Center which interpreted the slides					
Lab 6	1352	137 (10.1)		421 (31.1)	
Lab 1	1299	58 (4.4)		350 (26.9)	
Lab 2	1397	147 (10.5)		389 (27.8)	
Lab 4	1197	89 (7.4)	0.000	220 (18.3)	0.000
Lab 3	1297	46 (3.5)		401 (30.9)	
Lab 7	1417	223 (15.7)		577 (40.7)	
Lab 5	1341	205 (15.2)		274 (20.4)	
Time between immunostaining and interpretation					
Recent	4661	467 (10)	0.347	1257 (26.9)	0.016
Old	4639	438 (9.4)		1375 (29.6)	
Time between sampling and immunostaining					
Recent	4656	351 (7.5)	0.000	1447 (31.1)	0.000
Old	4644	554 (11.9)		1185 (25.5)	

*women who did not undergo colposcopy or 1-year retest

Table 4. Univariate and multivariate analyses for inadequate report

Variables	Univariate*			Multivariate**		
	OR	95%IC	p	OR	95%IC	p
Age	1.01	(1.00 - 1.02)	0.05	1	(0.99 - 1.01)	0.64
Age						
25-34	1					
35-44	1.23	(0.91 - 1.68)	0.18			
45-54	1.17	(0.86 - 1.60)	0.32			
55+	1.63	(1.15 - 2.31)	0.01			
Presence of CIN2+						
Non-CIN2+	1			1		
CIN2+	0.46	(0.28 - 0.77)	0.00	0.46	(0.27 - 0.77)	0.00
No assesment[§]	1.12	(0.89 - 1.39)	0.33	1.13	(0.89 - 1.42)	0.32
Cytology result						
Negative	1			1		
ASC-US+	0.46	(0.36 - 0.58)	0.00	0.42	(0.33 - 0.54)	0.00
Inadequate	3.9	(2.32 - 6.54)	0.00	4.26	(2.45 - 7.41)	0.00
Missing	1.38	(0.42 - 4.58)	0.6	1.28	(0.43 - 3.77)	0.66
mRNA result						
Negative	1			1		
Positive	0.63	(0.53 - 0.74)	0.00	0.57	(0.48 - 0.68)	0.00
Missing	0.99	(0.35 - 2.80)	0.99	0.96	(0.38 - 2.45)	0.94
Typing						
Other HR types	1			1		
16 and/or18 (with or without other HRs)	0.94	(0.71 - 1.25)	0.67	0.95	(0.70 - 1.28)	0.73
Recruiting centre						
Lab 2	1					
Lab 6	0.71	(0.57 - 0.88)	0.00			
Lab 7	0.49	(0.39 - 0.62)	0.00			
Lab 5	0.74	(0.58 - 0.95)	0.02			
Lab 3	1.14	(0.56 - 2.29)	0.72			
Centre which interpreted the slides						
Lab 2	1					
Lab 6	0.97	(0.77 - 1.22)	0.81			
Lab 1	0.41	(0.31 - 0.52)	0.00			
Lab 7	1.61	(1.29 - 2.01)	0.00			
Lab 4	0.69	(0.55 - 0.88)	0.00			
Lab 5	1.55	(1.25 - 1.93)	0.00			
Lab 3	0.32	(0.23 - 0.44)	0.00			

Variables	Univariate*			Multivariate**		
	OR	95%IC	p	OR	95%IC	p
Time between immunostaining and interpretation						
Recent	1			1		
Old	0.92	(0.80 - 1.06)	0.25	0.73	(0.60 - 0.90)	0.00
Time between sampling and immunostaining						
Recent	1			1		
Old	1.63	(1.38 - 1.93)	0.00	1.41	(1.15 - 1.73)	0.00

*Adjusted for age

**Model for age, time between immunostaining and interpretation and time between sampling and immunostaining, adjusted for recruting centre, reading centre. Model for presence of CIN2+, citology result, mRNA result and typing, adjusted for age, recruting centre, reading centre, time between immunostaining and interpretation and time between sampling and immunostaining

[§]women who did not undergo colposcopy or 1-year test repeat

Table 5. Univariate and multivariate analyses for positive report (split model for CIN2+)

		Univariate for CIN2+*			Multivariate for CIN2+**				Univariate for not CIN2+*			Multivariate for not CIN2+**				Univariate overall*			Multivariate overall**		
Variables	**n**	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***	**n**	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***	**n#**	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Age	503	0.98	0.94 -1.02	0.34	1	0.95 - 1.05	0.98	6637	0.99	0.98 - 1.00	0.05	0.99	0.98 - 1.00	0.04	7140	0.99	0.98 - 1.00	0.00	0.99	0.98 - 0.99	0.00
Age																					
25-34	32	1						600	1						632	1					
35-44	260	0.23	0.05 - 0.97	0.05				2629	1.07	0.81 - 1.43	0.62				2889	1.12	0.85 - 1.47	0.43			
45-54	193	0.18	0.04 - 0.77	0.02				2398	0.94	0.70 - 1.26	0.68				2591	0.95	0.72 - 1.25	0.7			
55+	18	0.53	0.08 - 3.50	0.51				1010	0.83	0.60 - 1.15	0.26				1028	0.75	0.55 - 1.03	0.08			
Cytology result																					
Negative	161	1			1			4908	1			1			5069	1			1		
ASC-US+	341	6.34	3.42 - 11.76	0.00	7.85	3.93 - 15.68	0.00	1656	3.54	2.98 - 4.20	0.00	4.04	3.36 - 4.84	0.00	1997	4.34	3.69 - 5.09	0.00	4.99	4.21 - 5.93	0.00
Inadequate	1	1	-	-	1	-	-	64	1.79	0.85 - 3.76	0.13	2.07	0.97 - 4.42	0.06	65	1.65	0.79 - 3.48	0.18	1.82	0.86 - 3.88	0.12
Missing	0	-	-	-	-	-	-	9	2.93	0.36 - 23.92	0.32	2.73	0.22 - 34.06	0.43	9	2.79	0.34 - 22.74	0.34	2.64	0.23 - 29.84	0.43
mRNA result																					
Negative	18	1			1			2222	1			1			2240	1			1		
Positive	485	18.93	4.35 - 82.30	0.00	19.79	2.81 - 139.14	0.00	4407	5.61	4.58 - 6.88	0.00	5.86	4.75 - 7.25	0.00	4892	6.63	5.42 - 8.10	0.00	7.11	5.78 - 8.76	0.00
Missing	0	-	-	-	-	-	-	8	8.76	1.46 - 52.55	0.02	12.9	2.28 - 72.89	0.00	8	8.61	1.46 - 50.86	0.02	13.23	2.28 - 76.70	0.00
Typing																					
Other HR types	52	1			1			2230	1			1			2282	1			1		
16 and/or 18 (with or without other HRs)	91	4.52	1.30 - 15.72	0.02	12.6	2.66 - 59.60	0.00	757	1.59	1.24 - 2.03	0.00	1.63	1.25 - 2.12	0.00	848	1.9	1.50 - 2.40	0.00	1.95	1.52 - 2.51	0.00
Presence of CIN2+																					
Not CIN2+															6637	1			1		
CIN2+															503	8.3	6.15 -11.19	0.00	10.19	7.49 - 13.87	0.00
Recruiting center																					
Lab 2	99	1						1912	1						2011	1					
Lab 6	213	0.53	0.25 - 1.14	0.10				1690	0.57	0.46 - 0.70	0.00				1903	0.68	0.56 -0.82	0.00			
Lab 7	158	0.86	0.36 - 2.05	0.74				1765	0.83	0.68 - 1.02	0.08				1923	0.89	0.73 - 1.08	0.22			
Lab 5	28	5.82	0.35 - 52.34	0.12				1253	0.78	0.61 - 0.99	0.04				1281	0.73	0.58 -0.92	0.01			
Lab 3	5	0.9	0.10 - 8.10	0.93				17	1.8	0.41 - 7.99	0.44				22	2.17	0.61 - 7.70	0.23			

	Univariate for CIN2+*				Multivariate for CIN2+**			Univariate for not CIN2+*				Multivariate for not CIN2+**			Univariate overall*				Multivariate overall**		
Variables	**n**	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***	**n**	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***	**n#**	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Reading center																					
Lab 2	53	1						1002	1						1055	1					
Lab 6	105	0.52	0.21 - 1.28	0.15				950	1.11	0.93 - 1.31	0.25				1055	1.18	1.00 - 1.38	0.05			
Lab 1	44	1.51	0.55 - 4.11	0.42				979	0.83	0.73 - 0.95	0.01				1023	0.83	0.73 - 0.94	0.00			
Lab 7	87	1.31	0.45 - 3.82	0.62				944	2.01	1.68 -2.41	0.00				1031	2.03	1.71 - 2.40	0.00			
Lab 4	77	0.24	0.11 - 0.55	0.00				866	0.5	0.41 - 0.60	0.00				943	0.55	0.46 - 0.65	0.00			
Lab 5	70	0.26	0.10 - 0.67	0.01				904	0.66	0.54 - 0.81	0.00				974	0.68	0.56 - 0.82	0.00			
Lab 3	67	0.52	0.20 - 1.33	0.17				992	1.02	0.85 - 1.23	0.83				1059	1.02	0.85 - 1.21	0.86			
Time between immunostaining and interpretation																					
Recent	200	1			1			3319	1			1			3519	1			1		
Old	303	0.67	0.40 - 1.11	0.12	1.11	0.68 - 1.83	0.67	3318	1.13	1.00 - 1.28	0.05	1.61	1.38 - 1.89	0.00	3621	1.17	1.04 - 1.31	0.01	1.15	1.31 - 1.74	0.00
Time between sampling and immunostaining																					
Recent	274	1			1			3367	1			1			3641	1			1		
	229	0.57	0.32 - 1.02	0.06	0.67	0.33 - 1.36	0.27	3270	0.88	0.75 - 1.03	0.12	0.85	0.70 - 1.02	0.08	3499	0.86	0.74 - 1.00	0.05	0.88	0.74 - 1.06	0.18

*Adjusted for age

**Model for age, time between immunostaining and interpretation and time between sampling and immunostaining, adjusted for recruiting center, reading center. Model for presence of CIN2+, cytology result, mRNA result and typing, adjusted for age, recruiting center, reading center, time between immunostaining and interpretation, and time between sampling and immunostaining

#Included all the evaluable reports of women who underwent colposcopy

DISCUSSION

In this large HPV-positive population, we observed that inadequacy rate (9.7%) was higher than in other studies (Wright TC Jr 2017: 3.7%; Wentsenzen N. 2012: 7.1%; Wentsenzen N. 2015: 6.3%; Wentzensen N. 2019: 2.5%) [4, 6, 13, 14], although this value was similar or even lower than that obtained in some other studies (Ebish RM.2017: 46%; Clarke MA. 2019: 8.2%; Stanczuk GA. 2017: 7-8%; Stoler MH. 2020: 8%). [3, 5, 12, 15] The proportion of inadequate slides was only slightly associated with the time interval between sampling and slide preparation and not associated at all with time between slide preparation and its interpretation. It should be noted that our inadequate rate includes both specimens with insufficient residual liquid-based cytology and slides with significant technical defects that make it impossible to interpret them. On the contrary, some studies reported these two types of inadequate cases separately. [15]

The discrepancies that we observed between our study and others may be due to the different populations under study, regarding the age, the prevalence of disease, or the rate of cytologic atypia. It is worth noting that the proportion of inadequate slides increased with increasing age and was strongly associated with unsatisfactory cytology, but the of unsatisfactory cytologic slides accounted for only a small proportion of p16/ki67 inadequate slides. As expected, these observations highlight the close link between cytology and p16/ki67 assay. In particular, the typical aspects of cytology in menopausal women may make it difficult to interpret the dual staining. Moreover, our data show that a lower prevalence of disease corresponds with a higher inadequate rate. This is not surprising because unclear or weak staining can increase the probability of an inadequate report, and characteristics such as the presence of CIN2+, E6/E7 mRNA overexpression, and HPV type 16 are surely associated with a higher presence of the targets of immunostaining, as shown in Table 5.

The positivity rate we observed (28.3% overall, 31.3% considering only p16/ki67 evaluable cases) was lower than that observed in other studies conducted in HPV-positive populations (Ebish RM. 2017: 55%; Clarke MA. 2019: 45.5%; Wentzensen N. 2015: 46%; Wentzensen N. 2019: 50%) [5, 6, 12, 14] and comparable to that of the ATHENA study (Wright TC Jr. 2017: 28.4%; Stoler MH. 2020: 27.4%). [4, 15] We found that dual staining positivity was associated with abnormal cytology at the ASC-US+ threshold and with E6/E7 mRNA positivity, both in cases with and without a CIN2+, while the association with HPV type 16 or 18 was strong only in cases with a CIN2+. These observations are consistent with the known pathway of HR-HPV infection, in which E6/E7 expression may occur, leading to evident morphologic atypia (ASC-US+) and to the deregulation of the cell cycle, and thus to the resulting p16/ki67 positivity. However, as evidenced in the multivariate analysis, the infection with HPV16/18 increased the sensitivity for CIN2+ of p16/ki67 staining, i.e., the association with positivity was only present in the model restricted to women with CIN2+, probably because the molecular and morphologic changes in these infections were clearer and transformation toward high grade CIN2+ lesion was less equivocal.

Concerning both adequacy and positivity, we found great variability among the participating centers with regard to sampling, immunostaining, and interpretation, although we did not observe any statistically significant difference. These last findings are consistent with studies on interpretation reproducibility and confirm that this assay is influenced by a certain grade of subjectivity. [7-9, 11]

Strengths and Limitations

This study presents some limitations. First of all, p16/ki67 immunostaining was performed for the purpose of research; the results were not used for the management of the women. Thus, interpretation was not influenced by the responsibility of a clinical decision on which women to refer to colposcopy. Moreover, the research purpose also affected the time intervals between sampling, immunostaining, and interpretation, which were far longer than they would be in clinical practice, and they varied greatly among the participating labs. However, these variabilities seem to have had a small effect on adequacy and on positivity.

In addition, we adopted a split sample design in which the slide for immunocytochemistry was obtained after that for Pap test. As already reported, this may increase the number of cases with scant cellularity. [10, 11] However, adopting dual staining as the triage test should overcome this problem. Moreover, regarding inadequacy due to staining problems, in a screening setting it is possible to repeat uncertain cases, thereby decreasing the proportion of inadequate reports.

The main strengths of the study are that it included a large screening population sample, p16/ki67 dual staining was performed on all the HPV-positive women, and all slides were interpreted independently by three laboratories.

Implications for practice

Dual staining for p16/ki67 proved to be accurate in triaging HPV DNA-positive women, alone or in combination with typing. [18] However, its interpretation still requires a subjective microscopic evaluation. Even when an automatic immunostaining analysis system is introduced in the near future, we still expect to find a number of uncertain cases that will require human supervision. Indeed, there are several possible sources of misinterpretation linked to immunostaining, for instance, weak counterstaining, a background of weak p16 staining, or to the interpretation itself, as happens in the presence of cellular sheets. [10, 11]

For a test candidate for population screening, knowing the determinants of adequate as well as positive results is essential in order to monitor screening program performance indicators, to correctly interpret variability, and to better standardize the procedures in all respects. Concerning the monitoring of adequacy of p16/ki67 interpretation, our findings suggest that variability can be found in all stages: sampling, immunostaining, and interpretation. Increases in the number of inadequate cases could be due to changes in the composition of the screened population in terms of age, or the prevalence of disease, or cytologic modifications.

When monitoring the positivity rate, increases can obviously be due to an increase in disease prevalence, whereas age has almost no effect. Both sampling and reading can act as a determinant of positivity.

The probability of a true positive seems lower in women aged >35 years, when the slide is old, or when cytology is negative, whereas it is higher when HPV16/18 infection is present and when E6/E7 mRNA is overexpressed. The probability of a false positive seems unaffected by age and decreases with cytology positivity and mRNA positivity, whereas it increases only slightly with HPV16/18 infection and with old slides.

Conclusion

Our data show that dual staining interpretation may be influenced by a number of different variables relating to all the steps of the procedure, i.e., sampling, immunostaining, and interpretation, and by the characteristics of the screened population. These factors should be considered in order to better interpret monitoring data and to implement appropriate measures of intervention in cases of anomalies in positivity or inadequacy rates. Intra-laboratory quality control programs should be applied to make each step reliable. Moreover, the variability among the centers both in terms of adequacy and of positivity highlights the need for inter-laboratory quality control programs to standardize criteria.

Bibliography

1. Ronco G, Arbyn M, Meijer CJLM, Snijders PJF, Cuzick J. Screening for cervical cancer with primary testing for human papillomavirus. In: Anttila A, Arbyn M, de Vuyst H, et al, eds. European Guidelines for Quality Assurance in Cervical Cancer Screening. 2nd ed, suppl. Office for Official Publications of the European Communities; 2015:1-68
2. US Preventive Services Task Force, Curry SJ, Krist AH, et al. Screening for Cervical Cancer: US Preventive Services Task Force Recommendation Statement. *JAMA.* 2018;320(7):674-686. doi:10.1001/jama.2018.10897
3. Stanczuk GA, Baxter GJ, Currie H, et al. Defining Optimal Triage Strategies for hrHPV Screen-Positive Women-An Evaluation of HPV 16/18 Genotyping, Cytology, and p16/Ki-67 Cytoimmunochemistry. *Cancer Epidemiol Biomarkers Prev.* 2017;26(11):1629-1635. doi:10.1158/1055-9965.EPI-17-0534
4. Wright TC Jr, Behrens CM, Ranger-Moore J, et al. Triaging HPV-positive women with p16/Ki-67 dual-stained cytology: Results from a sub-study nested into the ATHENA trial. *Gynecol Oncol.* 2017;144(1):51-56. doi:10.1016/j.ygyno.2016.10.031.
5. Ebisch RM, van der Horst J, Hermsen M, et al. Evaluation of p16/Ki-67 dual-stained cytology as triage test for high-risk human papillomavirus-positive women. *Mod Pathol.* 2017;30(7):1021-1031. doi:10.1038/modpathol.2017.16
6. Wentzensen N, Clarke MA, Bremer R, et al. Clinical Evaluation of Human Papillomavirus Screening with p16/Ki-67 Dual Stain Triage in a Large Organized Cervical Cancer Screening Program [published correction appears in *JAMA Intern Med.* 2019 Jul 1;179(7):1007]. *JAMA Intern Med.* 2019;179(7):881-888. doi:10.1001/jamainternmed.2019.0306
7. Allia E, Ronco G, Coccia A, et al. Interpretation of p16(INK4a) /Ki-67 dual immunostaining for the triage of human papillomavirus-positive women by experts and nonexperts in cervical cytology. *Cancer Cytopathol.* 2015;123(4):212-218. doi:10.1002/cncy.21511
8. Benevolo M, Allia E, Gustinucci D, et al. Interobserver reproducibility of cytologic p16INK4a /Ki-67 dual immunostaining in human papillomavirus-positive women. *Cancer Cytopathol.* 2017;125(3):212-220. doi:10.1002/cncy.21800
9. Wentzensen N, Fetterman B, Tokugawa D, et al. Interobserver reproducibility and accuracy of p16/Ki-67 dual-stain cytology in cervical cancer screening. *Cancer Cytopathol.* 2014;122(12):914-920. doi:10.1002/cncy.21473
10. McMenamin M, McKenna M, McDowell A, Dawson C, McKenna R. Intra- and inter-observer reproducibility of CINtec® PLUS in ThinPrep® cytology preparations. *Cytopathology.* 2017;28(4):284-290. doi:10.1111/cyt.12426
11. Benevolo M, Mancuso P, Allia E, et al. Interlaboratory concordance of p16/Ki-67 dual-staining interpretation in HPV-positive women in a screening population. *Cancer Cytopathol.* 2020;128(5):323-332. doi:10.1002/cncy.22248
12. Clarke MA, Cheung LC, Castle PE, et al. Five-Year Risk of Cervical Precancer Following p16/Ki-67 Dual-Stain Triage of HPV-Positive Women. *JAMA Oncol.* 2019;5(2):181-186. doi:10.1001/jamaoncol.2018.4270
13. Wentzensen N, Schwartz L, Zuna RE, et al. Performance of p16/Ki-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population. Clin Cancer Res. 2012;18(15):4154-4162. doi:10.1158/1078-0432.CCR-12-0270
14. Wentzensen N, Fetterman B, Castle PE, et al. p16/Ki-67 Dual Stain Cytology for Detection of Cervical Precancer in HPV-Positive Women. *J Natl Cancer Inst.* 2015;107(12):djv257. Published 2015 Sep 15. doi:10.1093/jnci/djv257
15. Stoler MH, Baker E, Boyle S, et al. Approaches to triage optimization in HPV primary screening: Extended genotyping and p16/Ki-67 dual-stained cytology-Retrospective insights from ATHENA. *Int J Cancer.* 2020;146(9):2599-2607. doi:10.1002/ijc.32669
16. Giorgi Rossi P, Bisanzi S, Allia E, et al. Determinants of Viral Oncogene E6-E7 mRNA Overexpression in a Population-Based Large Sample of Women Infected by High-Risk Human Papillomavirus Types. *J Clin Microbiol.* 2017;55(4):1056-1065. doi:10.1128/JCM.01794-16
17. Solomon D, Davey D, Kurman R, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. *JAMA.* 2002;287(16):2114-2119. doi:10.1001/jama.287.16.2114
18. Cuzick J, Adcock R, Carozzi F, et al. Combined use of cytology, p16 immunostaining and genotyping for triage of women positive for high-risk human papillomavirus at primary screening [published online ahead of print, 2020 Mar 14]. *Int J Cancer.* 2020;10.1002/ijc.32973. doi:10.1002/ijc.32973

3.5. PhD Candidate contribution to research articles in Chapter 3

Research article #03 p16/ki67 and E6/E7 mRNA Accuracy and Prognostic Value in Triaging HPV DNA-Positive Women.

The PhD Candidate contributed to conceptualization, definition of statistical plan, data collection from the recruiting centres of the New Technology for Cervical Cancer 2 (NTCC2), and data analysis. The PhD Candidate also discussed results presentation and gave a main contribution to the draft of each paper.

Research articles #04 Interlaboratory Concordance of p16/Ki-67 Dual-Staining Interpretation in HPV-Positive Women in a Screening Population and #05 Determinants of p16/Ki-67 Adequacy and Positivity in HPV-Positive Women From a Screening Population.

The PhD Candidate contributed to data collection from the recruiting centres of the New Technology for Cervical Cancer 2 (NTCC2) and data analysis, discussed results presentation, and contributed to the draft of each paper.

4. Chapter 4: Evidence synthesis and recommendations development for cervical cancer screening

4.1. Rationale

The actual impact of emerging technologies on population health and healthcare systems sustainability comes through a timely development of evidence-based recommendations allowing patients, clinicians, and health services decisionmakers to make better choices.

A rigorous and transparent methodology is required, to provide recommendations based on the best available evidence, appraised by a multidisciplinary panel of experts and patients, easily implementable in different contexts and timely updatable if required.

In Italy, the National Institute of Health provided methodological standards to scientific societies to develop national guidelines, including the Grading of Recommendations Assessment, Development and Evaluation (GRADE) as referent methodology.

In this context, nine Italian scientific societies involved in tackling cervical cancer, coordinated by the Italian Group for Cervical Cancer Screening (GISCi) and endorsed by the National Centre for Screening Monitoring, started a project to develop evidence-based recommendations in areas of cervical cancer prevention not covered by existing international guidelines.

The fourth chapter provide a description of the methodology used by guidelines development and report the process and results of the first recommendation published on the official database of the Italian National Institute of Health.

4.2. Research article #06: Developing evidence-based Multisociety Italian guidelines for cervical cancer prevention: rationale, methods, and development process.

The findings of this research article were published in Italian the Italian National System of Guidelines (SNLG) online database. [8]. The English version is invited for submission in *European Journal of Gynaecological Oncology* by March 31, 2021.

Ventureli F, Garutti P, Giorgi Rossi P. Writing committee on behalf of the Multisociety Italian guidelines for cervical cancer prevention Working Group. Developing evidence-based Multisociety Italian guidelines for cervical cancer prevention: rationale, methods, and development process. Invited submission in *Eur J Gynaecol Oncol.*

Abstract

Cervical Cancer prevention shows a variability across Italian Regions unjustified by available evidence, with high health, economic and organizational impact. Evidence-based recommendations on areas not covered by international guidelines are needed to tackle existing inequalities. This article describes the rationale, methods, and process for development of the Multisociety Italian guidelines for cervical cancer prevention. The Italian legislative framework requires Guidelines to be consistent with the methodological standards set by the National System for Guidelines (SNLG) of the National Institute of Health. All the nine scientific societies involved in cervical cancer prevention participate to the project, including clinicians, policy makers, methodologists, and researchers. Patients were involved as full voting panel members. The GRADE (Grading of Recommendations, Assessment, Development and Evaluations) approach was adopted to assess the certainty of evidence collected by systematic reviews. The GRADE Evidence-to-Decision framework (EtD) was used to structure the appraisal of evidence and to formulate final recommendations. The EtD and a conflict-of-interest management policy were adopted to minimize the influence of competing interests. A full transparency guided the reporting of each step of the process, to support the implementation of recommendations in each context and the future updating process. Considerations for subgroups, monitoring and evaluation of recommendations implementation and research priorities were also provided. To increase publication timeliness, guidelines were also organised in chapters grouping sets of meaningful recommendations to be published independently. Finally, a two-step review process by external experts and reviewers by the SNLG, prior to online publication, ensured the methodological robustness underling final recommendations.

Ethics approval: The work here report does not need ethical approval. The guideline development project has been submitted to and approved by the Italian National System of Guidelines (SNLG) of the National Institute of Health (ISS) (Sistema Nazionale Linee Guida, Istituto Superiore di Sanità). This specific recommendation has been submitted to the SNLG and published on the web platform of the Italian recognized guidelines database: https://snlg.iss.it/

BACKGROUND

The European framework for breast and cervical cancer screening was set up by the European Council 2003 recommending to all member countries to implement screening programs for these two cancers according to European guidelines for quality assurance. [1] In 2001, the Italian National Health Service included cervical cancer screening in the core benefit package (Essential Levels of Care - LEA) to ensure uniform level of preventive services across the country. [2]

The European guidelines on cervical cancer screening were published in 2008 and updated in 2015. [3, 4] They provided recommendations on the age of screening, on the test to be adopted and on the screening intervals for women with negative tests. The guidelines also developed recommendations for the management of women with positive primary tests, including the triage, providing a large spectrum of options, to be adapted to national contexts. Conversely, the European guidelines did not consider new biomarkers for tirage, follow-up of women treated for lesions identified by screening, and the introduction of the post-treatment HPV vaccine for cervical lesions.

Up to date, there are no national reference guidelines on these areas, and their management is very heterogeneous and inconsistent across Italian regions. Furthermore, new scientific evidence, useful to support specific recommendations in the topic, has recently emerged. [5, 6]

To standardize clinical behaviours, it was therefore necessary to produce updated evidence-based recommendations on these areas to be implemented in the Italian screening programs.

The purpose of the " Multisociety Italian guidelines for cervical cancer prevention." working group was to define recommendations on cervical cancer screening, endorsed by all scientific societies involved in tackling cervical cancer.

In Italy, the legal framework for the production of accredited guidelines was represented by the Law No. 24, March 8, 2017, "Provisions on the safety of care and of the assisted person, as well as in matters of professional liability of health professions ". [7]

The Italian group for cervical cancer screening (GISCi), a national scientific society including all health professions participating in cervical cancer screening programs, promoted the guideline development process inviting all the Italian scientific societies involved in cervical cancer prevention.

Eight scientific societies accredited to the Ministry of Health have been formally invited to draft shared guidelines and all agreed to participate establishing the Technical Scientific Committee (CTS).

The CTS decided to adopt a methodology compliant with the National Institute of Health (Istituto Superiore di Sanità ISS) guidance on the production of guidelines to be included in the Italian National System of Guidelines (SNLG). [8] According to the recent law on legal responsibility of health professionals, guidelines included in the SNLG are the reference to define appropriate or non-appropriate clinical behaviours of healthcare professionals particularly if called in legal trials. [7]

Therefore, CTS adopted the GRADE approach (Grading of Recommendations Assessment, Development and Evaluation) to develop recommendations. This method has been defined in the past decades and it is now adopted by most of the international and national agencies producing recommendations. [9]

The methodology is inspired by the principle that recommendations should be based on the best available evidence and developed through a transparent process, considering all the points of view of experts from different disciplines and of the patients and policy makers. [9-14]

AIMS

This article describes the methods and process adopted to develop the " Multisociety Italian guidelines for cervical cancer prevention." in the context of existing international and national recommendations for cervical cancer screening. [4, 15, 16].

GUIDELINE METHODOLOGY AND DEVELOPMENT

The guidelines development process follows the phases provided for by the "Methodological manual for the production of clinical practice guidelines" edited by the CNEC (National Centre for Clinical Excellence, Quality and Safety of Care), in charge from the Italian National Institute of Health. [17]

As mentioned before, the process was started by the GISCi with the sponsorship of the National Centre for Screening Monitoring (ONS). The first action was to invite all other involved scientific societies to join the project. (Table 1) The representatives of the scientific societies constituted the CTS. After the constitution of the CTS all the participating societies were equally involved, and the promoter does not have any prominent status.

Table 1 – Scientific societies involved in the Guidelines Development Group, sorted alphabetically

- AIO Italian Association of Obstetrics (Associazione Italiana Ostetricia);
- AOGOI Italian Association of Hospital Obstetricians and Gynaecologists (Associazione Italiana Ostetrici e Ginecologi Ospedalieri);
- GISCi Italian group for cervical cancer screening (Gruppo Italiano per lo Screening del Cervicocarcinoma);
- SIAPEC-IAP Italian Society for Pathological Anatomy and Diagnostic Cytology. Italian Division of the International Academy of Pathology (Società Italiana Anatomia Patologica e Citologia Diagnostica. Divisione Italiana dell'International Accademy of Pathology);
- SICi Italian Society of Cytology (Società Italiana di Citologia);
- SICPCV Italian Society for Colposcopy and Cervico-Vaginal Pathology (Società Italiana di Colposcopia e Patologia Cervico-Vaginale);
- SIGO Italian Society of Gynecology and Obstetrics (Società Italiana Ginecologia e Ostetricia);
- SItI Italian Society of Hygiene, Preventive Medicine and Public Health (Società Italiana di Igiene, Medicina Preventiva e Sanità Pubblica);
- SIV-ISV Italian Society for Virology (Società Italiana di Virologia)

Roles:

Technical Scientific Committee

The CTS has the following tasks:

- definition of the structure of the guidelines development group (GDG) and the roles, tasks and relationships between the teams involved in accordance with the CNEC methodology;
- outline of the budget and expected costs of the process;
- definition of the scope that guides the PICOs definition by the GDG, for each topic and chapter;
- selection and recruitment of the members of the Panel of Experts, the Evidence Review Team (ERT) and of the independent external reviewers;
- definition of the specific Conflict-of-Interest (COI) identification and management policy;
- participation in all the plenary sessions, with contribution to the discussion, but without voting.

The GDG members were selected by the CTS in collaboration with the participating scientific societies, with the purpose to include representatives with the necessary background, expertise, and knowledge.

The GDG includes women as end users of the recommendations, health care professionals, epidemiologists, and guideline methodologists with previous experience of GRADE approach.

The participation is voluntary, and these individuals are assigned to the Panel of Experts and the ERT.

Panel of Experts

Panel members are selected by the CTS starting from the list of panellists who already participated in the last guidelines produced by GISCi and from other independent Italian experts in the field. All the experts who accept to participate and timely send the Conflict-of-Interest disclosure are considered part of the panel.

The panel includes experts from all the medical fields that could be interested in cervical cancer prevention (gynaecologists, oncologists, pathologists, cytologists, cytotechnologists, midwives, epidemiologists, evidence-base medicine methodologists, public health and hygiene experts, virologists), patients, local and regional decisionmakers.

Patients and other lay members of the panel are full voting members.

For each minimum set of recommendations to be developed, the panellists are asked to confirm the participation and to sign a PICO specific Conflict-of-Interest form.

The CTS checks that the participants cover all the needed expertise and, if needed, stimulates the participation of relevant professionals.

Within the Panel a Chair is elected to neutrally lead the Panel of Experts in the Guidelines development process. The Chair should be qualified, authoritative, and with experience in group coordination, in optimizing teamwork and in techniques of reaching consensus. In addition, a Methodological Co-Chair is identified and elected considering the expertise in the application of the guideline development methods required by CNEC.

Evidence Review Team

For each guideline chapter and topic, the CTS selects individuals from the GDG to be part of the ERT, including Evidence-based Medicine (EBM) specialists and panellists with clinical background.

The ERT produces systematic reviews of existing evidence following the Cochrane Collaboration standards, assesses the quality and certainty of evidence, and presents the results using the Summary of Findings (SoF) tables, according to the GRADE approach. [9, 18, 19]

The SoF tables are sent to the Panellists one week before plenary sessions and presented during each meeting to collect feedback and to provide the necessary information to support evidence-based recommendations.

Synthesised evidence and plenary discussion guide the appraisal of Evidence-to-Decision (EtD) framework criteria by the Panel of Experts and the formulation of each recommendation. [10]

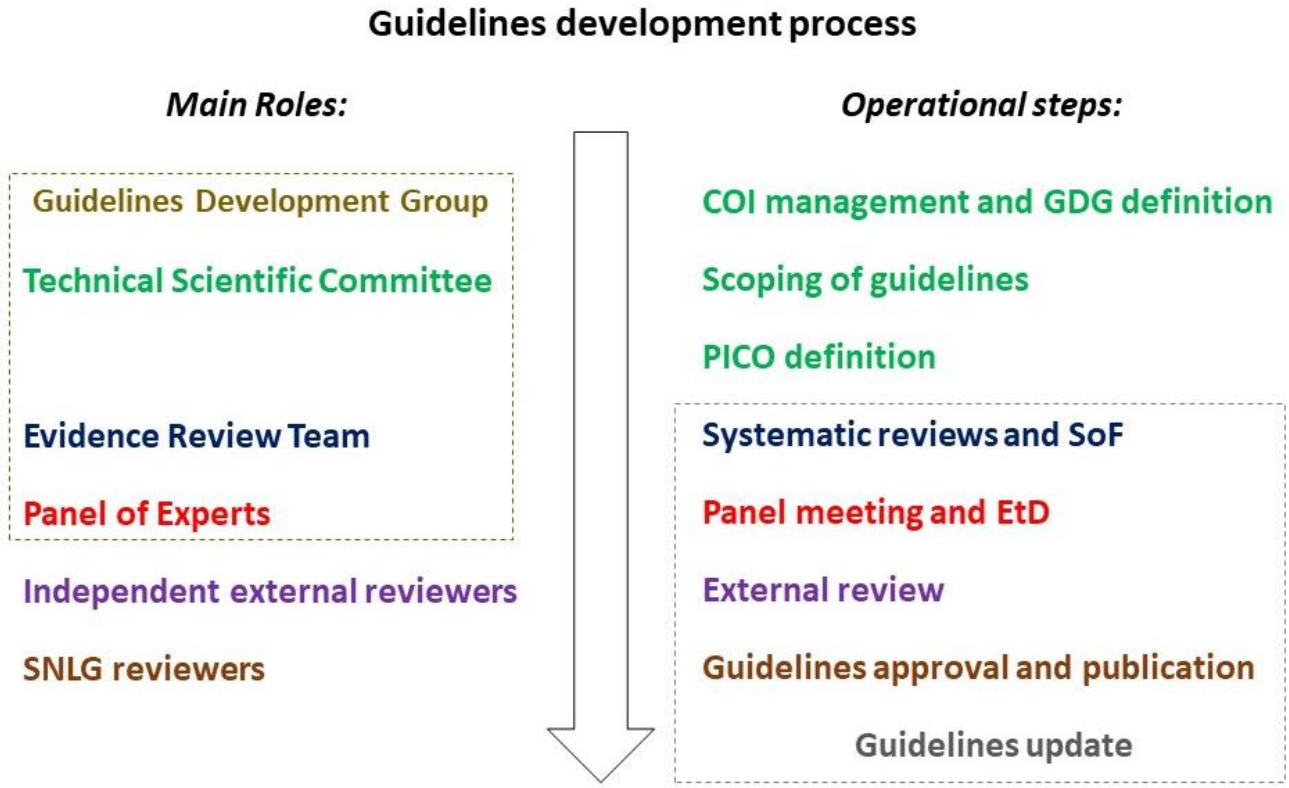
Independent external reviewers

For each chapter and topic, the CTS invites three experts in the field, with complementary expertise, to make a peer review of the recommendations' development process. Each expert is identified for her/his expertise and is asked to sign a Conflict-of-Interest form, assessed by the CTS according to the COI policy.

Operational steps:

The operational steps in the development of recommendations are executed as follows (Figure 1):

Figure 1 – Guidelines development process including operational steps e main roles, leading each step. COI: Conflict of Interest; EtD: Evidence-to-Decision framework; GDG Guidelines Development Group; PICO: population, intervention, comparison, outcomes; SNLG: Italian National System of Guidelines; SoF: Summary of Findings.



Conflict-of-Interest management

The CTS applied the COI policy included in the CNEC methodology, which is consistent with international recommendations. [9, 17, 20]

The COI disclosure is used to assess the eligibility of each member of the GDG by the CTS and it is PICO specific for Panel members.

Moreover, the COI declared by each panellist is classified in 3 degrees of relevance, to each of those a specific measure is applied. COIs classified as of “minimal or insignificant relevance” pose no limitation to participation in all the recommendation development process phases. Panellists which COI is classified as “potentially relevant” are admitted to all the process phases but requiring a public disclosure in the final document or in the SNLG website. Finally, COIs classified as “Relevant” led to a partial exclusion from the participation to discussion and voting of the criteria potentially influenced by the specific COI, up to the complete exclusion from the Guidelines Development process. [17]

Moreover, the use of the GRADE Evidence-to-Decision framework minimizes the influence of COI on recommendations formulations, leading the experts to make informed choices on the bases of predefined and transparent criteria. [10, 17]

Finally, panellists refusing to fulfil and sign the COI disclosure form or not participating to the plenary session are excluded from the authors' list of each specific recommendation.

Scoping of guidelines

The CTS agreed on a wide scope of the guidelines covering all the aspects of primary and secondary prevention of cervical cancer. The CTS recognized that for public health interventions, national recommendations produced by scientific societies must respect European Commission guidelines and must follow the National recommendations for country specific implementation of the European guidelines.

The CTS agreed on a gradual development of the Guidelines through chapters prioritization.

Within each chapter a more specific scoping phase is conducted to identify the relevant and important clinical questions.

In the first scoping phase, the topics included in the guideline's chapters were:

- Human Papillomavirus (HPV) Vaccination in women treated for CIN2/CIN3;
- Follow-up of women treated for CIN2/CIN3 within organized screening programs.
- Use of biomarkers in HPV based cervical cancer screening programs;
- Management of low risk CIN2: treatment vs follow up.

According to the CNEC methodology [17], the criteria used for topic prioritization were:

- National variability in healthcare professional practices not justified by available evidence;
- Inequalities of care processes and outcomes;
- Availability and quality of evidence;
- Health practices with high costs for the NHS and high organizational or technological burden;
- Social demands and needs perceived by the population;
- Lack of up-to-date guidelines of high methodological quality, directly implementable in the Italian context

The first chapter, including only one recommendation on HPV vaccination in women treated for Cervical Intraepithelial Neoplasia (CIN) 2 and CIN3, has been concluded and published on the SNLG website in July 2020. [8] The chapter on the follow up of women treated for CIN2/CIN3 ended the voting phases and it is currently under review by external reviewers, while the chapter on the use of biomarkers is in the scoping phase.

PICO definition

Each clinical question prioritized is framed in PICOs (population, intervention, comparison, outcomes) by the panel. According to the GRADE approach to guideline development, the panellists list and prioritise the PICOs according to the indications given by the CTS, using the GRADEpro online platform. [21] For each PICO or group of similar PICOs included in the chapter, the panel identifies the possible patient relevant outcomes through an online brainstorming process. Then the proposed outcomes are grouped in for the clinical underlying condition that they intend to measure and are prioritized as critical, important, and non-important in the judgement, through a voting and consensus process. [21, 22]

Systematic review of evidence

For each PICO or group of PICOs, the ERT conducts systematic reviews and summarises the findings for each critical and important outcome, grading the certainty of evidence and quantifying the effects in relative and absolute differences through the SoF table proposed by the GRADEpro. [21]

Evidence is graded according to the risk of bias, the indirectness, the reproducibility/heterogeneity of results, the precision of the estimates, the presence of publication bias, the strength of association, and eventually the presence of dose response effect or of known biases going in the direction of underestimating the effect. [22]

Panel meeting and Evidence-To-Decision framework

During panel meetings, the SoF is reviewed and the evidence on health outcomes is integrated with context specific evidence provided by panellist on how women value the outcomes, costs/resources, eventually cost effectiveness, equity, feasibility, and acceptability. These integrations may be simply added as additional consideration during the plenary session or can require an integration of systematic review outputs.

In some cases, the panel or the external reviewers can ask for a specific systematic review on some of the aspects if they think it is worth trying. This was the case of cost effectiveness for the PICO on vaccine after CIN2 and CIN3 treatment, where the external reviewers asked for such a systematic review, but no studies were found.

All the evidence was then integrated and reported to panellists in a systematic way using the EtD Table proposed by the GRADEpro. [10, 21, 22] Two kinds of EtD are used for these guidelines: the intervention framework and the diagnostic framework. (Table 2) In the first one, two interventions (or one intervention to no intervention) are compared based on their desired and undesired effects. In the diagnostic framework, we compare two tests or combinations of tests for their accuracy, then, starting from estimated true positives, true negatives, false positives, and false negatives, the panel compares the estimated downstream consequences obtained with the two tests. [10, 21, 22]

The panel makes its judgement on each of the specific domain of the EtD framework: priority of the question, magnitude of the desirable effects, and undesirable effects, certainty of the evidence, and

balance of the two, values given to the outcome by patients, resource required, cost effectiveness, certainty of evidence on costs, equity, feasibility, and acceptability. If required by at least one of the panel members, the judgement is expressed through voting.

Voting options may be restricted by the panellists to only those that are considered plausible options after the evidence evaluation.

Table 2 – Summary of Judgements on Evidence-to-Decision Criteria, layouts by GRADEpro. [21]

a. Framework used for questions comparing two managements.

	JUDGEMENT						
PROBLEM	No	Probably no	Probably yes	Yes		Varies	Don't know
DESIRABLE EFFECTS	Trivial	Small	Moderate	Large		Varies	Don't know
UNDESIRABLE EFFECTS	Large	Moderate	Small	Trivial		Varies	Don't know
CERTAINTY OF EVIDENCE	Very low	Low	Moderate	High			No included studies
VALUES	Important uncertainty or variability	Possibly important uncertainty or variability	Probably no important uncertainty or variability	No important uncertainty or variability			
BALANCE OF EFFECTS	Favors the comparison	Probably favors the comparison	Does not favor either the intervention or the comparison	Probably favors the intervention	Favors the intervention	Varies	Don't know
RESOURCES REQUIRED	Large costs	Moderate costs	Negligible costs and savings	Moderate savings	Large savings	Varies	Don't know
CERTAINTY OF EVIDENCE OF REQUIRED RESOURCES	Very low	Low	Moderate	High			No included studies
COST EFFECTIVENESS	Favors the comparison	Probably favors the comparison	Does not favor either the intervention or the comparison	Probably favors the intervention	Favors the intervention	Varies	No included studies
EQUITY	Reduced	Probably reduced	Probably no impact	Probably increased	Increased	Varies	Don't know
ACCEPTABILITY	No	Probably no	Probably yes	Yes		Varies	Don't know
FEASIBILITY	No	Probably no	Probably yes	Yes		Varies	Don't know

b. Framework used for questions comparing two diagnostic procedures

	JUDGEMENT						
PROBLEM	No	Probably no	Probably yes	Yes		Varies	Don't know
TEST ACCURACY	Very inaccurate	Inaccurate	Accurate	Very accurate		Varies	Don't know
DESIRABLE EFFECTS	Trivial	Small	Moderate	Large		Varies	Don't know
UNDESIRABLE EFFECTS	Large	Moderate	Small	Trivial		Varies	Don't know
CERTAINTY OF THE EVIDENCE OF TEST ACCURACY	Very low	Low	Moderate	High			No included studies
CERTAINTY OF THE EVIDENCE OF TEST'S EFFECTS	Very low	Low	Moderate	High			No included studies
CERTAINTY OF THE EVIDENCE OF MANAGEMENT'S EFFECTS	Very low	Low	Moderate	High			No included studies
CERTAINTY OF THE EVIDENCE OF TEST RESULT/MANAGEMENT	Very low	Low	Moderate	High			No included studies
CERTAINTY OF EFFECTS	Very low	Low	Moderate	High			No included studies
VALUES	Important uncertainty or variability	Possibly important uncertainty or variability	Probably no important uncertainty or variability	No important uncertainty or variability			
BALANCE OF EFFECTS	Favors the comparison	Probably favors the comparison	Does not favor either the intervention or the comparison	Probably favors the intervention	Favors the intervention	Varies	Don't know
RESOURCES REQUIRED	Large costs	Moderate costs	Negligible costs and savings	Moderate savings	Large savings	Varies	Don't know
CERTAINTY OF EVIDENCE OF REQUIRED RESOURCES	Very low	Low	Moderate	High			No included studies
COST EFFECTIVENESS	Favors the comparison	Probably favors the comparison	Does not favor either the intervention or the comparison	Probably favors the intervention	Favors the intervention	Varies	No included studies
EQUITY	Reduced	Probably reduced	Probably no impact	Probably increased	Increased	Varies	Don't know
ACCEPTABILITY	No	Probably no	Probably yes	Yes		Varies	Don't know
FEASIBILITY	No	Probably no	Probably yes	Yes		Varies	Don't know

The same procedure is adopted for the final recommendation. (Table 3) Strong recommendation can be given only if at least 75% of the panel votes for it and after the panel assessed that the presented evidence satisfies the conditions for a strong recommendation. This is particularly important when the certainty of the evidence is low or very low.

Table 3 – Type of Recommendation table layout from GRADEpro [21]

TYPE OF RECOMMENDATION

Strong recommendation against the intervention	Conditional recommendation against the intervention	Conditional recommendation for either the intervention or the comparison	Conditional recommendation for the intervention	Strong recommendation for the intervention
○	○	○	○	○

Adolopment of existing recommendations developed with GRADE method

Whether the CTS identifies recommendations produced by other international or national groups that adopted a rigorous and transparent methodology on areas that fall within the scope of these guidelines, it can evaluate the opportunity to start an adoloptment process, according to the procedures provided for by GRADE method. [23]

To this purpose, the CTS should ask the group that developed the recommendations to be adopted for all the original material used for the review of the evidence and the development of the recommendations.

Only if the original materials are available, the CTS will decide whether the recommendations have been developed robustly enough based on the following criteria:

1. Multidisciplinary composition of the panel
2. Evidence collected and analysed systematically, according to standard methods
3. Policy for management of conflicts of interest transparent and coherent with the SNLG requirements
4. Use of the Evidence-to-Decision framework
5. Consistency with higher-order recommendations: European guidelines and Italian guidelines issued by the Ministry of Health, National Observatory for Screening and National Prevention Plan.

Once the eligibility of the recommendations has been ascertained, the CTS will assess, for each individual recommendation, whether it can be "adopted" or "adapted":

- adoption: the direction and strength of the recommendation are not changed, the panel can integrate the text of the justification of the recommendation, the implementation considerations, and of monitoring considerations. In the case of “conditional” recommendations, in which the conditions for preferring an option are linked to contextual factors (availability of resources, cost effectiveness, organizational impact), the working group

can decline what the current Italian context conditions are; Substantial changes in the context could lead to an adaptation of the recommendation (see adaptation)

- adaptation: the direction and / or strength of the recommendation is changed.

In order to adopt a recommendation, the CTS, with the collaboration of the ERT, should evaluates whether an update of the systematic review of the literature is necessary. The need for updating is assessed not only on the basis of a chronological criterion, but also on the plausibility that an update can substantially change the results in terms of the strength of the association (or test accuracy in the case of diagnostics) and certainty of the estimate.

The CTS submits the PICOs to the Panel including a preliminary assessment according to the consistency criteria mentioned above and giving one of two possible indications: adoption or adaptation.

In any case, the CTS submits one or more PICOs to the Panel to go through the EtD criteria in order to update and integrate the judgments provided by the original panel as an update or adaptation to the current Italian context. These are the operational steps that should be followed:

1. The ERT prepares the material for presentation to the panel in plenary meeting, acquiring and uploading it to GRADEPro, if it has not already been done by the developers of the previous recommendations. To do this:
   1.1. the ERT Evaluate whether the included literature is updated and complete and if there is a need of updating and / or contextualization.
   1.2. the ERT Carries out a preliminary assessment of any additional evidence included following the GRADE criteria for the certainty of evidence, feasibility, acceptability, values and preferences, and costs.
2. The ERT drafts the adopted or adapted EtD framework and send it to the panel experts at least one week prior to the plenary meeting.
3. The panel, coordinated by the chairs, proceeds to formulate the recommendation according to the standard procedures indicated by the GRADE method. [23]

The panel, on its own initiative or on the proposal of the ERT, may request changing the PICO if it is no longer considered useful or relevant to the needs of clinical decisions. The reformulation of the PICO requires a new systematic review and therefore must undergo the prioritization process.

At the time of implementation of the new methodological requirements for guidelines development by the Italian National Institute of Health, six recommendations have already been developed by a Panel of Experts from the GISCi society, following the GRADE method, and published online in the GISCi website. [24] Since the methodology used by GISCi was consistent with the one required by the ISS, the CTS decided to present them to the new Panel of Experts to be voted, revised, and included in the " Multisociety Italian guidelines for cervical cancer prevention.", within the chapter "Follow-up of women treated for cervical intraepithelial neoplasia grade 2/3 (CIN2/3)", whether a consensus is reached. Adopted recommendations undergo the same review process required for publication in the

SNLG database. Up to February 2021, these recommendations completed the adoloptment process and are under review by external experts.

External review

The methodology of systematic literature review, the process of evaluating the evidence, and of the development of the recommendation undergoes external review by the experts in the field identified by the CTS.

The main aim of the external review is to assess the correctness of methodology implementation and the quality of reporting for each development process phase.

The comments from the review phase are debated in plenary with panel members to decide on how to integrate them into the final documents.

Reviewers' comments and panel decisions are attached to the documentation sent to the SNLG for final approval.

Guidelines approval and publication

The document including scope, PICOs, systematic reviews and plenary sessions outputs in SoF and EtD tables and the final recommendations, together with COI and external reviewers' contribution is sent to the SNLG for assessment and approval.

Comments from the reviewers of the SNLG and related answers and revisions are integrated in the final document in an iterative process, which could imply supplementary plenary sessions for discussion with panel members, until SNLG approval.

The approved recommendations are then published on the SNLG ISS Web site, available for free by all healthcare professional and patients. [8]

Guidelines updating process

The GDG is committed to reviewing and updating the recommendations when required by new European Guidelines, under request by the Italian Ministry of Health, or every 3 years to assess whether new evidence becomes available.

DISCUSSION

The process to develop the " Multisociety Italian guidelines for cervical cancer prevention." started. The first recommendation, which is in a single chapter, on HPV vaccination has already been published, and other chapters on post treatment follow up, use of biomarkers in triaging HPV-positive women, and conservative management of low risk CIN2 are in the pipeline. [8]

The guidelines are the product of a collaboration between all the scientific societies involved and endorsed by The National Centre for Screening Monitoring (ONS). This endorsement, the

multidisciplinary participation, and the rigorous methodology adopted brought to the recognition by the SNLG.

Given their public health nature, the guidelines were born in the framework of the recommendation of higher level, developed by European Commission and by national governmental agencies (Ministry of Health and ONS). This was the case of recommendations for screening, about the ages, the test to be used and the intervals, determined by European Guideline published in 2008 and updated in 2015 and contextualised to our country by the Italian HTA report and by the National Prevention Plan. [3, 4, 15, 25] For HPV vaccination, the basic recommendations were provided by the National Immunization Plan drafted by the Italian Ministry of Health. [25]

Moreover, in Italy both HPV vaccination and cervical cancer screening are included in the Essential Levels of Care set by the Ministry of Health. [2]

Therefore, the "Multisociety Italian guidelines for cervical cancer prevention" must develop recommendations in accordance with the recommendations given by higher level documents for all those clinical questions that were not afforded by these documents.

Strengths and limitations

As reported by Schunemann et al. "*Guidelines are criticized for taking too long to become available and for not being tailored to key users*." [9]

The GRADE approach and the CNEC methodology supported the GDG in overcoming these challenges.

Among the strengths of the GRADE approach, extensively described elsewhere [9, 22], the GDG emphasised the following:

- role of the multidisciplinary experts in all the phases, from evidence synthesis to development of the final recommendations;
- inclusion of patients as full voting members in the Panel of Experts;
- inclusion of methodological experts to guide clinical experts in the guideline's development method;
- transparency on all the phases, criteria, and justification, that makes the final recommendation easy to be adopted in each context and easy to be updated in future;
- flexibility in clinical implementation with inclusion of subgroup and implementation considerations;
- support for decision makers and healthcare manager with Monitoring and evaluation considerations;
- support for future development and to overcome existing gap of knowledge, enlisting Research priorities.

Among the limitations, in the GDG lacked a specific expertise in cost effectiveness analysis, which impact was limited by the experience of panel experts in different fields including health policy and management and health technology assessment.

To overcome the issues related to the timing for guidelines availability, this process will produce guidelines organized in chapters, grouping sets of meaningful recommendations. This implies that it will never be available a final book, but an ongoing online publication, as a results of a continuous production process that follows the topics timely prioritized.

Bibliography

1. Council Recommendation of 2 December 2003 on cancer screening OJ L 327, 16.12.2003, p. 34–38 2003/878/EC. Available at: http://data.europa.eu/eli/reco/2003/878/oj (Accessed on March 2021)
2. Decreto del Presidente del Consiglio dei Ministri 29 novembre 2001. Definizione dei livelli essenziali di assistenza. Gazzetta Ufficiale Serie Generale n.33 del 08-02-2002 - Suppl. Ordinario n. 26. Available at: https://www.gazzettaufficiale.it/eli/id/2002/02/08/02A00907/sg (Accessed on March 2021)
3. Arbyn M, Anttila A, Jordan J, Schenck U, Ronco G, Segnan N, et al (eds.). European guidelines for quality assurance in cervical cancer screening. Second edition. European Commission, Office for Official Publications of the European Communities, 2008 Luxembourg.
4. Anttila A, Arbyn A, De Vuyst H, Dillner J, Dillner L, Franceschi S, et al. (eds.). European guidelines for quality assurance in cervical cancer screening. Second edition, Supplements. Office for Official Publications of the European Union, 2015 Luxembourg, pp. XIII–XXIV.
5. Cuschieri K, Ronco G, Lorincz A, Smith L, Ogilvie G, Mirabello L, Carozzi F, Cubie H, Wentzensen N, Snijders P, Arbyn M, Monsonego J, Franceschi S. Eurogin roadmap 2017: Triage strategies for the management of HPV-positive women in cervical screening programs. Int J Cancer. 2018 Aug 15;143(4):735-745. doi: 10.1002/ijc.31261. Epub 2018 Feb 8. PMID: 29341110.
6. Tornesello ML, Buonaguro L, Giorgi-Rossi P, Buonaguro FM. Viral and cellular biomarkers in the diagnosis of cervical intraepithelial neoplasia and cancer. Biomed Res Int. 2013;2013:519619. doi: 10.1155/2013/519619. Epub 2013 Dec 9. PMID: 24383054; PMCID: PMC3872027.
7. LEGGE 8 marzo 2017, n. 24. Disposizioni in materia di sicurezza delle cure e della persona assistita, nonche' in materia di responsabilita' professionale degli esercenti le professioni sanitarie. (17G00041) (Gazzetta Ufficiale Serie Generale n.64 del 17-03-2017. Available at: https://www.gazzettaufficiale.it/ (Accessed on March 2021)
8. Istituto Superiore di Sanità. Sistema Nazionale Linee Guida dell'Istituto Superiore di Sanità. Website: https://snlg.iss.it/
9. Schünemann HJ, Lerda D, Dimitrova N, et al. Methods for Development of the European Commission Initiative on Breast Cancer Guidelines: Recommendations in the Era of Guideline Transparency. Ann Intern Med. 2019;171(4):273-280. doi:10.7326/M18-3445
10. Schünemann HJ, Wiercioch W, Brozek J, et al. GRADE Evidence to Decision (EtD) frameworks for adoption, adaptation, and de novo development of trustworthy recommendations: GRADE-ADOLOPMENT. J Clin Epidemiol. 2017;81:101-110. doi:10.1016/j.jclinepi.2016.09.009
11. Schünemann HJ, Best D, Vist G, et al; GRADE Working Group. Letters, numbers, symbols and words: how to communicate grades of evidence and recommendations. CMAJ. 2003;169:677-80. [PMID: 14517128]
12. Schünemann HJ, Wiercioch W, Etxeandia I, et al. Guidelines 2.0: systematic development of a comprehensive checklist for a successful guideline enterprise. CMAJ. 2014;186:E123-42. [PMID: 24344144] doi:10.1503/cmaj.131237
13. Schünemann HJ, Woodhead M, Anzueto A, et al; ATS/ERS Ad Hoc Committee on Integrating and Coordinating Efforts in COPD Guideline Development. A guide to guidelines for professional societies and other developers of recommendations: introduction to integrating and coordinating efforts in COPD guideline development. An official ATS/ERS workshop report. Proc Am Thorac Soc. 2012;9:215-8. [PMID: 23256161] doi:10.1513/pats.201208-053ST
14. Woolf S, Schünemann HJ, Eccles MP, et al. Developing clinical practice guidelines: types of evidence and outcomes; values and economics, synthesis, grading, and presentation and deriving recommendations. Implement Sci. 2012;7:61. [PMID: 22762158] doi:10 .1186/1748-5908-7-61
15. Ronco G, Biggeri A, Confortini M, et al. Health technology assessment report: ricerca del DNA di papillomavirus umano (HPV) come test primario per lo screening dei precursori del cancro del collo dell'utero [Health technology assessment report: HPV DNA based primary screening for cervical cancer precursors]. Epidemiol Prev. 2012;36(3-4 Suppl 1):e1-e72.
16. Italian Ministry of Health. Screening oncologici. Raccomandazioni per la pianificazione e l'esecuzione degli screening di popolazione per la prevenzione del cancro della mammella, del cancro della cervice uterina e del cancro del colon

retto. 2006. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_774_allegato.pdf (Accessed on March 2021)

17. SNLG- ISS. Methodological manual for the production of clinical practice guidelines. National Centre for Clinical Excellence, Quality and Safety of Care (CNEC), 2019. Available at: https://snlg.iss.it/wp-content/uploads/2019/04/MM_v1.3.2_apr_2019.pdf (Accessed March 2021)
18. Higgins JPT, Thomas J, Chandler J, Cumpston M, Li T, Page MJ, Welch VA, eds. Cochrane Handbook for Systematic Reviews of Interventions, Second Edition. The Cochrane Collaboration. First published:20 September 2019. DOI:10.1002/9781119536604
19. Schünemann HJ, Higgins JPT, Vist GE et al. on behalf of the Cochrane GRADEing Methods Group (formerly Applicability and Recommendations Methods Group) and the Cochrane Statistical Methods Group. Completing 'Summary of findings' tables and grading the certainty of the evidence. pp. 375-402 Doi: https://doi.org/10.1002/9781119536604.ch14
20. Schünemann HJ, Al-Ansary LA, Forland F, et al; Board of Trustees of the Guidelines International Network. Guidelines International Network: principles for disclosure of interests and management of conflicts in guidelines. Ann Intern Med. 2015;163:548-53. [PMID: 26436619] doi:10.7326/M14-1885
21. GRADEpro GDT online platform. 2020, McMaster University and Evidence Prime Inc. Available at: https://gradepro.org/ (Accessed on March 2021)
22. Schünemann HJ, Brożek J, Guyatt G, and Andrew Oxman, eds. Handbook for grading the quality of evidence and the strength of recommendations using the GRADE approach. Updated October 2013. Available at: https://gdt.gradepro.org/app/handbook/handbook.html (Accessed on March 2021)
23. Schünemann HJ, Wiercioch W, Brozek J, et al. GRADE Evidence to Decision (EtD) frameworks for adoption, adaptation, and de novo development of trustworthy recommendations: GRADE-ADOLOPMENT. J Clin Epidemiol. 2017;81:101-110. doi:10.1016/j.jclinepi.2016.09.009
24. GISCI Working Group. "Utilizzo del test HPV-HR nel triage delle ASC-US, delle LSIL in donne con più di 35 anni, nel follow-up delle donne con citologia ASC-US+ dopo un approfondimento di secondo livello negativo per CIN2+ e nel follow-up dopo trattamento delle lesioni CIN2-3: aggiornamento 2018" 2018. Available at: https://gisci.it/documenti/documenti_gisci/Utilizzo_test_HPV-HR_2018_def2.pdf (Accessed on March 2021)
25. Italian Ministry of Health. Piano Nazionale della Prevenzione 2014-2019. 2014. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_2285_allegato.pdf (Accessed on March 2021)
26. Italian Ministry of Health. Piano Nazionale della Prevenzione Vaccinale 2017-2019. 2017. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_2571_allegato.pdf (Accessed on March 2021)

4.2. Research article #07: HPV vaccination in women treated for CIN 2 or 3: evidence-based recommendation from the Multisociety Italian guidelines for cervical cancer prevention.

The findings of this research article were published in Italian the Italian National System of Guidelines (SNLG) online database. [8]. The English version is invited for submission in European Journal of Gynaecological Oncology by March 31, 2021.

Garutti P, Ventureli F, Ghini I, Giorgi Rossi P. Writing committee on behalf of the Multisociety Italian guidelines for cervical cancer prevention Working Group. HPV vaccination in women treated for CIN 2 or 3: evidence-based recommendation from the Multisociety Italian guidelines for cervical cancer prevention. Invited submission in *Eur J Gynaecol Oncol.*

Abstract

BACKGROUND: Women treated for Cervical Intraepithelial Neoplasia (CIN) grade 2 or 3 are at higher risk of CIN and cervical cancer than the general population. Human Papillomavirus (HPV) Vaccination is effective in preventing CIN in women who are not infected by HPV. Some studies suggested a reduction of CIN2 or 3 in vaccinated women treated for CIN. A working group including all Italian scientific societies involved in tackling cervical cancer answered the question: “Should women treated for CIN2 and CIN3 be vaccinated for HPV?”

MATERIALS AND METHODS: The group conducted a systematic review of literature on efficacy published from January 2006 to May 2019. Evidences on safety outcomes were retrieved by a recent Cochrane Review on vaccination in the general population. To develop the final recommendation, evidences were appraised and integrated by a Panel of Experts using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) Evidence to Decision frameworks.

RESULTS: Six eligible studies were included. Four were RCTs and 2 cohort studies, with different timing of vaccination. An additional study, published in October 2019, was added after external review. A reduction of 70% CIN2+ in the treatment group was estimated; the vaccine was considered safe.

CONCLUSIONS: The GDG recommends the use of HPV vaccination in women treated for CIN2 or 3. The strong recommendation is based on large estimated desirable effects and trivial anticipated undesirable effects (moderate certainty of evidence), negligible costs and savings (not included studies), and a positive judgment in terms of feasibility, acceptability, and impact on equity.

ETHICS APPROVAL: The work here report does not need ethical approval. The guideline development project has been submitted to and approved by the Italian National System of Guidelines (SNLG) of the National Institute of Health (ISS) (Sistema Nazionale Linee Guida, Istituto Superiore di Sanità). This specific recommendation has been submitted to the SNLG and published on the web platform of the Italian recognized guidelines database: https://snlg.iss.it/

BACKGROUND

The main Italian scientific societies (Appendix - Table 1) involved in tackling cervical cancer started a project to develop recommendations on cervical cancer prevention. The project aims at integrating the European guidelines for quality assurance in screening and the Italian Ministry of Health guidelines for screening on all those specific aspects that are not included in the scope of these two frameworks setting guidelines. [1-4] In the first chapter afforded the prioritized clinical question was "should women treated for CIN2 or CIN3 be vaccinated against HPV".

In Italy, approximately 1,700,000 women are screened for cervical cancer every year within organized programs with a detection rate of 3.4 per thousand preneoplastic lesions, for a total of more than seven thousand treatments. [5] In addition, data from the Italian nationwide surveillance PASSI suggest that almost the same amount of cervical preneoplastic lesions are identified and treated every year in Italy outside organised screening programs. [6]

The HPV vaccine was shown to be effective in preventing new infections and CIN associated with HPV 16 and 18 infection. Instead, the vaccine was shown to have no therapeutic effect on infections and lesions already present at the time of vaccination. [7] Thus, vaccine effectiveness in women treated for CIN2 and CIN3 is uncertain and challenging to be assessed given the difficulty of classifying lesions identified after treatment as a new pathology or persistence of the treated lesion.

The rationale for vaccinating women treated for CIN2 and CIN3 starts from the evidence of an increased risk of CIN lesions and cancer that persists for many years in these women. [8,9]

A meta-analysis conducted by the Italian Group for Cervical Cancer Screening (GISCi), reported an average viral clearance of 76.5% at 6 months after treatment, and an 8.4% risk of developing CIN2 + at 24 months in women treated for CIN2 and CIN3. [10] The risk of recurrence of CIN2+ lesions, differs by HPV test, and cytology results, ranging from a 0.9% to 30% at 24 months after treatment, in negative and positive women to the first HPV test after treatment, respectively. [11]

Aim

To develop, following a transparent and rigorous methodology, an evidence-based recommendation answering the question: should women treated for CIN2 and CIN3 be vaccinated for HPV?

MATERIALS AND METHODS

Structured question and outcomes prioritization

The clinical question "Should HPV vaccination be used in women treated for Cervical Intraepithelial Neoplasia 2 or 3?" was structured according to the Population, Intervention, Comparison and Outcomes (PICO) framework (Table 1). The outcomes were also prioritized by the GDG as suggested by the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach. [12-14]

Table 1 – Clinical question

Population: women treated for CIN2 or CIN3

Intervention: HPV vaccine (2-, 4-, 9-valent), including full vaccination course with 3 doses and incomplete vaccination courses

Comparator: no HPV vaccination

Outcomes prioritised in the scoping phase:

- Incidence of invasive cervical cancers (Rating: CRITICAL)
- Incidence of CIN2 and CIN3 (Rating: CRITICAL)
- Safety Outcomes (Proposed in the prioritization phase)[7]
    - Local /injection site adverse events
    - Overall systematic event and general symptoms
    - Serious adverse events
    - Deaths

All the outcomes proposed in the prioritization phase were included and none were excluded.

Systematic review

Review protocol and amendments

The review protocol was registered in PROSPERO - International prospective register of systematic reviews del National Institute for Health Research with ID CRD42019135870 [15]

According to external reviewers' comments, the Guidelines Development Group (GDG) integrated the protocol on 17/01/2020 to include the description of sources and methods to collect background information, safety outcomes and timing of vaccination.

Data sources and searches

To quantify the actual population potentially targeted by the intervention in the background section of the Evidence-To-Decision (EtD) table, two nationwide sources were used. Data on organised cervical cancer screening programs were taken from the 2017 Report of The National Centre for Screening Monitoring (ONS). [5] While lesions and treatments from spontaneous screening were estimated starting from coverage data collected by the PASSI surveillance. [6]

To synthesize evidence on effectiveness and safety of the intervention, a systematic search was performed on May 31, 2019 in the following databases: MEDLINE (accessed through PubMed), The Cochrane Library, EMBASE (accessed through embase.com) and Scopus (accessed through Scopus.com). A pre-defined search strategy was adapted to achieve the requirements of each database. (Appendix – Table 2)

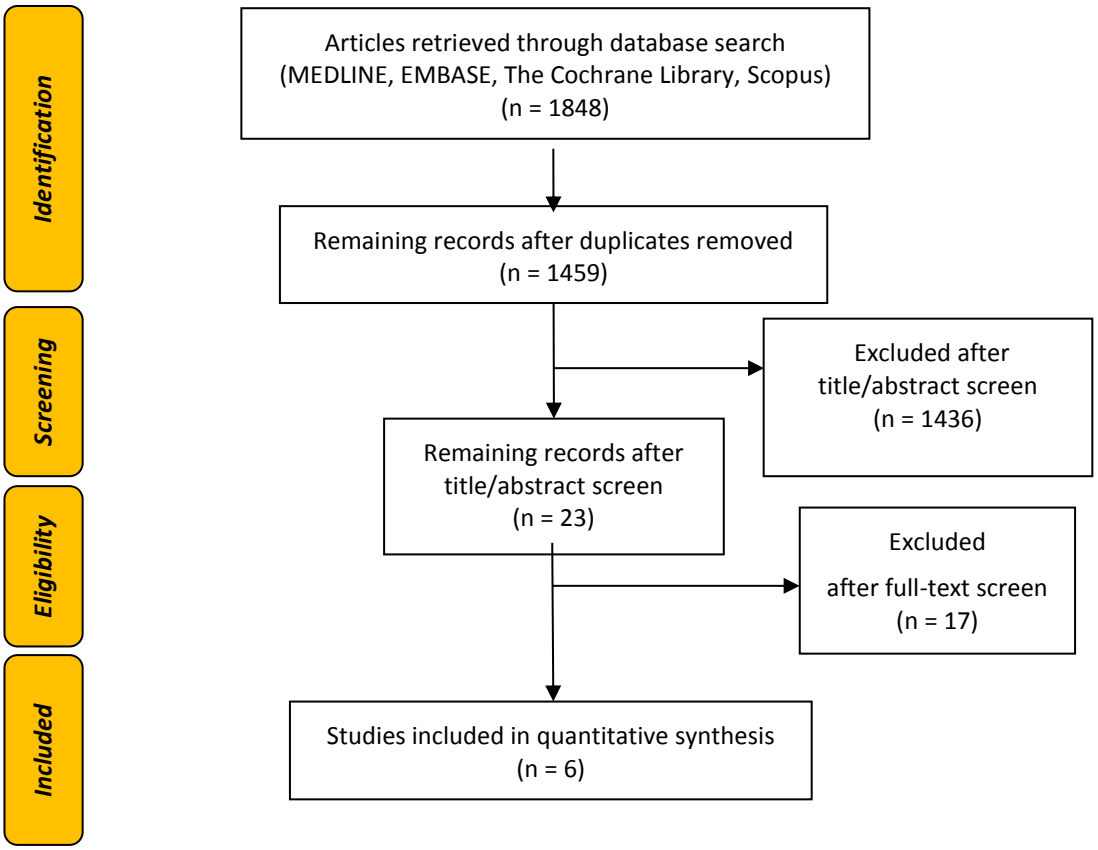
Study selection

Studies were included if published after 2006, the year of publication of the first trials on HPV vaccines marketed in the European Union.

All titles/abstracts of studies identified using the search strategy, and those from the reference lists, were screened independently by two reviewers for eligibility. The full texts of potentially eligible studies were then assessed by two Evidence Review team (ERT) members. Any disagreement or uncertainty over eligibility was discussed between the reviewers and, when consensus was not reached, the project leader was involved in the discussion.

The result of this process is reported in the PRISMA flowchart (Figure 1).

Figure 1 - PRISMA Flow chart



Data extraction

Data extraction was done by one reviewer using predefined data extraction form including: first author and year of publication, country, study design, study aims, study population and sample size, description of intervention, descriptions of comparators, study outcomes and times of measurement, main results, results in population subgroups, and the quality of the study. It was not necessary to contact study authors to retrieve additional information.

Data analysis

The efficacy outcomes of interest were the risks of cervical intraepithelial neoplasia grade 2 or grade 3 and of invasive cervical cancer in vaccinated and non-vaccinated women after treatment of CIN2 and CIN3. As categorical outcomes, the effect measure was synthesized calculating the pooled Risk Ratio and the Absolute Risk Difference. The 95% confidence intervals were calculated to report the effect size with its precision.

The pooled estimates were reported for each outcome in the Summary of Findings table using the GRADEpro online software tool, along with the certainty of the evidence items. [16]

The evidence on safety outcomes were retrieved as reported by the Cochrane review retrieved.

A narrative synthesis was performed for the costs and resource consumption, not included in the meta-analysis.

Risk of bias assessment and Certainty of the evidence

The risk of bias was assessed using The Cochrane risk of bias RoB 2.0 tool [17] for randomized controlled trials, and the ROBINS-I tool for non-randomized studies. [18] The quality assessment results were reported in the “Risk of bias”, “Inconsistency”, “Indirectness”, and “Imprecision” items of the 'Summary-of-Findings' (SoF) table using the GRADEpro software tool. [16]

The overall certainty of evidence per outcome was rated using the GRADE approach and presented to the Panel of Experts using the SoF table. [16, 19 -21]

Evidence to decision framework and recommendation development

The process used by GDG to formulate recommendations was described in a dedicated article published elsewhere. [12] In brief, a Technical Scientific Committee (CTS) with representatives from nine scientific societies involved in tackling cervical cancer defined the scope of the process and set up the GDG subgroups, assessing conflict of interest (COI) disclosures. One of the GDG subgroups, the Evidence Review Team, including experts on the topic and methodologists, was responsible for the systematic review and completion of the first draft of the SoF table and the EtD framework. [19-21] The tables were presented in the plenary meetings to the Panel of Experts including clinicians and informed patients, supporting the appraisal of EtD criteria and the recommendation formulation. Subsequently, the whole process was reviewed by Independent External Reviewers invited by the CTS, and feedbacks were used to improve the quality of recommendation. Then, a final document was sent to the Italian National System of Guidelines (SNLG) reviewers, starting an iterative process of feedbacks and revisions done by the GDG, until final approval by SNLG reviewers and publication of the final recommendation on the Italian SNLG guidelines database. [22]

RESULTS

Results from the systematic review of efficacy

Included studies

Six eligible studies were included in the systematic review of the literature, including 1 Randomized Clinical Trial (RCT) [23] and 2 cohort studies [24, 25] evaluating the effectiveness of post-treatment HPV vaccination and 3 RCTs evaluating the effectiveness of pre-treatment HPV vaccination in reducing recurrences. [26-28] (Appendix – Table 3)

The total number of women included in the systematic review was 1427 in the intervention group and 1663 in the control group. In the included studies, no invasive cancers were identified, and this outcome was excluded from the quality assessment of the evidence on efficacy.

On January 17, 2020, during the external review process, a study published after the literature search date was noticed, showing a smaller protection than previous studies (Hazard Ratio 0.86; 95%CI 0.67-1.09). [29] In Plenary meeting, the GDL expressed 13 out of 16 votes in favour of the inclusion of the study in the additional considerations without updating the systematic review neither changing the criteria of the recommendation.

Evidence synthesis

By comparing the risks of recurrences measured using CIN2 and CIN3 as endpoints, the intervention was associated with a significant reduction in risk in all included studies, with consistent estimates. The pooled estimates showed a Relative Risk (RR) of 0.32 (95%CI 0.15 – 0.66) for RCTs assessing the effectiveness of pre-treatment HPV vaccination, a RR of 0.00 (0.00 – 1.45) for the RCT and RR 0.30 (0.15 – 0.58) for the cohort studies assessing of post-treatment HPV vaccination.

The pooled estimates of RR for lesions related to HPV types included in the vaccine, showed a higher reduction of risk in vaccinated women compared to non-vaccinated. (Table 2)

The overall certainty of evidence assessed according to GRADE approach was rated as moderate.

Table 2 – Summary of findings table

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	HPV vaccination	No intervention	Relative (95% CI)	Absolute (95% CI)		
Cervical cancer incidence												
6 [23-28]	observational studies	serious [a]	not serious	serious [b]	very serious [c]	none	0/1427 (0.0%)	0/1663 (0.0%)	not estimable		⨁◯◯◯ VERY LOW	CRITICO
Incidence of CIN2 and CIN3 (for persistent and non-persistent infections) (follow up: range 16 months to 27 months; assessed with: pre-treatment vaccination; lesion associated with all hr-HPV types)												
3 [26,27,28]	randomised trials	not serious	not serious	very serious [d]	not serious	strong association	9/806 (1.1%)	36/1025 (3.5%)	**RR 0.32** (0.15 to 0.66)	**24 fewer per 1.000** (from 30 fewer to 12 fewer)	⨁⨁⨁◯ MODERATE	CRITICO
Incidence of CIN2 and CIN3 (for persistent and non-persistent infections) (follow up: median 36 months; assessed with: post-treatment vaccination; lesion associated with all hr-HPV types)												
1 [23]	randomised trials	not serious	not serious	not serious	very serious [e,f]	strong association	0/89 (0.0%)	4/89 (4.5%)	**RR 0.00** (0.00 to 1.45)	**-- per 1.000** (from -- to 20 more)	⨁⨁⨁◯ MODERATE	CRITICO
Incidence of CIN2 and CIN3 (for persistent and non-persistent infections) (follow up: range 36 months to 42 months; assessed with: post-treatment vaccination; lesion associated with all hr-HPV types)												
2 [24, 25]	observational studies	serious [a]	not serious	not serious	not serious	strong association	11/532 (2.1%)	38/549 (6.9%)	**RR 0.30** (0.15 to 0.58)	**48 fewer per 1.000** (from 59 fewer to 29 fewer)	⨁⨁◯◯ LOW	CRITICO

Research article #07: HPV vaccination in women treated for CIN 2 or 3: evidence-based recommendation from the Multisociety Italian guidelines for cervical cancer prevention.

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	HPV vaccination	No intervention	Relative (95% CI)	Absolute (95% CI)		
Incidence of CIN2 and CIN3 (for persistent and non-persistent infections) (follow up: range 16 months to 27 months; assessed with: pre-treatment vaccination; only lesions associated with hr-HPV types coved by vaccine)												
3 [26,27,28]	randomised trials	not serious	not serious	very serious [d]	not serious	strong association	1/806 (0.1%)	7/1026 (0.7%)	**RR 0.18** (0.02 to 1.48)	**6 fewer per 1.000** (from 7 fewer to 3 more)	⊕⊕⊕◯ MODERATE	CRITICO
Incidence of CIN2 and CIN3 (for persistent and non-persistent infections) (follow up: median 36 months; assessed with: post-treatment vaccination; only lesions associated with hr-HPV types coved by vaccine)												
1 [23]	randomised trials	not serious	not serious	not serious	very serious [e,f]	strong association	0/89 (0.0%)	4/89 (4.5%)	**RR 0.00** (0.00 to 1.45)	**-- per 1.000** (from -- to 20 more)	⊕⊕⊕◯ MODERATE	CRITICO
Incidence of CIN2 and CIN3 (for persistent and non-persistent infections) (follow up: range 36 months to 42 months; assessed with: post-treatment vaccination; only lesions associated with hr-HPV types coved by vaccine)												
2 [24, 25]	observational studies	serious [a]	not serious	not serious	not serious	strong association	5/532 (0.9%)	26/549 (4.7%)	**RR 0.20** (0.08 to 0.51)	**38 fewer per 1.000** (from 44 fewer to 23 fewer)	⊕⊕◯◯ LOW	CRITICO

CI: Confidence interval; **RR:** Risk ratio

Explanations

a. Non-randomized studies with selection and attrition bias in Ghelardi 2018 [25]

b. In some studies the HPV vaccination wad administered before diagnosis and treatment of cervical lesions

c. The expected incidence of cervical cancer in screened women (population of the included studies) is too low to be compared between the two groups.

d. In these studies the HPV vaccination wad administered before diagnosis and treatment of cervical lesions

e. Limited number of events and limited sample size of included studies.

f. The assessed imprecision led to a define strong, rather than very strong, the overall effect

GRADE Working Group grades of evidence [19]

High quality: Further research is very unlikely to change our confidence in the estimate of effect. *The attribution of "high quality" depends on the following conditions: well-conducted randomized trials, with consistent findings, direct outcome, precise estimates (narrow confidence intervals), absence of reporting bias.

Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.

Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.

Very low quality: We are very uncertain about the estimate.

Results from the systematic review of safety

Included studies

The studies included in the systematic review of efficacy had too small sample size to observe any serious adverse effect. Nevertheless, none of the included study reported serious adverse effects in women vaccinated after treatment. [23-28]

Given the availability of a recent Cochrane review on the safety of HPV vaccination, the evidence synthesised in this review was used, as considered transferable to the population included in the PICO defined by the GDG. [7] (Table 3)

Evidence synthesis

The Cochrane review on the safety of HPV vaccination showed an increased risk of local adverse events for vaccination compared to placebo (RR 1.18; 95% CI 1.16 - 1.20) assessed on 8 studies and 18113 women, with moderate certainty of evidence. Conversely, no differences in the risk of systemic adverse events were reported (RR1.02 95%CI 0.98-1.07) assessed on 8 studies and 18191 women, with a moderate certainty of evidence. Finally, there was no statistically significant increase in the risk of death (RR 1.25 CI95% 0.81-1.93) assessed in 23 studies and 71452 women, with an overall low certainty of evidence. [7] (Table 3)

Table 3 – Evidence synthesis on safety from a Cochrane Systematic Review by Arbyn et al. 2018. [7]

Outcomes	Absolute risk / per 10,000		Relative effect (95% CI)	No of Participants (studies)	Quality of the evidence (GRADE)
	placebo	vaccinated			
Overall local/injection site adverse events	6847	8080	**1.18** (1.16 to 1.20)	18,113 (8 studies)	⨁⨁⨁⨀ moderate
Pain at injection site	6505	8782	**1.35** (1.23 to 1.49)	25,691 (13 studies)	⨁⨁⨁⨀ moderate
Swelling at injection site	1582	2737	**1.73** (1.32 to 2.27)	22,106 (9 studies)	⨁⨁⨁⨀ moderate
Redness at injection site	1938	3333	**1.72** (1.50 to 1.97)	19,996 (6 studies)	⨁⨁⨁⨀ moderate
Overall systematic event and general symptoms	6102	6224	**1.02** (0.98 to 1.07)	18,191 (8 studies)	⨁⨁⨁⨀ moderate
Serious adverse events	605	611	**1.01** (0.95 to 1.07)	6978 (21studies)	⨁⨁⨁⨁ high
Deaths	11	13	**1.25** (0.81 to 1.93)	71,452 (23 studies)	⨁⨁⨀⨀ low
CI: Confidence interval**; RR:** Risk Ratio					

Certainty of evidence

The overall certainty of the evidence was rated as moderate.

The main concerns across studies were related to the timing of vaccination and study design. Three of the four RCTs included assessed the effectiveness of pre-diagnosis and pre-treatment HPV vaccination [26-28] and evidence on post-treatment were mainly available from cohort studies [24, 25], with potential selection and attrition biases. [25] (Appendix Figures 1 and 2)

The evidence was consequently downgraded for indirectness because the largest randomized studies were conducted on a pre-treatment vaccination and then restricting the analysis to a sub-population of treated women, so the evidence was not direct considering the intervention under evaluation, i.e. treatment of women who already have been treated or who are undergoing the treatment. On the other hand, the GDG noted that there was not heterogeneity between results independently from the timing of the intervention. Thus, evidence was not downgraded for heterogeneity. The effect was considered large in all subgroups thus the certainty was upgraded to moderate.

Moreover, no evidence of difference in the risk of cervical cancer were retrieved due to the sample size of included studies and low expected incidence of cancer in this population. Notwithstanding, the pooled estimates showed consistent results toward reduction of CIN2 and CIN3 risk in the intervention group, internationally validated endpoints as proxy for cervical cancer.

Results for economic evidence

A systematic review of the literature on economic evidence was not performed.

The appraisal of the economics criteria was conducted during plenary meeting, starting from data on costs retrieved by public tenders for 4-valent (€33.5 per dose) 9-valent HPV vaccine (€63 per dose) purchase by regional healthcare services and costs for CIN treatment reported by an Italian Health Technology Assessment (HTA) report on cervical cancer screening. [3, 30, 31]

The GDG highlighted that costs did not include counselling on vaccination and dose administration.

From an approximate estimate, every avoided CIN2 + costs 2500/5000 euros considering the current costs of the HPV vaccine. The estimates considering CIN2+ incidence at 6% and vaccine protection at 60%, ranged from 2792 € if a vaccine dose costs 33.5 €, up to 5250 € if vaccine costs 63 € per dose. Considering a CIN2 + incidence of 8% and vaccine protection at 60%, the cost of each avoided CIN2+ ranged from € 2094 (with vaccine costs € 33.5) to € 3938 (with vaccine cost of € 63 per dose).

The GDG reported that there were also unquantified avoided costs (retreatments and treatment related to obstetric outcomes). According the Italian HTA report, the cost of treating a CIN2 +, including follow up, was € 1785 (2010 currency) [3], while costs related to reproductive outcomes avoided by reducing retreatments were not quantified.

The Panel of Experts judged as negligible the costs for each event avoided with the HPV vaccination, leading to a judgment of cost-effectiveness in favour of intervention. [21] (Table 4)

The GDG also stated that a more in-depth economic analysis should be conducted, since the current judgments were based on a rapid cost-consequences analysis and on the opinion of the panel's experts assessing available data.

Table 4 – Summary of judgments from the Evidence-to-Decision framework by Panel of experts [16]

CRITERI	SUMMARY OF JUDGEMENTS						IMPORTANCE FOR DECISION
PROBLEM	No	Probably no	Probably yes	**Yes**	Varies	Don't know	
DESIRABLE EFFECTS	Trivial	Small	Moderate	**Large**	Varies	Don't know	
UNDESIRABLE EFFECTS	Large	Moderate	Small	**Trivial**	Varies	Don't know	
CERTAINTY OF EVIDENCE	Very low	Low	**Moderate**	High	No included studies		
VALUES	Important uncertainty or variability	Possibly important uncertainty or variability	**Probably no important uncertainty or variability**	No important uncertainty or variability			
BALANCE OF EFFECTS	Favors the comparison	Probably favors the comparison	Does not favor either the intervention or the comparison	Probably favors the intervention	**Favors the intervention**	Varies	Don't know
RESOURCES REQUIRED	Large costs	Moderate costs	**Negligible costs and savings**	Moderate savings	Large savings	Varies	Don't know
CERTAINTY OF EVIDENCE OF REQUIRED RESOURCES	Very low	Low	Moderate	High	No included studies		
COST EFFECTIVENESS	Favors the comparison	Probably favors the comparison	Does not favor either the intervention or the comparison	Probably favors the intervention	**Favors the intervention**	Varies	No included studies
EQUITY	Reduced	Probably reduced	Probably no impact	Probably increased	**Increased**	Varies	Don't know
ACCEPTABILITY	No	Probably no	Probably yes	**Yes**	Varies	Don't know	
FEASIBILITY	No	Probably no	Probably yes	**Yes**	Varies	Don't know	

Equity, acceptability, and feasibility

Equity was considered to increase since a free access to vaccine with active proposal by the screening service would decrease economic and health literacy barriers, in analogy to the documented effect of population-based screening programs [32, 33] and active invitation to HPV vaccination in Italy in reducing health inequalities. [34]

The intervention was considered as largely acceptable by all the three relevant involved groups: health professionals were in large part in favour of the intervention as reflected by the panellists; included studies showed that most women accepted the intervention; finally, in Italy several regional health services were already providing the intervention for free, showing that also policy makers accepted the intervention. (Table 4)

DISCUSSION

Recommendation and justification

The GDG expressed a strong recommendation in favour of the use of HPV vaccination in women treated for CIN2 / 3, mainly considering the large expected desired effects, ant the negligible undesirable events. (Table 4)

Although the outcome assessed as "incidence of invasive cancers" had a "very low" quality of evidence, the panel unanimously in plenary discussion assessed that the outcomes "CIN2 and CIN3 lesions" were valid proxies of the risk of invasive cancer. This decision was consistent with decisions taken by other panels in the development of international recommendations for the prevention of

cervical cancer. [35-37] Furthermore, given the preventive efficacy of post-treatment follow-up, it was not reasonable to expect incident cancers in women treated for CIN2 and CIN3 in controlled studies.

Desirable effects

The GDG judged as large the desirable effects expected from the implementation of HPV vaccination in women treated for CIN2 / 3, mainly based on the results of the three studies included assessing vaccination performed after treatment, thus with population and intervention consistent with the PICO. [23-25]

The GDG also considered the increased risk of premature births and negative pregnancy outcomes resulting from repeated cervical treatments in women of childbearing age. [38]

Finally, the potential increase in the desired effects compared to those reported in the included studies, resulting from the introduction of the nonavalent HPV vaccine was considered for the final judgment.

Undesirable effects

The GDL judged as trivial the undesirable effects of HPV vaccination in women treated for CIN2 / 3 based on the evidence reported by a Cochrane review published in 2018 on HPV vaccination safety. Despite the Cochrane review assessed safety outcomes in the general population, [7] the GDG considered the results transferable to the target population of the recommendation. (Table 4)

Resources required

Although direct assessments of all the costs and savings anti-HPV vaccination in women treated for CIN2 / 3 was not available, the GDG considered the costs related to the HPV vaccination implementation to be negligible. This judgment was based on the opinion of the Panel of Experts, considering the limited costs related to the vaccination purchase and counseling, and the avoided costs, not quantified, resulting from the reduction in repeated treatments in the management of related obstetric outcomes. (Table 4)

Finally, although no direct assessment of the cost-effectiveness of HPV vaccination was available in women treated for CIN2/3, the GDG evaluated the cost-effectiveness in favour of the intervention.

Strengths and weaknesses of the study

A systematic review of cost-effectiveness of the intervention, neither a proper cost effectiveness analysis was conducted. A rapid cost consequence estimate was produced by the panellists, which results suggested that a proper cost effectiveness analysis should be conducted, even if the budget impact of the intervention would not be large in terms of absolute cost for the NHS.

Similarly, no systematic review was conducted for evidence on how women value the included outcomes. Nevertheless, cancer incidence and reproductive outcomes were considered hard outcomes that most women would rate as critical.

Finally, the systematic review search has been closed on May 2019 even if studies published after that date have been considered in the final evaluation [29], according to external reviewers' suggestion. After the publication of the recommendation, two studies were published and identified by the ERT, which results go in the same direction of the included studies. [39, 40]

Subgroup considerations

Although not considered in the PICO, the populations of the included trials include also women treated for AIS (adenocarcinoma in situ) and microinvasive cancers. The GDG stated that recommendation can also be extended to these women.

In women of non-reproductive age, the overall impact of the intervention is lower, making the balance between desirable and undesirable effects, as much as the balance between benefits and costs, less favourable.

Implementation considerations

The timing of vaccination in trials was close to treatment (before or within three months after surgery). There was no evidence for vaccinations after this time frame.

Thus, to ensure timeliness in HPV vaccination, it is advisable to identify a care pathway, defining the roles of the health services involved. The steps to be defined include the identification of target women, the referral to vaccination services, the counselling and HPV vaccine administration. In this regard, the GDG recalled how such problems emerged in other vaccinations targeting high risk groups as hepatitis B vaccination. [41, 42] Moreover, the GDG highlighted how issues related to care pathway organization can be greater in some opportunistic screening settings.

Monitoring and evaluation

The GDL stated that HPV vaccination coverage in women treated for CIN2/3 should be calculated in organised screening programs.

Relation to other guidelines

The ERT found that the most important agencies and scientific societies do not mention the use of HPV vaccine in women treated for CIN2 or CIN3. The American Cancer Society and the CDC recommend recently not to vaccinate women over 26, actually excluding the vast majority of the potential target of the proposed intervention. [43, 44] Similarly, ECDC limits the vaccination to women below 26, but it mentions the need to study strategies to vaccinate high-risk groups in addition to mass vaccination of girls and boys. [45]

At National level, Spain introduced free vaccination of women treated for CIN2 and CIN3 in 2017. [46]

The Austrian HTA agency in 2020 issued a report concluding that there "is moderate evidence that HPV vaccination in women treated for high-grade cervical cancer lesions reduces the risk of future HPV related high-grade CIN and is more effective than usual care", but it is also cautious about safety in this group, since vaccine has been administered mostly to younger women, and on long term effectiveness. [47]

Finally, the European society of Gynaecologic Oncology and the European Federation for colposcopy published in 2019 a position paper on HPV vaccine in which they suggest offering vaccination, on individual basis, to women with HPV-related disease and prior local treatment. [48]

Unanswered questions and future research

The main gap of knowledge emerged during the recommendation development process was related to the optimal timing for vaccination, for which evidence from new studies is needed.

Moreover, to have stronger evidence on the effectiveness of HPV vaccination in the natural history of the disease after treatment of CIN2/3, it is important to be able to distinguish recurrences due to inadequate treatment and/or persistence of the HPV infection that caused the treated lesion, from recurrences due to new infections. To this end, it would be advisable in future studies or in re-analyses of already published studies, to perform HPV typing on samples collected before and after treatment.

Finally, the GDG reported that an updated of the recommendation will be necessary when new evidence coming from ongoing or planned trials will be available. Relevant evidence will come from the Hope9 Trial planned in Italy [49] and from the NOVEL trial in Sweden and UK. [50]

Bibliography

1. Arbyn M, Anttila A, Jordan J, Schenck U, Ronco G, Segnan N, et al (eds.). European guidelines for quality assurance in cervical cancer screening. Second edition. European Commission, Office for Official Publications of the European Communities, 2008 Luxembourg.
2. Anttila A, Arbyn A, De Vuyst H, Dillner J, Dillner L, Franceschi S, et al. (eds.). European guidelines for quality assurance in cervical cancer screening. Second edition, Supplements. Office for Official Publications of the European Union, 2015 Luxembourg, pp. XIII–XXIV.
3. Ronco G, Biggeri A, Confortini M, et al. Health technology assessment report: ricerca del DNA di papillomavirus umano (HPV) come test primario per lo screening dei precursori del cancro del collo dell'utero [Health technology assessment report: HPV DNA based primary screening for cervical cancer precursors]. Epidemiol Prev. 2012;36(3-4 Suppl 1):e1-e72.
4. Italian Ministry of Health. Screening oncologici. Raccomandazioni per la pianificazione e l'esecuzione degli screening di popolazione per la prevenzione del cancro della mammella, del cancro della cervice uterina e del cancro del colon retto. 2006. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_774_allegato.pdf (Accessed on March 2021)
5. Osservatorio Nazionale Screening (ONS). Rapporto Nazionale 2017. Available at: https://www.osservatorionazionalescreening.it/sites/default/files/allegati/ons%20rapporto%202017.pdf (Accessed on March 2021)
6. Gruppo Tecnico Nazionale PASSI. I dati per l'Italia: lo screening cervicale. 2015-2018. Available at: https://www.epicentro.iss.it/passi/dati/ScreeningCervicale (Accessed on March 2021)
7. Arbyn M, Xu L, Simoens C, Martin-Hirsch PP. Prophylactic vaccination against human papillomaviruses to prevent cervical cancer and its precursors. *Cochrane Database Syst Rev*. 2018;5(5):CD009069. Published 2018 May 9. doi:10.1002/14651858.CD009069.pub3
8. Strander B, Hällgren J, Sparén P. Effect of ageing on cervical or vaginal cancer in Swedish women previously treated for cervical intraepithelial neoplasia grade 3: population based cohort study of long term incidence and mortality. BMJ. 2014 Jan 14;348:f7361. doi: 10.1136/bmj.f7361.
9. Rebolj M, Helmerhorst T, Habbema D, Looman C, Boer R, van Rosmalen J, van Ballegooijen M. Risk of cervical cancer after completed post- treatment follow-up of cervical intraepithelial neoplasia: population based cohort study. BMJ. 2012 Oct 31;345:e6855. doi: 10.1136/bmj.e6855.
10. Prime 6 Raccomandazioni GISCI per il follow up post trattamento CIN2 e CIN3 riportate nel capitolo 5 del: Gruppo trasversale GISCi sul follow up dopo trattamento. Utilizzo del test HPV-HR nel triage delle ASC-US, delle LSIL in donne con più di 35 anni, nel follow-up delle donne con citologia ASC-US+ dopo un approfondimento di secondo livello negativo per CIN2+ e nel follow-up dopo trattamento delle lesioni CIN2-3: aggiornamento 2018. 2018 Available at: http://gisci.it/documenti/documenti_gisci/Utilizzo_test_HPV-HR_2018_def2.pdf (Accessed on March 2021)
11. Onuki M, Matsumoto K, Sakurai M, Ochi H, Minaguchi T, Satoh T, Yoshikawa H. Posttreatment human papillomavirus testing for residual or recurrent high-grade cervical intraepithelial neoplasia: a pooled analysis. J Gynecol Oncol. 2016 Jan;27(1):e3. doi: 10.3802/jgo.2016.27.e3.
12. Multisociety Italian guidelines for cervical cancer prevention Working Group. Developing evidence-based Multisociety Italian guidelines for cervical cancer prevention: rationale, methods, and development process. Unpublished paper, invited for submission by the European Journal of Gynaecological Oncology (EJGO) by March 31 2021
13. Guyatt GH, Oxman AD, Kunz R, Atkins D, Brozek J, Vist G, et al. GRADE guidelines: 2. Framing the question and deciding on important outcomes. Journal of clinical epidemiology. 2011;64(4):395-400.
14. Alonso-Coello P, Oxman AD, Moberg J, et al. GRADE Evidence to Decision (EtD) frameworks: a systematic and transparent approach to making well informed healthcare choices. 2: Clinical practice guidelines. BMJ 353:i2089, 2016.
15. Ilaria Ghini, Francesco Venturelli, Paolo Giorgi Rossi, Anna Iossa, Paola Garutti. HPV vaccination and the risk of clinical disease recurrence in women treated for high-grade cervical intraepithelial neoplasia (CIN2 or CIN3). PROSPERO 2019 CRD42019135870 Available from: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42019135870 (Accessed on March 2021)

16. GRADEpro GDT online platform. 2020, McMaster University and Evidence Prime Inc. Available at: https://gradepro.org/ (Accessed on March 2021)
17. Sterne JAC, Savović J, Page MJ, et al. RoB 2: a revised tool for assessing risk of bias in randomised trials. *BMJ*. 2019;366:l4898. Published 2019 Aug 28. doi:10.1136/bmj.l4898
18. Sterne JA, Hernán MA, Reeves BC, et al. ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. *BMJ*. 2016;355:i4919. Published 2016 Oct 12. doi:10.1136/bmj.i4919
19. Schünemann HJ, Brożek J, Guyatt G, and Andrew Oxman, eds. Handbook for grading the quality of evidence and the strength of recommendations using the GRADE approach. Updated October 2013. Available at: https://gdt.gradepro.org/app/handbook/handbook.html (Accessed on March 2021)
20. Schünemann HJ, Higgins JPT, Vist GE et al. on behalf of the Cochrane GRADEing Methods Group (formerly Applicability and Recommendations Methods Group) and the Cochrane Statistical Methods Group. Completing 'Summary of findings' tables and grading the certainty of the evidence. pp. 375-402 Doi: https://doi.org/10.1002/9781119536604.ch14
21. Schünemann HJ, Wiercioch W, Brozek J, et al. GRADE Evidence to Decision (EtD) frameworks for adoption, adaptation, and de novo development of trustworthy recommendations: GRADE-ADOLOPMENT. J Clin Epidemiol. 2017;81:101-110. doi:10.1016/j.jclinepi.2016.09.009
22. Istituto Superiore di Sanità. Sistema Nazionale Linee Guida dell'Istituto Superiore di Sanità. Website: https://snlg.iss.it/
23. Pieralli A, Bianchi C, Auzzi N, Fallani MG, Bussani C, Fambrini M, Cariti G, Scarselli G, Petraglia F, Ghelardi A. Indication of prophylactic vaccines as a tool for secondary prevention in HPV-linked disease. Arch Gynecol Obstet. 2018 Dec;298(6):1205-1210. doi: 10.1007/s00404-018-4926-y. Epub 2018 Oct 10. PMID: 30306310.
24. Kang WD, Choi HS, Kim SM. Is vaccination with quadrivalent HPV vaccine after loop electrosurgical excision procedure effective in preventing recurrence in patients with high-grade cervical intraepithelial neoplasia (CIN2-3)?. *Gynecol Oncol*. 2013;130(2):264-268. doi:10.1016/j.ygyno.2013.04.050
25. Ghelardi A, Parazzini F, Martella F, et al. SPERANZA project: HPV vaccination after treatment for CIN2. *Gynecol Oncol*. 2018;151(2):229-234. doi:10.1016/j.ygyno.2018.08.033
26. Joura EA, Garland SM, Paavonen J, et al. Effect of the human papillomavirus (HPV) quadrivalent vaccine in a subgroup of women with cervical and vulvar disease: retrospective pooled analysis of trial data. *BMJ*. 2012;344:e1401. Published 2012 Mar 27. doi:10.1136/bmj.e1401
27. Garland SM, Paavonen J, Jaisamrarn U, et al. Prior human papillomavirus-16/18 AS04-adjuvanted vaccination prevents recurrent high grade cervical intraepithelial neoplasia after definitive surgical therapy: Post-hoc analysis from a randomized controlled trial. *Int J Cancer*. 2016;139(12):2812-2826. doi:10.1002/ijc.30391
28. Hildesheim A, Gonzalez P, Kreimer AR, et al. Impact of human papillomavirus (HPV) 16 and 18 vaccination on prevalent infections and rates of cervical lesions after excisional treatment. *Am J Obstet Gynecol*. 2016;215(2):212.e1-212.e15. doi:10.1016/j.ajog.2016.02.021
29. Sand FL, Kjaer SK, Frederiksen K, Dehlendorff C. Risk of cervical intraepithelial neoplasia grade 2 or worse after conization in relation to HPV vaccination status. *Int J Cancer*. 2020;147(3):641-647. doi:10.1002/ijc.32752
30. Intercent-ER. Agenzia per lo sviluppo dei mercati telematici. Vaccino HPV Gardasil 9. Regione Emilia-Romagna. Available at: https://intercenter.regione.emilia-romagna.it/servizi-pa/convenzioni/convenzioni-scadute-e-o-esaurite/2017/vaccino-hpv-gardasil-9 (Accessed on June 2019)
31. Intercent-ER. Agenzia per lo sviluppo dei mercati telematici. Vaccini vari ad uso umano 2016/2018 "esclusivi". Regione Emilia-Romagna. Available at: https://intercenter.regione.emilia-romagna.it/servizi-pa/convenzioni/convenzioni-scadute-e-o-esaurite/2016/vaccini-vari-ad-uso-umano-2016-2018-201cesclusivi201d/vaccini-vari-ad-uso-umano-2016-2018-201cesclusivi201d (Accessed on June 2019)
32. Petrelli A, Giorgi Rossi P, Francovich L, et al. Geographical and socioeconomic differences in uptake of Pap test and mammography in Italy: results from the National Health Interview Survey. BMJ Open. 2018;8(9):e021653. Published 2018 Sep 19. doi:10.1136/bmjopen-2018-021653
33. Carrozzi G, Sampaolo L, Bolognesi L, et al. Cancer screening uptake: association with individual characteristics, geographic distribution, and time trends in Italy. *Epidemiol Prev*. 2015;39(3 Suppl 1):9-18.

34. Venturelli F, Baldacchini F, Campari C, et al. Association between mothers' screening uptake and daughters' HPV vaccination: a quasi-experimental study on the effect of an active invitation campaign. *BMJ Open*. 2017;7(9):e016189. Published 2017 Sep 25. doi:10.1136/bmjopen-2017-016189
35. Perkins RB, Guido RS, Castle PE, et al. 2019 ASCCP Risk-Based Management Consensus Guidelines for Abnormal Cervical Cancer Screening Tests and Cancer Precursors. J Low Genit Tract Dis. 2020;24(2):102-131. doi:10.1097/LGT.0000000000000525
36. Public Health England. Guidance Cervical screening: programme and colposcopy management. NHS cervical screening (CSP) programme. Published: 3 May 2010. Last updated:5 February 2020. Available at: https://www.gov.uk/government/publications/cervical-screening-programme-and-colposcopy-management (Accessed on March 2021)
37. SICPCV Raccomandazioni SICPCV 2019. Italian Society for Colposcopy and Cervico-Vaginal Pathology. 2019. Available at http://www.colposcopiaitaliana.it/interna04.asp.htm (Accessed on March 2021)
38. Kyrgiou M, Athanasiou A, Paraskevaidi M, et al. Adverse obstetric outcomes after local treatment for cervical preinvasive and early invasive disease according to cone depth: systematic review and meta-analysis. *BMJ*. 2016;354:i3633. Published 2016 Jul 28. doi:10.1136/bmj.i3633
39. Del Pino M, Martí C, Torras I, et al. HPV Vaccination as Adjuvant to Conization in Women with Cervical Intraepithelial Neoplasia: A Study under Real-Life Conditions. Vaccines (Basel). 2020;8(2):245. Published 2020 May 23. doi:10.3390/vaccines8020245
40. Karimi-Zarchi M, Allahqoli L, Nehmati A, Kashi AM, Taghipour-Zahir S, Alkatout I. Can the prophylactic quadrivalent HPV vaccine be used as a therapeutic agent in women with CIN? A randomized trial. BMC Public Health. 2020;20(1):274. Published 2020 Feb 27. doi:10.1186/s12889-020-8371-z
41. Saslow D, Andrews KS, Manassaram-Baptiste D, Smith RA, Fontham ETH; American Cancer Society Guideline Development Group. Human papillomavirus vaccination 2020 guideline update: American Cancer Society guideline adaptation. CA Cancer J Clin. 2020;70(4):274-280. doi:10.3322/caac.21616
42. Meites E, Szilagyi PG, Chesson HW, Unger ER, Romero JR, Markowitz LE. Human Papillomavirus Vaccination for Adults: Updated Recommendations of the Advisory Committee on Immunization Practices. *MMWR Morb Mortal Wkly Rep*. 2019;68(32):698-702. Published 2019 Aug 16. doi:10.15585/mmwr.mm6832a3
43. European Centre for Disease Prevention and Control. Introduction of HPV vaccines in EU countries – an update. Stockholm: ECDC; 2012. doi 10.2900/60814 Available at: https://www.ecdc.europa.eu/sites/default/files/media/en/publications/Publications/20120905_GUI_HPV_vaccine_update.pdf (Accessed on March 2021)
44. Lu PJ, Byrd KK, Murphy TV, Weinbaum C. Hepatitis B vaccination coverage among high-risk adults 18-49 years, U.S., 2009. Vaccine. 2011;29(40):7049-7057. doi:10.1016/j.vaccine.2011.07.030
45. François G, Hallauer J, Van Damme P. Hepatitis B vaccination: how to reach risk groups. Vaccine. 2002;21(1-2):1-4. doi:10.1016/s0264-410x(02)00440-1
46. Martínez-Gómez X, Curran A, Campins M, et al. Multidisciplinary, evidence-based consensus guidelines for human papillomavirus (HPV) vaccination in high-risk populations, Spain, 2016. *Euro Surveill*. 2019;24(7):1700857. doi:10.2807/1560-7917.ES.2019.24.7.1700857
47. Schiller-Frühwirth I, Wilbacher I. HPV vaccination after treatment for high-grade cervical intraepithelial neoplasia. Österreichische Sozialversicherung. 2019. HTA Report available at https://www.sozialversicherung.at/cdscontent/load?contentid=10008.723534&version=1570024134 (Accessed on March 2021)
48. Joura EA, Kyrgiou M, Bosch FX, et al. Human papillomavirus vaccination: The ESGO-EFC position paper of the European society of Gynaecologic Oncology and the European Federation for colposcopy. Eur J Cancer. 2019;116:21-26. doi:10.1016/j.ejca.2019.04.032
49. Ghelardi A. Impact on Disease Relapse of HPV Vaccination in Women Treated With LEEP for Cervical Intraepithelial Neoplasia. HOPE9 (HOPE9). HOPE9 Study protocol. 2019. ClinicalTrials.gov Identifier: NCT03848039. Available at: https://clinicaltrials.gov/ct2/show/study/NCT03848039 (Accessed on March 2021)
50. Kyrgiou M. Nonavalent Prophylactic HPV Vaccine (GARDASIL9) After Local Conservative The NOVEL Trial (NOVEL). NOVEL study protocol. 2019. ClinicalTrials.gov Identifier: NCT03979014 Available at: https://clinicaltrials.gov/ct2/show/NCT03979014 (Accessed on March 2021)

4.3. PhD Candidate contribution to research articles in Chapter 4

Research article #06 Developing evidence-based Multisociety Italian guidelines for cervical cancer prevention: rationale, methods, and development process, and Research article #07 HPV vaccination in women treated for CIN 2 or 3: evidence-based recommendation from the Multisociety Italian guidelines for cervical cancer prevention.

The PhD Candidate is a member of the Evidence Review Team of the Guidelines Development Group. He participated to all the steps of the recommendation development process, coordinated the systematic reviews of the literature, synthesised and appraised the certainty of evidence, and presented results to the Panel of Experts during plenary meetings. The PhD Candidate also drafted the documents for the SNLG, including revision and approval process by the reviewers from the National Institute of Health. Finally, the PhD Candidate drafted the research articles to be submitted for publication on Scientific Journals.

5. Chapter 5: Discussion

5.1. Cervical cancer screening in the Italian context

The conceptual framework on cancer screening was developed within a multicentre project funded by the Italian Ministry of Health. It is an attempt to provide a view on how the findings of the PhD research project are linked each other with the final common aim of increasing the positive effect of screening on population health. [1] Understanding the elements influencing the causal chain that links the actions to the effect on populations is of crucial importance to increase the value of secondary prevention of cancer. It allows to choose the most appropriate indicators and to better understand how their changes are linked to the implemented actions, allowing timely changes when necessary and a continuous improvement.

Using the DPSEEA framework to describe the path of the PhD research activities, it can be said that after measuring the Exposure, the aim was to support the Actions that improve the screening Performance, acting also through Pressures. (Figure 1)

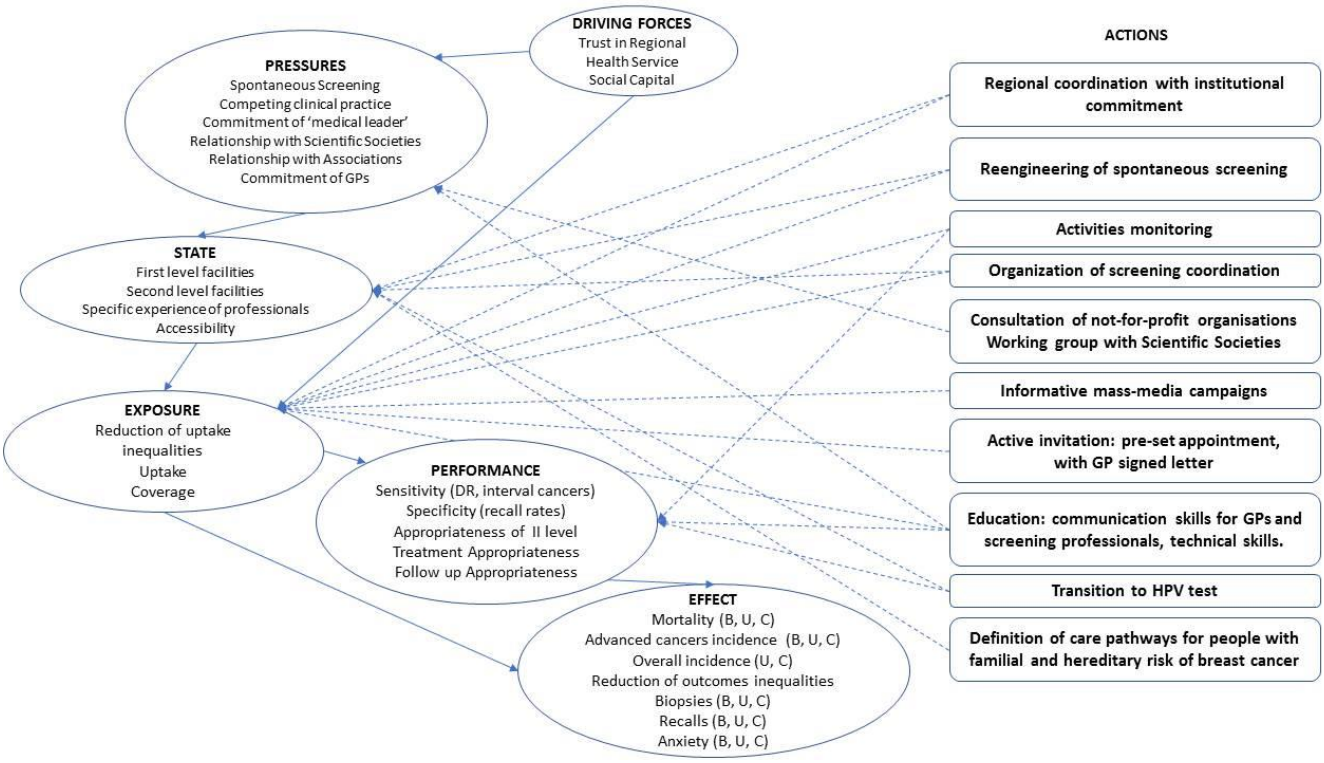


Figure 1. Application of the DPSEEA framework to cancer screening programmes. Acronyms: B, Breast cancer; C, Colorectal cancer; DR, Detection Rate; GPs, General Practitioners, HPV Human Papillomavirus; U, Cervical cancer. [1]

The Exposure to cervical cancer screening, in organized programs and spontaneous access, was measured analysing the data provided by more than 40 thousand women interviewed between 2014 and 2016 by the Italian Behavioural Risk Factor Surveillance System (PASSI). [2] The results showed an overall cervical cancer screening uptake of 78.9%, of which 44.9% within organised screening

programs and 34.0% in spontaneous screening. Data showed socio-economic inequalities in screening uptake, with a higher extent in spontaneous screening. Screening uptake was positively correlated with education level (i.e. from a 61.3% in women with Elementary level of education up to an 83.3% among graduated women) and with income (i.e. from 69.0% in women with high economic difficulties up to 84.4% among those with no economic issues). As reported in previous studies, another driver of socioeconomic differences in screening coverage in Italy was the migrant status. [3, 4] The PASSI surveillance showed an overall uptake of 79.3% for Italian citizens and foreign women from high-income countries, and a 73.5% among foreign women from middle and low income countries. Interestingly, these differences were mainly due to access to spontaneous screening suggesting a positive effect of organised screening programs with active invitation on screening inequalities. [2] It must be said that differences in the incidence of cervical cancer were mainly due to the different screening uptake that represents the most effective preventive intervention. However, the lower uptake among women coming from countries with high HPV prevalence may have a synergic effect in increasing the burden of disease. [4] This is emphasized by the different access to HPV vaccination, the second main preventive strategy, which was relevant in our context before the implementation of an active invitation. [5] Lower screening uptake was also associated with unhealthy behaviours and chronic conditions, even after taking into account socioeconomic differences, leading to a potential synergic negative impact on health outcomes (Effect). This evidence highlighted equity as one of the priorities in tackling cervical cancer. At the same time, this work showed the relevance and the potentiality of national surveillance data for monitoring the impact of policies and actions. Monitoring is one of the main Actions needed to improve both screening Exposure and Performance as reported in the DPSEEA framework. [1]

The Performance of cervical cancer screening is measured in terms of accuracy and appropriateness of screening protocols, starting from primary test to treatment and follow up. Data from the NTTC2 study reported in Chapter 3 provides robust evidence on how the implementation of new biomarkers in HPV-based organised screening could impact on accuracy, safety, and overall efficiency of screening protocols. [6-8]

Similarly, the multisociety guidelines development process presented in Chapter 4 aimed to improve the Performance increasing follow up appropriateness, promoting the appropriate implementation of HPV-test even after treatment, and the definition of care pathways for high-risk subgroups of women. Moreover, the active involvement of all the Italian scientific societies in the guidelines development process represented a successful example of consultation of not-for-profit organisations in working groups (Action) that facilitated the actual implementation of recommendations in first and second level facilities by increasing the competences of involved professionals (State), by strengthening the commitment of “medical leaders”, and by reinforcing the relationships between scientific societies (Pressures). [9, 10]

Moving from a theoretical framework to real-world impact, the implication of the PhD research project results on current practice are reported in Chapter 5.2.

5.2. Implication for practice of main findings

5.2.1. Biomarkers for triage in HPV-based cervical cancer screening

The first point emerged from the results of the PhD research project with implication for practice is related to the impact of difference triage strategies on HPV-based screening efficiency.

Whichever biomarker or combination of biomarkers is used to triage HPV positive women, the overall impact on referral rate and Positive Predictive Value (PPV) is limited if HPV positive / triage negative women are referred to HPV retesting after one year.

In the Italian context, as in many other European countries, the follow up of HPV positive / triage negative women includes HPV-DNA retesting, stand-alone or with cytology (co-testing). The only exception is for Netherlands in which the follow up is made with cytology only. The Dutch algorithm has been considered unacceptable by many Italian experts in both the implementation phase of existing European guidelines [11-15] and in the scoping phase of the new Italian Guidelines, for the residual 5-year risk of progression in women with persistent HPV infections even if cytology negative. [16, 17]

Thus, the new perspective for triage in HPV-based screening aims to longer interval for retesting of HPV positive/ triage negative women, to increase clearance rate then reduce second step and overall referral to colposcopy but maintaining at the second step a test with very high negative predictive value, as the HPV DNA. To be safe, longer intervals require a high “prospective” Negative Predictive Value (NPV) of the triage test, reducing the risk of missing a prevalent precancer and the risk that missed lesions progress to cancer in the re-testing interval.

In this context, the second point highlighted by the PhD research project is about the importance of the prognostic value of biomarkers, meaning a lower risk of progression for biomarker negative precancerous cervical lesions, undetected by triage and referred to re-testing.

The results of the PhD research project showed $p16^{ink4a}$ / ki-67 immunostaining as the most promising test for triage of HPV-DNA positive women. $p16^{ink4a}$ / ki-67 cross-sectional accuracy resulted slightly better than high-quality cytology, with higher sensitivity and similar specificity. Despite this, the results on screening efficiency showed a similar overall referral rate with overlapping PPV for $p16^{ink4a}$ / ki-67 and cytology. What $p16^{ink4a}$ / ki-67 immunostaining adds is a high prospective NPV, showed by the 2-fold 1-year clearance rate of HPV-infections in $p16^{ink4a}$ / ki-67 negative women compared to $p16^{ink4a}$ / ki-67 positive, measured in HPV-DNA positive / Cytology negative women randomised in the NTCC2 study.

The results of the PhD research project on E6/E7 mRNA overexpression showed a high sensitivity but low specificity, resulting in a too high immediate referral rate of HPV-DNA positive women to be efficient as triage test. However, as for $p16^{ink4a}$ / ki-67, when negative E6/E7 mRNA showed a good prognostic value identifying HPV infections at 2-fold 1-year clearance rate. Compared to $p16^{ink4a}$ / ki-

67, E6/E7 mRNA showed a higher prognostic value also for CIN2+ regressive lesions measured at 1-year retesting.

The combination of cytology or p16ink4a / ki-67 with HPV typing showed no improvement in screening efficiency yet may result in high NPV, making it a promising strategy for having longer re-testing intervals in HPV positive/tirage negative women.

5.2.2. Multisociety Italian guidelines for cervical cancer prevention

The first recommendation of the updated "Multisociety Italian guidelines for cervical cancer prevention" guidelines has been published in June 2020 on the Italian National System of Guidelines (SNLG) online database. [18]

Developing guidelines in Public Health requires dealing with different decision-making levels, including political ones. The Italian framework for cervical cancer guidelines was set up by the Ministry of Health, including cancer screening in the Essential Levels of Care (LEA) in 2003 and providing objectives to Italian Regions through the National Prevention Plans 2014-2019 and 2020-2025. [14, 15, 19] A contribution to the Italian framework also came from technical reports developed in an era in which guidelines development methodology was less rigorous and transparent than currently required. These reports include the Italian Ministry of Health and National Centre for Screening Monitoring (ONS) 2006 guidelines [20], and an Italian Health Technology Assessment Report published in 2012. [11] Moreover, Italian Guidelines should be developed in accordance with European guidelines 2008, updated in 2015 following a process based on systematic reviews but without a predefined and transparent framework to appraise evidence and develop recommendations. [21, 22] Despite these limitations, to date technical reports on cervical cancer screening provided recommendations consistent with available evidence and quite coherent worldwide, especially in high income countries. As an example, both European guidelines 2015 and American guidelines 2012 recommend HPV-DNA as primary test for cervical cancer, with 5-year interval starting from the age of 30. Nevertheless, up to 2020 only a few countries actually implemented HPV screening and in Italy, in 2018, less than one third of women invited for screening were invited for a HPV test. [23, 24]

Thus, rigorous and transparent methodology and involvement of decision makers at different levels are two main points to produce and timely implement evidence-based recommendations in Public Health.

In the PhD research project, the GRADE methodology was applied, as required by the Italian SNLG, to develop the "Multisociety Italian guidelines for cervical cancer prevention". The scoping phase prioritised 18 clinical questions for the follow up of women treated for CIN2 and CIN3, a topic not covered in detail by existing international guidelines. The first recommendation on HPV vaccination of women treated for CIN2 and CIN3 was published on the Italian SNLG database in June 2020 and, up to January 2021, several Italian Regions already implemented it. [18]

The remaining recommendations on clinical questions related to the tests, number of episodes and intervals of retesting in the follow up are in the external revision phase and will be submitted to SNLG reviewers by the mid of 2021.

Although guidelines development methodology improved, the process is still time and resource consuming leading to a delay in recommendation availability. Nevertheless, the transparency of the process definitely favours the updating process, when new evidence will be available, improving timeliness in keeping up to date the existing recommendations. Moreover, the involvement of healthcare decision makers in the development process reduces the time between recommendation publication and its implementation in real world practice.

Consistently to the upcoming evidence and emerging issues, the "Multisociety Italian guidelines for cervical cancer prevention" guidelines development group already prioritized clinical questions on the use of biomarkers in cervical cancer screening to be answered with the next recommendations.

5.3. Bibliography Chapter 5

1. Zappa M, Bevere F, Braga M, et al. Sviluppo di un modello concettuale di riferimento per l'interpretazione degli indicatori di monitoraggio degli screening oncologici nel Piano nazionale della prevenzione [Development of a conceptual model for interpretation of monitoring indicators of cancer screenings from the Italian National Prevention Plan]. Epidemiol Prev. 2019;43(5-6):354-363. doi:10.19191/EP19.5-6.P354.105
2. Venturelli F, Sampaolo L, Carrozzi G; PASSI Working Group, Zappa M, Giorgi Rossi P. Associations between cervical, breast and colorectal cancer screening uptake, chronic diseases and health-related behaviours: Data from the Italian PASSI nationwide surveillance [published correction appears in Prev Med. 2019 Sep;126:105788]. Prev Med. 2019;120:60-70. doi:10.1016/j.ypmed.2019.01.007
3. Campari C, Fedato C, Petrelli A, et al. HPV prevalence and risk of pre-cancer and cancer in regular immigrants in Italy: results from HPV DNA test-based screening pilot programs. Infect Agent Cancer. 2015;10:14. Published 2015 May 7. doi:10.1186/s13027-015-0009-x
4. Di Felice E, Caroli S, Paterlini L, Campari C, Prandi S, Giorgi Rossi P. Cervical cancer epidemiology in foreign women in Northern Italy: role of human papillomavirus prevalence in country of origin. Eur J Cancer Prev. 2015;24(3):223-230. doi:10.1097/CEJ.0000000000000137
5. Venturelli F, Baldacchini F, Campari C, et al. Association between mothers' screening uptake and daughters' HPV vaccination: a quasi-experimental study on the effect of an active invitation campaign. BMJ Open. 2017;7(9):e016189. Published 2017 Sep 25. doi:10.1136/bmjopen-2017-016189
6. Giorgi Rossi P, Carozzi F, Ronco G, et al. p16/ki67 and E6/E7 mRNA Accuracy and Prognostic Value in Triaging HPV DNA-Positive Women. J Natl Cancer Inst. 2021;113(3):292-300. doi:10.1093/jnci/djaa105
7. Benevolo M, Mancuso P, Allia E, et al. Interlaboratory concordance of p16/Ki-67 dual-staining interpretation in HPV-positive women in a screening population. Cancer Cytopathol. 2020;128(5):323-332. doi:10.1002/cncy.22248
8. Benevolo M, Mancuso P, Allia E, et al. Determinants of p16/Ki-67 adequacy and positivity in HPV-positive women from a screening population [published online ahead of print, 2020 Nov 3]. Cancer Cytopathol. 2020;10.1002/cncy.22385. doi:10.1002/cncy.22385
9. Multisociety Italian guidelines for cervical cancer prevention Working Group. Developing evidence-based Multisociety Italian guidelines for cervical cancer prevention: rationale, methods, and development process. Invited for submission by the European Journal of Gynaecological Oncology (EJGO), under review
10. Multisociety Italian guidelines for cervical cancer prevention Working Group. HPV vaccination in women treated for CIN 2 or 3: evidence-based recommendation from the Multisociety Italian guidelines for cervical cancer prevention. I Invited for submission by the European Journal of Gynaecological Oncology (EJGO), under review

11. Ronco G, Biggeri A, Confortini M, et al. Health technology assessment report: ricerca del DNA di papillomavirus umano (HPV) come test primario per lo screening dei precursori del cancro del collo dell'utero [Health technology assessment report: HPV DNA based primary screening for cervical cancer precursors]. Epidemiol Prev. 2012;36(3-4 Suppl 1):e1-e72.
12. Italian Ministry of Health. Screening oncologici. Raccomandazioni per la pianificazione e l'esecuzione degli screening di popolazione per la prevenzione del cancro della mammella, del cancro della cervice uterina e del cancro del colon retto. 2006. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_774_allegato.pdf (Accessed on March 2021)
13. Italian Ministry of Health. Piano Nazionale della Prevenzione 2010-2012. 2010. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_1383_allegato.pdf (Accessed on March 2021)
14. Italian Ministry of Health. Piano Nazionale della Prevenzione 2014-2019. 2014. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_2285_allegato.pdf (Accessed on March 2021)
15. Italian Ministry of Health. Piano Nazionale della Prevenzione 2020-2025. 2020. Available at: http://www.salute.gov.it/imgs/C_17_notizie_5029_0_file.pdf (Accessed on March 2021)
16. Del Mistro A, Giorgi Rossi P, Frayle H, et al. Five-year risk of CIN3 after short-term HPV-DNA negativity in cytology-negative women: a population-based cohort study. *BJOG*. 2019;126(11):1365-1371. doi:10.1111/1471-0528.15893
17. Polman NJ, Veldhuijzen NJ, Heideman DAM, Snijders PJF, Meijer CJLM, Berkhof J. HPV-positive women with normal cytology remain at increased risk of CIN3 after a negative repeat HPV test. *Br J Cancer*. 2017;117(10):1557-1561. doi:10.1038/bjc.2017.309
18. Istituto Superiore di Sanità. Sistema Nazionale Linee Guida dell'Istituto Superiore di Sanità. Website: https://snlg.iss.it/
19. Decreto del Presidente del Consiglio dei Ministri 29 novembre 2001. Definizione dei livelli essenziali di assistenza. Gazzetta Ufficiale Serie Generale n.33 del 08-02-2002 - Suppl. Ordinario n. 26. Available at: https://www.gazzettaufficiale.it/eli/id/2002/02/08/02A00907/sg (Accessed on March 2021)
20. Italian Ministry of Health. Screening oncologici. Raccomandazioni per la pianificazione e l'esecuzione degli screening di popolazione per la prevenzione del cancro della mammella, del cancro della cervice uterina e del cancro del colon retto. 2006. Available at: http://www.salute.gov.it/imgs/C_17_pubblicazioni_774_allegato.pdf (Accessed on March 2021)
21. Arbyn M, Anttila A, Jordan J, Schenck U, Ronco G, Segnan N, et al (eds.). European guidelines for quality assurance in cervical cancer screening. Second edition. European Commission, Office for Official Publications of the European Communities, 2008 Luxembourg.
22. Anttila A, Arbyn A, De Vuyst H, Dillner J, Dillner L, Franceschi S, et al. (eds.). European guidelines for quality assurance in cervical cancer screening. Second edition, Supplements. Office for Official Publications of the European Union, 2015 Luxembourg, pp. XIII–XXIV.
23. Chrysostomou AC, Stylianou DC, Constantinidou A, Kostrikis LG. Cervical Cancer Screening Programs in Europe: The Transition Towards HPV Vaccination and Population-Based HPV Testing. *Viruses*. 2018;10(12):729. Published 2018 Dec 19. doi:10.3390/v10120729
24. Osservatorio Nazionale Screening (ONS). Rapporto Nazionale 2019. Available at: https://www.osservatorionazionalescreening.it/sites/default/files/allegati/Rapporto%202019_0.pdf (Accessed on March 2021)

6. Appendix - Supplementary material of research articles

6.1. Supplementary material – Research article #02: Associations between cervical, breast and colorectal cancer screening uptake, chronic diseases and health-related behaviours: data from the Italian PASSI nationwide surveillance.

Supplementary material – Table A.1 Description of each variable included in the analysis and related categories. Definitions are taken by the Italian Behavioral Risk Factor Surveillance System (PASSI) website. For more details see: http://www.epicentro.iss.it/passi/default.asp (Accessed February 22, 2018).

OUTCOME DEFINITION (SCREENING UPTAKE)

Screening program	Definition
Cervical cancer	Women aged 25-64 that self-report a Pap test uptake in the last three years and/or a HPV-DNA test uptake in the last 5 years.
Breast cancer	Women aged 50-69 that self-reported a mammogram uptake in the last two years.
Colorectal cancer	People aged 50-69 that self-reported a FOBT uptake in the last two years or a colonoscopy or flex sigmoidoscopy uptake in the last five years.
Proxy for type of screening uptake:	
Within organised programme	People self-reporting the last screening test uptake for free.
Spontaneous uptake	People self-reporting the last screening test uptake was paid out of pocket.

BEHAVIOURAL RISK FACTORS

Behaviours	Definition
Smoking	
Non-smokers	People aged 18-69 self-reporting fewer than 100 cigarettes (5 packets of 20 cigarettes each) smoked in their entire life or responding “I don’t know/don’t remember”.
Former smokers	People aged 18-69 self-reporting at least 100 cigarettes (5 packets of 20 cigarettes each) smoked in their entire life and quitting smoking at least 6 months prior.
Smokers	People aged 18-69 self-reporting at least 100 cigarettes (5 packets of 20 cigarettes each) smoked in their entire life and who are active smokers or who have quit smoking less than 6 months ago.
Physical activity level	
Active	People aged 18-69 self-reporting a physically demanding job and/or who have performed moderate or intense physical activity (PA) in the last 30 days at the levels recommended by the WHO (moderate physical activity of at least 150 minutes per week or intense physical activity of at least 75 minutes or a combination of the two. Physical activity lasting under 10 minutes is not considered).

Partially active	People aged 18-69 self-reporting a moderately physically demanding job and/or having performed some physical activity in the last 30 days but less than the recommendations.
Sedentary	People aged 18-69 self-reporting a sedentary job and/or having not having performed moderate or intense physical activity in the last 30 days.
Alcohol consumption	
High-risk drinking behaviour	People aged 18-69 self-reporting in the last 30 days a high daily alcohol consumption (men: a mean of more than 2 alcohol units a day; women: a mean of more than 1 alcohol unit a day) and/or binge drinking (men: at least one episode of 5 or more alcohol units consumed at one time; women: at least one episode of 4 or more alcohol units consumed at one time) and/or alcohol consumption mainly or exclusively between meals (as self-reported by specific question). [An alcohol unit is defined as 12 grams of ethanol, almost equivalent to the intake from a can of beer (330 ml), a glass of wine (125 ml) or a small glass of spirit (40 ml), considering respective mean alcohol concentrations.]
Daily fruit and vegetable intake	
0; 1-2; 3-4 portions	People aged 18-69 self-reporting a usual daily consumption of 0, 1-2 or 3-4 portions of fruit and vegetables.
≥5 portions	People aged 18-69 self-reporting a usual daily consumption of 5 or more portions of fruit and vegetables.
Road safety behaviours	
Driving after drinking alcohol	People aged 18-69 self-reporting having driven a car or motorcycle in the last 30 days within an hour after drinking 2 or more alcohol units.
Front seat belt (always used)	People aged 18-69 self-reporting “always” using a seat belt when travelling by car (on urban or extra-urban roads) when seated in the front.
Back seat belt (always used)	People aged 18-69 self-reporting “always” using a seat belt when travelling by car (on urban or extra-urban roads) when seated in the back.
Helmet (always used)	People aged 18-69 self-reporting having travelled on a motorcycle, as driver or as a passenger, in the last 12 months and having “always” worn a helmet (on urban or extra-urban roads).
Preventive intervention uptake	
Influenza vaccination uptake	People interviewed between April 1 and September 30, aged over 64 or aged 18-64 that self-reported at least one chronic disease (see below for list of investigated chronic diseases) and self-reporting influenza vaccination uptake in the last 12 months.

CHRONIC CONDITIONS AND DISEASES

Nutritional status	
Underweight/normal weight	People aged 18-69 with a body mass index (BMI) < 25.0 calculated by self-reported height and weight.
Overweight	People aged 18-69 with a BMI between 25.0 and 29.9 calculated by self-reported height and weight.
Obese	People aged 18-69 with a BMI ≥ 30.0 calculated by self-reported height and weight.
Cholesterolemia	
Hypercholesterolemia	People aged 18-69 self-reporting having ever received a diagnosis of hypercholesterolemia (i.e. high blood cholesterol level).
Blood pressure	
Hypertension	People aged 18-69 self-reporting having ever received a diagnosis of hypertension (i.e. high blood pressure).
Chronic diseases	
None	People aged 18-69 self-reporting never having received a diagnosis of one of the investigated chronic diseases (see below, including chronic renal and liver diseases).
Diabetes	People aged 18-69 self-reporting having ever received a diagnosis of diabetes.
Cancer (current or past)	People aged 18-69 self-reporting having ever a diagnosis of cancer (current or in the past).
Depressive symptoms	
	Symptoms of depression as detected with Patient-Health Questionnaire-2 (PHQ-2). The PHQ-2 is composed of two internationally validated questions that detect the number of days in the last two weeks during which the respondent has presented the following symptoms: 1) Little interest or pleasure in doing things; 2) Feeling down, depressed or hopeless. Based on the number of days for the two questions, a score ranging from 0 to 6 is calculated; people with a score ≥3 are classified as "people with symptoms of depression".
Self-perceived health status	
Very well/well; Normal; Bad/Very bad	People aged 18-69 answering “Very well”, “Well”, “Normal”, “Bad”, “Very bad” to the question “How is your health in general?”.

6.2. Supplementary material – Research article #03: p16/ki67 and E6/E7 mRNA Accuracy and Prognostic Value in Triaging HPV DNA-Positive Women

Supplementary Table S1. Number of women randomized in the two arms, E6/E7 mRNA and p16/ki67 positivity rate, by type of HPV DNA test and NTCC2 study recruiting centre

Randomized women	Immediate colposcopy	One-year HPV retesting
Overall		
Recruited women	1077 (46.7)	1229 (53.3)
Age mean (SD)	44.9 (8.3)	44.5 (8.3)
E6/E7 mRNA positive/tested	657/1073	732/1224
% E6/E7 mRNA positive	61.2	59.8
p16/ki67 positive/adequate	192/1002	213/1130
% p16/ki67 positive	19.2	18.8
HPV DNA assay- Cobas		
Recruited women	482	550
Age mean (SD)	45.8 (9.0)	45.2 (8.9)
E6/E7 mRNA positive/tested	321/479	377/545
% E6/E7 mRNA positive	67.0	69.2
p16/ki67 positive/adequate	70/435	94/490
% p16/ki67 positive	16.1	19.2
HPV DNA assay- HC2		
Recruited women	595	679
Age mean (SD)	44.2 (7.5)	43.9 (7.8)
E6/E7 mRNA positive/tested	336/594	355/679
% E6/E7 mRNA positive	56.6	52.3
p16/ki67 positive/adequate	122/567	119/640
% p16/ki67 positive	21.5	18.6
Centre		
Umbria	286 (44.7)	353 (55.3)
Veneto	202 (46.3)	234 (53.7)
Florence	307 (49.7)	311 (50.3)
Turin	282 (46.0)	331 (54.0)

Supplementary Table S2. Accuracy of different triage tests. Sensitivity for CIN2+ and CIN3 and specificity for CIN2+, with relative 95% confidence interval (95% CI), of cytology high-grade and combinations of biomarkers. TP, true positive; FP, false positive; TN, true negative; FN, false negative.

	Tested*	Test positive	TP	FP	TN	FN	Sensitivity for CIN2+ (95% CI)		Specificity for CIN2+ (95% CI)		Sensitivity for CIN3+ (95% CI)	
							Raw	Adj	Raw	Adj	Raw	Adj
High-grade Cytology†	2636	170	82	88	2375	91	47.4% (39.8% - 55.1%)	43.7% (36.4% - 51.0%)	96.4% (95.6% - 97.1%)	95.6% (94.5% - 96.6%)	54.2% (43.7% - 64.4%)	51.4% (41.3% - 61.6%)
Cytology ASC-US+ or p16/ki67	2502	1056	144	912	1420	26	84.7% (78.4% - 89.8%)	80.1% (72.9% - 85.7%)	60.9% (58.9% - 62.9%)	57.7% (54.9% - 60.5%)	89.5% (81.5% - 94.8%)	83.1% (74.3% - 90.5%)
Cytology high-grade or p16/ki67	2463	755	140	615	1678	30	82.4% (75.8% - 87.8%)	78.0% (70.8% - 84.0%)	73.2% (71.3% - 75.0%)	67.7% (65.1% - 70.1%)	87.1% (78.0% - 93.4%)	72.2% (61.8% - 81.1%)
p16/ki67 and E6/E7 mRNA	2546	658	130	528	1850	38	77.4% (70.3% - 83.5%)	72.6% (65.1% - 79.4%)	77.8% (76.1% - 79.5%)	75.1% (72.8% - 77.3%)	85.1% (76.3% - 91.6%)	78.1% (68.1% - 86.0%)

*Al women with valid test and complete assessment

† High-grade cytology including ASC-H, H-SIL, AIS, and cancer

Supplementary Table S3. Estimated referral rate and positive predictive value (PPV) immediate, at one-year retesting, and overall, for high-grade cytology and combinations of biomarkers.

			Immediate		1y retesting		Overall	
Colposcopy referral	**Tested**	**Test positive**	**Referral (%)**	**PPV (%)**	**Referral (%)**	**PPV (%)**	**Referral (%)**	**PPV (%)**
High-grade cytology†	3100	186	6.0%	48.2%	50.2%	5.1%	56.2%	9.7%
Cytology ASC-US+ or p16/ki67	2934	1207	41.1%	13.9%	28.7%	-	69.9%	-
Cytology high-grade or p16/ki67	2887	865	30.0%	18.7%	37.3%	-	67.3%	-
p16/ki67 and E6/E7 mRNA	2997	755	25.2%	20.3%	35.9%	-	61.0%	-

*Only valid test included

† High-grade cytology including ASC-H, H-SIL, AIS, and cancer

PPV positive predictive value. One-year retesting and overall PPV are not computed for biomarkers combinations.

Supplementary methods.

1. Single and combined biomarker results categories

Cytology ASC-US+	Positive (ASC-US, L-SIL, AGC, ASC-H, H-SIL, Ca, Adenocarcinoma In Situ**)** Negative (NILM) Inadequate Missing
Cytology high grade	Positive (ASC-H, H-SIL, Ca, Adenocarcinoma In Situ) Negative (NILM, ASC-US, L-SIL, AGC) Inadequate Missing
E6/E7 mRNA	Positive Negative Missing
p16/ki67	Positive Negative Inadequate Missing
HPV 16/18 typing	Positive (16 or 18 or 16/18 or HR/16 or HR/18 or HR/16/18) Negative (negative or other hrHPV)
Cytology ASC-US+ or p16/ki67	Positive (cytology ASC-US+ positive or p16/ki67 positive) Negative (other) Inadequate
Cytology high grade or p16/ki67	Positive (cytology high grade positive or p16/ki67 positive) Negative (other) Inadequate
p16/ki67 and E6/E7 mRNA	Positive (p16/ki67 positive and E6/E7 mRNA positive) Negative (other) Inadequate
Cytology ASC-US+ or HPV 16/18 typing	Positive (cytology ASC-US+ positive or HPV 16/18 typing positive) Negative (other) Inadequate
p16/ki67 or HPV 16/18 typing	Positive (p16/ki67 positive or HPV 16/18 typing positive) Negative (other) Inadequate
1-year HPV-DNA retesting	Positive Negative Missing
Colposcopy	CIN3+ (CIN3, Adenocarcinoma In Situ, Ca) CIN2+ (CIN2, CIN3, Adenocarcinoma In Situ, Ca) CIN1/negative Missing

2. Definitions of positive, negative and indeterminate results for combination of biomarkers

Legend: **Red cells** mean **positive** combination results
Green cells mean **negative** combination results
Yellow cells mean combination results **inadequate**

a. Cytology ASC-US+ OR HPV16/18 typing

	HPV 16/18 typing		
Cytology ASC-US+	Negative	Positive	**Total**
Negative	791	241	1,032
Positive	247	140	387
Inadequate	12	9	21
Missing	5	1	6
Total	1,055	391	1,446

b. Cytology ASC-US+ OR p16/ki67

	p16/ki67				
Cytology ASC-US+	Negative	Positive	Inadequate	Missing	**Total**
Negative	1,727	405	133	41	2,306
Positive	333	409	20	32	794
Inadequate	19	7	10	1	37
Missing	4	1	1	4	10
Total	2,083	822	164	78	3,147

c. High-grade cytology OR p16/ki67

	p16/ki67				
High-grade cytology	Negative	Positive	Inadequate	Missing	**Total**
Negative	2,022	671	150	71	2,914
Positive	38	143	3	2	186
Inadequate	19	7	10	1	37
Missing	4	1	1	4	10
Total	2,083	822	164	78	3,147

d. p16/ki67 OR HPV 16/18 typing

	HPV 16/18 typing		
p16/ki67	Negative	Positive	**Total**
Negative	729	208	937
Positive	223	137	360
Inadequate	65	24	89
Missing	38	22	60
Total	1,055	391	1,446

e. p16/ki67 AND E6/E7 mRNA

	E6/E7 mRNA			
p16/ki67	Negative	Positive	Missing	**Total**
Negative	880	1,201	2	2,083
Positive	65	755	2	822
Inadequate	68	96	0	164
Missing	26	40	12	78
Total	1,039	2,092	16	3,147

3. Adjustment process

The adjustment process intends to precisely estimate the accuracy and performance indicators of each biomarker or combination of biomarker results. In order to do that, we must estimate women who would be immediately referred to colposcopy (positivity to biomarker), number of lesions found in biomarker positive, number of women testing negative and number of lesions in these women, number of women biomarker negative and still HPV DNA-positive at one year, in case that all women had been managed according to biomarker or biomarker combinations.

These estimates assume the general algorithm that HPV DNA-positive women undergo triage test (the biomarker or combination of biomarkers), if positive for the triage test are immediately referred to colposcopy, if negative are referred to 1-year re-testing with HPV DNA, then if positive are referred to colposcopy, if negative to standard re-screening. See also supplementary figure 1.

In this section we present the adjustment formulae to take into account the randomization ratio and the proportion of cytology-positive women that have been referred to colposcopy.

Immediate colposcopy randomization proportion (Rand%)

$$\boldsymbol{Rand}\% = \frac{Cyto\ neg\ imm}{Cyto\ neg} = \frac{1077}{2306} = 0.467 \qquad (1.1)$$

Number of cytology-negative women randomized to immediate colposcopy (*Cyto neg imm*) divided by the overall number of cytology-negative women (*Cyto neg*).

Cytology-negative adjusted women (Cyto neg adj)

$$\boldsymbol{Cyto\ \ neg\ adj} = \frac{Cyto\ neg\ imm}{Rand\%} \qquad (1.2)$$

Number of cytology-negative women randomized to immediate colposcopy (*Cyto neg imm*) divided by the randomization proportion factor (*Rand%*).

CIN2+ adjusted (CIN2plus adj)

$$\boldsymbol{CIN2plus\ adj} = (CIN2plus \mid Cyto\ pos) + \frac{(CIN2plus \mid Cyto\ neg\ imm)}{Rand\%} \qquad (1.3)$$

Number of colposcopy-positive for CIN2+ (*CIN2plus*) among cytology-positive women (*Cyto pos*) plus number of colposcopy-positive women among cytology-negative women randomized to immediate colposcopy (*Cyto neg imm*) divided by randomization proportion. The same is done for CIN3+.

Colposcopy negative adjusted (Colpo neg adj)

$$\boldsymbol{Colpo\ neg\ adj} = (Colpo\ neg\ |Cyto\ pos) + \frac{(Colpo\ neg \mid Cyto\ neg\ imm)}{Rand\%} \qquad (1.4)$$

Number of women with colposcopy negative for CIN2+ (*Colpo neg*) among cytology-positive women (*Cyto pos*) plus number of women with colposcopy negative for CIN2+ (*Colpo neg*) among cytology-negative women randomized to immediate colposcopy (*Cyto neg imm*) divided by randomization proportion (*Rand%*)

4. Statistical formulae to calculate biomarker accuracy

In this section we present the formulae to estimate row and adjusted sensitivity and specificity of each biomarker or combination of biomarkers used as triage test. Formulae are different for cytology and for other biomarkers. In fact, women positive to cytology were all referred to colposcopy, thus there is no need to estimate the number of CIN2+ in cytology positive women. For other biomarkers it is necessary to take into account that those positive also to cytology were referred to colposcopy, but those negative to cytology were randomized, so opportune weight should be applied.

ROW SENSITIVITY

Cytology

$$\boldsymbol{Row\ Cyto\ Sens} = \frac{(CIN2plus\ |Cyto\ pos)}{(CIN2\ plus\ |Cyto\ adequate)} \qquad (2.1.1)$$

Where "CIN2 plus | Cyto pos" means number of colposcopy-positive women for CIN2+ among cytology positive-women and "CIN2 plus |Cyto adequate" means number of colposcopy-positive women for CIN2+ among cytology-positive and cytology-negative women (ie excluding women with not adequate or missing cytology). The same is done for CIN3+.

Other Biomarkers (mRNA, p16/ki67, HPV16/18 typing, combinations of biomarkers)

$$\boldsymbol{Row\ Biomarker\ Sens} = \frac{(CIN2plus\ |\ Biomarker\ pos)}{(CIN2plus\ |Biomarker\ adequate)} \qquad (2.1.2)$$

Where "CIN2plus | Biomarker pos" means number of colposcopy-positive women for CIN2+ among biomarker-positive women and "CIN2 plus | Biomarker adequate" means number of colposcopy-positive women for CIN2+ among biomarker-positive and biomarker-negative women (ie excluding women with not adequate or missing biomarker). The same is done for CIN3+.

ADJUSTED SENSITIVITY

Cytology

$$\boldsymbol{Adj\ Cyto\ Sens} = \frac{(CIN2plus\ |Cyto\ pos)}{(CIN2\ plus\ adj\ |Cyto\ adequate)} \qquad (2.2.1)$$

Where "CIN2 plus | Cyto pos" means number of colposcopy-positive women for CIN2+ among cytology-positive women and "Adj CIN2 plus | Cyto adequate" means the adjusted number of colposcopy-positive women for CIN2+ among cytology-positive and cytology-negative women (ie excluding women with not adequate or missing cytology [see CIN2+ adjusted formula 1.3]). The same is done for CIN3+. This adjustment excludes inadequate cytology from numerator and denominator and estimates CIN2+ present at baseline in cytology-negative women from the arm randomized at immediate colposcopy only, thus eliminating the bias due to regressive lesions.

Other Biomarkers (mRNA, p16/ki67, HPV16/18 typing, combinations of biomarkers)

$$\boldsymbol{Adj\ Biomarker\ Sens} = \frac{(Adj\ CIN2plus \mid Biomarker\ pos)}{(Adj\ CIN2plus \mid Biomarker\ adequate)} \qquad (2.2.2)$$

Where "Adj CIN2plus | Biomarker pos" means number of colposcopy-positive women for CIN2+ among biomarker-positive women and "Adj CIN2 plus |Biomarker adequate" means the adjusted number of colposcopy-positive women for CIN2+ [see CIN2+ adjusted formula 1.3] among biomarker-positive and biomarker-negative women (i.e. excluding women with not adequate or missing biomarker). The same is done for CIN3+. As above, this adjustment excludes inadequate biomarker determinations from numerator and denominator and estimates CIN2+ present at baseline in biomarker-negative women from that cytology positive and from those randomized at immediate colposcopy only, thus eliminating the bias due to regressive lesions.

ROW SPECIFICITY

Cytology

$$\boldsymbol{Row\ Cyto\ Spec} = \frac{(Colpo\ neg \mid Cyto\ neg)}{(Colpo\ neg \mid Cyto\ adequate)} \qquad (2.3.1)$$

Where "Colpo neg | Cyto neg" means number of colposcopy-negative women (Colpo neg) among cytology-negative women (Cyto neg) and "Colpo neg |Cyto adequate" means number of colposcopy-negative women among cytology-positive and cytology-negative women (ie excluding women with not adequate or missing cytology).

Other Biomarkers (mRNA, p16/ki67, HPV16/18 typing, combinations of biomarkers)

$$\boldsymbol{Row\ Biomarker\ Spec} = \frac{(Colpo\ neg \mid Biomarker\ neg)}{(Colpo\ neg \mid Biomarker\ adequate)} \qquad (2.3.2)$$

Where "Colpo neg | Biomarker neg" means number of colposcopy-negative women (Colpo neg) among biomarker-negative women (Biomarker neg) and "Colpo neg |Biomarker adequate" means number of colposcopy-negative women among biomarker-positive and biomarker-negative women (ie excluding women with not adequate or missing biomarker).

ADJUSTED SPECIFICITY

Cytology

$$\boldsymbol{Adj\ Cyto\ Sens} = \frac{(Colpo\ neg \mid Cyto\ neg)}{(Adj\ Colpo\ neg \mid Cyto\ adequate)} \qquad (2.4.1)$$

Where "Colpo neg | Cyto neg" means number of colposcopy-negative women among cytology-negative women and "(Adj Colpo neg |Cyto adequate" means the adjusted number of colposcopy-negative women among cytology-positive and cytology-negative women (ie excluding women with not adequate or missing cytology) [see Colposcopy-negative adjusted formula 1.4]. The adjustment takes into account only the results observed in immediate colposcopy arm to assess true negative appropriately weighted for randomization ratio.

Other Biomarkers (mRNA, p16/ki67, HPV16/18 typing, combinations of biomarkers)

$$\boldsymbol{Adj\ Biomarker\ Sens} = \frac{(Adj\ Colpo\ neg \mid Biomarker\ neg)}{(Adj\ Colpo\ neg \mid Biomarker\ adequate)} \qquad (2.4.2)$$

Where "Adj Colpo neg | Biomarker neg" means number of colposcopy-negative women among biomarker-negative women and "Adj Colpo neg | Biomarker adequate" means the adjusted number of colposcopy-negative women [see Colposcopy negative adjusted formula 1.4] among biomarker-positive and biomarker-negative women (ie excluding women with not adequate or missing biomarker). The adjustment takes into account only the results observed in immediate colposcopy arm and in cytology positive women to assess true negative, appropriately weighted for randomization ratio and proportion of cyto-negative/biomarker-negative women.

5. Statistical formulae to calculate biomarker performance

CYTOLOGY REFERRAL

Estimated immediate referral

$$\boldsymbol{Imm\ Ref} = \frac{Cyto\ pos}{Cyto\ adequate} \qquad (3.1.1)$$

The estimated immediate referral is the proportion of women tested positive to cytology (*Cyto pos*) among the overall women with an adequate cytology test (*Cyto adequate*) (ie cytology-positive and cytology-negative women, excluding women with not adequate or missing cytology)

Estimated 1-year retesting referral

$$\boldsymbol{1yr\ Ref} = [(\frac{1yr\ DNA\ pos}{1yr\ DNA\ pos + 1\ yr\ DNA\ neg}) \mid Cyto\ neg] * [\frac{Cyto\ neg}{(Cyto\ adequate)}] \qquad (3.1.2)$$

The estimated 1-year referral is the proportion of HPV DNA-positive women at 1 year retesting ($\frac{1yr\ DNA\ pos}{1yr\ DNA\ pos+1\ yr\ DNA\ neg}$) among cytology-negative (*Cyto neg*) women retested at 1 year multiplied by the proportion of cytology-negative women on the overall women with an adequate cytology test (*Cyto adequate*) (ie cytology-positive and cytology-negative women, excluding women with not adequate or missing cytology).

Estimated overall referral

$$\boldsymbol{Overall\ Ref} = \boldsymbol{Imm\ Ref} + \boldsymbol{1yr\ Ref} \qquad (3.1.2)$$

The estimated overall referral is the sum of the estimated immediate referral and the estimated 1-year referral.

OTHER BIOMARKER REFERRALS (mRNA, p16/ki67, combinations of biomarkers)

Estimated immediate referral

$$\boldsymbol{Imm\ Ref} = \frac{Biom\ pos}{Biom\ adequate} \qquad (3.2.1)$$

The estimated immediate referral is the proportion of women tested positive to biomarker (*Biom pos*) among the total number of women with an adequate biomarker test (*Biom adequate*) (ie biomarker-positive and biomarker-negative women, excluding women with not adequate or missing cytology).

Estimated 1-year retesting referral

$$\boldsymbol{1yr\ Ref} = [(\frac{1yr\ DNA\ pos}{1yr\ DNA\ pos + 1\ yr\ DNA\ neg}) \mid Biom\ neg] * [\frac{Biom\ neg}{(Biom\ adequate)}] \qquad (3.2.2)$$

The estimated 1-year referral is the proportion HPV DNA-positive women at 1-year retesting ($\frac{1yr\ DNA\ pos}{1yr\ DNA\ pos+1\ yr\ DNA\ neg}$) among biomarker-negative (*Biom neg*) women multiplied by the proportion of biomarker-negative women out of the total number of women with a adequate biomarker test (*Biom adequate*) (ie cytology-positive and cytology-negative women, excluding women with not adequate or missing cytology).

Estimated overall referral

$$\boldsymbol{Overall\ Ref} = (\boldsymbol{Imm\ Ref}) + (\boldsymbol{1yr\ Ref}) \qquad (3.2.3)$$

The estimated overall referral is the sum of the estimated immediate referral and the estimated 1-year referral.

CYTOLOGY POSITIVE PREDICTIVE VALUE (PPV)

Immediate PPV

$$\boldsymbol{Imm\ PPV} = \left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg)}\right] \mid Cyto\ pos \qquad (3.3.1)$$

Where $\frac{CIN2plus}{(CIN2plus+Colpo\ neg)}$ means the proportion of colposcopy-positive women for CIN2+ among all women with a valid colposcopy (ie colposcopy-positive and colposcopy-negative women). This value is considered only among cytology-positive women (| *Cyto pos*).

1-year retesting PPV

$$\boldsymbol{1yr\ PPV} = \left\{\left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg)}\right] \mid 1yr\ DNA\ pos\right\} \mid Cyto\ neg \qquad (3.3.2)$$

Where $\frac{CIN2plus}{(CIN2plus+Colpo\ neg)}$ means the proportion of colposcopy-positive women for CIN2+ among all women with a valid colposcopy (ie colposcopy-positive and colposcopy-negative women). This value is considered only among cytology-negative women (| *Cyto neg*) retested at 1-year with HPV DNA and resulting positive ($|1yr\ DNA\ pos$).

Overall PPV

$$\boldsymbol{Overall\ PPV} = (Imm\ PPV * Imm\ Ref) + (1yr\ PPV * 1yr\ Ref) \qquad (3.3.3)$$

The Overall PPV is calculated as the weighted average of Immediate PPV (formula 3.3.1) weighted for immediate referral of the same test (formula 3.1.1) and 1-year retesting PPV (formula 3.3.2) weighted for 1yr referral of the same test (formula 3.1.2).

OTHER BIOMARKERS' PPV (mRNA, p16/ki67, combinations of biomarkers)

Immediate PPV

$$\boldsymbol{Imm\ PPV} = \left[\frac{Adj\ CIN2plus}{(Adj\ CIN2plus + Adj\ Colpo\ neg)}\right] \mid Biom\ pos \qquad (3.4.1)$$

Where $\frac{CIN2plus}{(CIN2plus+Colpo\ neg)}$ means the proportion of colposcopy-positive women for CIN2+ among all women with a valid colposcopy (ie colposcopy-positive and colposcopy-negative women). This value is considered only among biomarker-positive women (| *Biom pos*).

1-year retesting PPV

$$\boldsymbol{1yr\ PPV} = \left\{\left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg)}\right] \mid 1yr\ DNA\ pos\right\} \mid Biom\ neg \qquad (3.4.2)$$

Where $\frac{CIN2plus}{(CIN2plus+Colpo\ neg)}$ means the proportion of colposcopy-positive women for CIN2+ among all women with a valid colposcopy (ie colposcopy-positive and colposcopy-negative women). This value is considered only among biomarker-negative women (| *Biom neg*) retested at 1-year with HPV DNA and resulting positive ($\mid 1yr\ DNA\ pos$).

Overall PPV

$$\boldsymbol{Overall\ PPV} = (Imm\ PPV * Imm\ Ref) + (1yr\ PPV * 1yr\ Ref) \qquad (3.4.3)$$

The Overall PPV is calculated as the weighted average of immediate PPV (formula 3.4.1) weighted for immediate referral of the same test (formula 3.2.1) and 1-year retesting PPV (formula 3.4.2) weighted for 1yr referral of the same test (formula 3.2.2).

6. Statistical formulae to calculate regression of CIN2+ lesions and HPV clearance overall and according to biomarker results

Overall 1-year clearance of HPV infection

$$\boldsymbol{Overall\ 1yr\ HPV\ Clear} = \left(\frac{1yr\ DNA\ neg}{1yr\ DNA\ pos + 1\ yr\ DNA\ neg}\right) \mid Cyto\ neg \qquad (4.1)$$

The overall 1-year clearance of HPV infection is the proportion of HPV negative women among cytology-negative women randomized and retested at 1-year with HPV DNA.

Biomarker negative Relative Risk of 1-year clearance of HPV infection

$$\boldsymbol{1yr\ Clear\ RR} = \left[\frac{\left(\frac{1yr\ DNA\ neg}{1yr\ DNA\ pos + 1\ yr\ DNA\ neg}\right) \mid Biom\ neg}{\left(\frac{1yr\ DNA\ neg}{1yr\ DNA\ pos + 1\ yr\ DNA\ neg}\right) \mid Biom\ pos}\right] \mid Cyto\ neg \qquad (4.2)$$

The biomarker negative women relative risk of 1-year clearance of HPV infections is the ratio between the proportion of HPV-negative women among biomarker-negative women randomized and retested at 1-year with HPV DNA and the proportion of HPV-negative women among biomarker-positive women randomized and retested at 1-year with HPV DNA.

Overall 1-year regression of CIN2+ lesions

$$\boldsymbol{1yr\ Reg} = 1 - \left\{\frac{\left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg +\ 1yr\ DNA\ neg\ )}\right] \mid Cyto\ neg_{1yr}}{\left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg)}\right] \mid Cyto\ neg_{imm}}\right\} \qquad (4.3)$$

The overall 1-year regression of CIN2+ lesions is the complement of the ratio of CIN2+ positive proportions among cytology-negative women randomized to immediate colposcopy vs 1-year retesting, including only those with complete follow up (ie a colposcopy if randomized to immediate colposcopy, and HPV DNA-negative results or a colposcopy in HPV DNA-positive women if randomized to 1-year retesting).

Biomarker positive 1-year regression of CIN2+ lesions

$$\mathbf{1}\boldsymbol{yr\ Biom\ pos\ Reg} = 1 - \frac{\left\{\left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg +\ 1yr\ DNA\ neg\ )}\right] \mid Cyto\ neg_{1yr}\right\} \mid Biom\ pos}{\left\{\left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg)}\right] \mid Cyto\ neg_{imm}\right\} \mid Biom\ pos} \qquad (4.4)$$

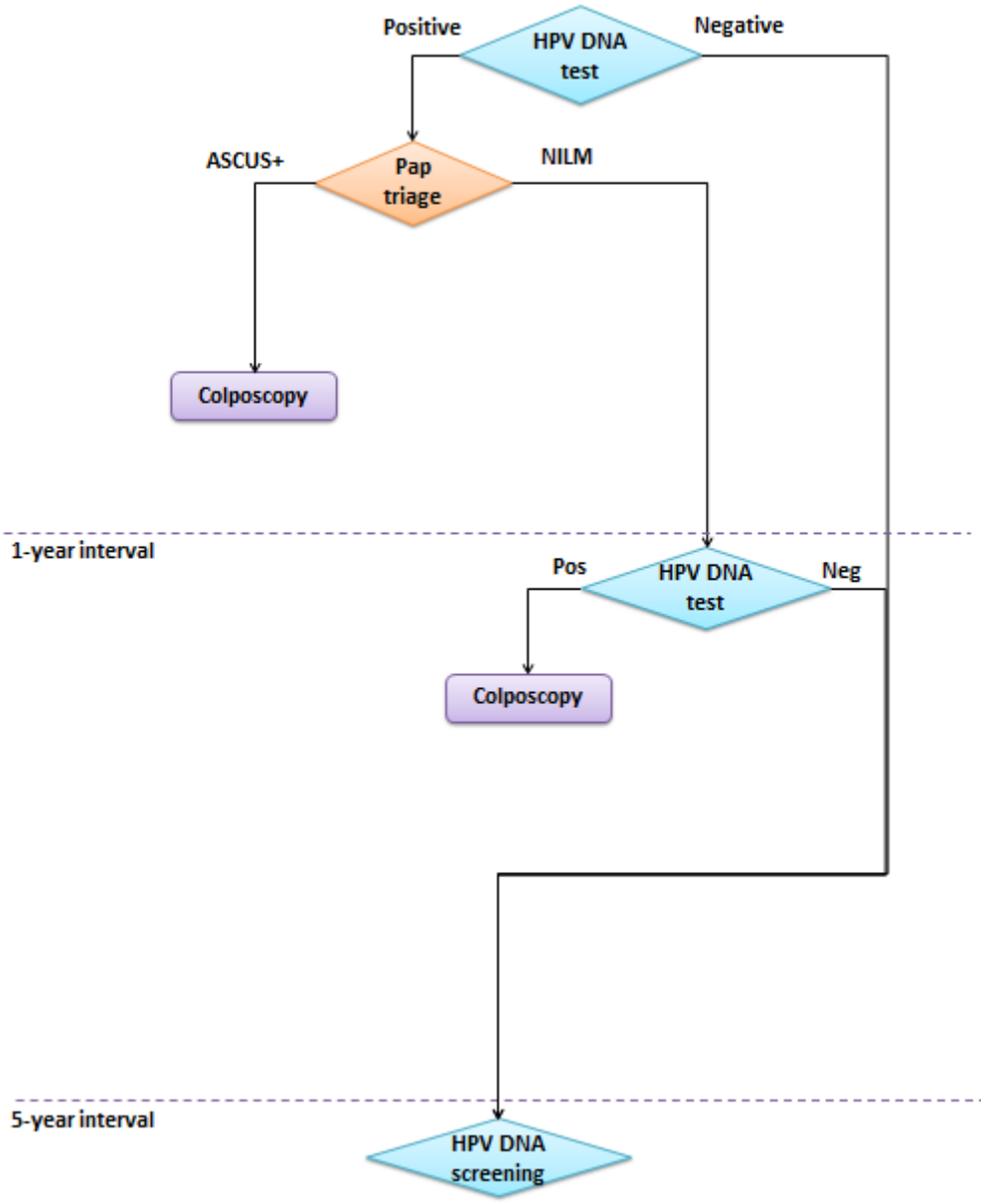
The overall 1-year regression of CIN2+ lesions is the complement of the ratio of CIN2+ positive proportions among cytology-negative biomarker-positive women randomized to immediate colposcopy vs 1-year retesting, including only those with complete follow up (ie a colposcopy if randomized to immediate colposcopy, and HPV DNA-negative results or a colposcopy in HPV DNA-positive women if randomized to 1-year retesting).

Biomarker negative 1-year regression of CIN2+ lesions

$$\mathbf{1}\boldsymbol{yr\ Biom\ neg\ Reg} = 1 - \frac{\left\{\left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg +\ 1yr\ DNA\ neg\ )}\right] \mid Cyto\ neg_{1yr}\right\} \mid Biom\ neg}{\left\{\left[\frac{CIN2plus}{(CIN2plus + Colpo\ neg)}\right] \mid Cyto\ neg_{imm}\right\} \mid Biom\ neg} \qquad (4.5)$$

The overall 1-year regression of CIN2+ lesions is the complement of the ratio of CIN2+ positive proportion among cytology-negative biomarker-negative women randomized to immediate colposcopy vs 1-year retesting, including only those with complete follow up (ie a colposcopy if randomized to immediate colposcopy, and HPV DNA-negative results or a colposcopy in HPV DNA-positive women if randomized to 1-year retesting).

Supplementary Figure S1. Italian HPV DNA-based screening flowchart



6.3. Supplementary material – Research article #05: Determinants of p16/Ki-67 Adequacy and Positivity in HPV-Positive Women From a Screening Population

Supplementary Table. Univariate and multivariate analyses for determinants of inadequate report excluding the reports from cases with inadequate (36 cases, 108 reports) or missing (9 cases, 27 reports) cytology.

Variables	Univariate			Multivariate		
	OR	95%CI	p	OR	95%CI	*P*
Age	1.01	(1.00 – 1.02)	0.03	1.00	(0.99 – 1.02)	0.40
Age						
25-34	1					
35-44	1.17	(0.86 – 1.59)	0.31			
45-54	1.15	(0.84 – 1.57)	0.38			
55+	1.58	(1.11 – 2.25)	0.01			
Presence of CIN2+						
Not CIN2+	1			1		
CIN2+	0.44	(0.26 – 0.75)	0.00	0.43	(0.25 – 0.75)	0.00
No assessment	1.11	(0.89 – 1.39)	0.37	1.11	(0.88 – 1.41)	0.38
Cytology result						
Negative	1			1		
ASC-US+	0.46	(0.37 – 0.58)	0.00	0.42	(0.33 – 0.54)	0.00
mRNA result						
Negative	1			1		
Positive	0.62	(0.52 – 0.73)	0.00	0.56	(0.47 – 0.67)	0.00
Missing	1.85	(0.77 – 4.44)	0.17	1.49	(0.63 – 3.55)	0.37
Typing						
Other HR types	1			1		
16 and/or 18 (with or without other HRs)	0.92	(0.68 – 1.23)	0.56	0.71	(0.37 – 1.34)	0.29
Recruiting center						
Lab 2	1					
Lab 6	0.75	(0.59 – 0.93)	0.01			
Lab 7	0.52	(0.41 – 0.65)	0.00			
Lab 5	0.80	(0.62 – 1.02)	0.07			
Lab 3	1.21	(0.60 – 2.44)	0.59			

Variables	Univariate			Multivariate		
	OR	**95%CI**	**p**	**OR**	**95%CI**	***P***
Center which interpreted the slides						
Lab 2	1					
Lab 6	1.02	(0.80 – 1.29)	0.89			
Lab 1	0.41	(0.32 – 0.54)	0.00			
Lab 7	1.67	(1.34 – 2.10)	0.00			
Lab 4	0.73	(0.57 – 0.93)	0.01			
Lab 5	1.62	(1.30 – 2.02)	0.00			
Lab 3	0.31	(0.22 – 0.44)	0.00			
Time between immunostaining and interpretation						
Recent	1			1		
Old	0.91	(0.78 – 1.05)	0.20	0.73	(0.60 – 0.90)	0.00
Time between sampling and immunostaining						
Recent	1			1		
Old	1.62	(1.37 – 1.92)	0.00	1.43	(1.16 – 1.76)	0.00

6.4. Supplementary material – Research article #07: HPV vaccination in women treated for CIN 2 or 3: evidence-based recommendation from the Multisociety Italian guidelines for cervical cancer prevention.

Table 1 – Scientific societies involved in the Guidelines Development Group, sorted alphabetically

- AIO Italian Association of Obstetrics (Associazione Italiana Ostetricia);
- AOGOI Italian Association of Hospital Obstetricians and Gynaecologists (Associazione Italiana Ostetrici e Ginecologi Ospedalieri);
- GISCi Italian group for cervical cancer screening (Gruppo Italiano per lo Screening del Cervicocarcinoma);
- SIAPEC-IAP Italian Society for Pathological Anatomy and Diagnostic Cytology. Italian Division of the International Academy of Pathology (Società Italiana Anatomia Patologica e Citologia Diagnostica. Divisione Italiana dell'International Accademy of Pathology);
- SICi Italian Society of Cytology (Società Italiana di Citologia);
- SICPCV Italian Society for Colposcopy and Cervico-Vaginal Pathology (Società Italiana di Colposcopia e Patologia Cervico-Vaginale);
- SIGO Italian Society of Gynecology and Obstetrics (Società Italiana Ginecologia e Ostetricia);
- SItI Italian Society of Hygiene, Preventive Medicine and Public Health (Società Italiana di Igiene, Medicina Preventiva e Sanità Pubblica);
- SIV-ISV Italian Society for Virology (Società Italiana di Virologia)

Table 2. Search strategy

MEDLINE ((vaccination* OR vaccine* OR vaccinating) AND (HPV OR human papillomavirus)) OR "Papillomavirus Vaccines"[MeSH] OR papillomavirus vaccine* OR HPV vaccine* AND (high-grade cervical intraepithelial neoplasia OR "Cervical Intraepithelial Neoplasia"[Mesh] OR CIN) AND (treatment OR secondary prevention OR therapy OR surg* OR recurren*) Filters: 2006-
EMBASE 'Wart virus vaccine'/exp OR papillomavirus vaccine* OR HPV vaccine* OR ((vaccination* OR vaccine* OR vaccinating) AND (HPV OR human papillomavirus)) AND 'uterine cervix carcinoma in situ'/exp OR high-grade cervical intraepithelial neoplasia OR CIN AND (treatment OR secondary prevention OR therapy OR surg* OR recurren*)
COCHRANE LIBRARY ((vaccination* OR vaccine* OR vaccinating) AND (HPV OR human papillomavirus)) OR papillomavirus vaccine* OR HPV vaccine* in Record Title AND (high-grade cervical intraepithelial neoplasia OR CIN) in Title Abstract Keyword AND (treatment OR secondary prevention OR therapy OR surg* OR recurren*) in Title Abstract Keyword - (Word variations have been searched)
SCOPUS (TITLE-ABS-KEY (((vaccination* OR vaccine* OR vaccinating) AND (hpv OR human AND papillomavirus)) OR papillomavirus AND vaccine* OR hpv AND vaccine*) AND TITLE-ABS-KEY (high-grade AND cervical AND intraepithelial AND neoplasia OR cin) AND TITLE-ABS-KEY (treatment OR secondary AND prevention OR therapy OR surg* OR recurren*))

Table 3 -Characteristics of included studies

First author, year	HPV vaccine	Study Design	Analysis	Allocation	Population	Sample size	Median follow up	Vaccination Timing
Joura, 2012 [26]	4-valent	RCT	Retrospective	randomized	Age: 15-26 years Follow- up starting 2 months after treatment	587v 763c	16 months	pre-diagnosis of CIN
Kang, 2013 [24]	4-valent	cohort	Retrospective	Non-randomized	Age: 20-45 years disease-free at 6 months after treatment	360v 377c	42 months	post-treatment
Garland, 2016 [27]	2-valent	RCT	Retrospective	randomized	Age: 15-25 years disease-free at 2 months after treatment	190v 264c	<24 months	pre-diagnosis of CIN
Hildesheim, 2016 [28]	2-valent	RCT	Retrospective	randomized	Age: 18-25 years Lesions associate with new infections after treatment	142v 169c	27 months	pre-diagnosis of CIN
Ghelardi, 2018 [25]	4-valent	cohort	Prospective	Non-randomized	Age: <45 years disease-free at 6 months after treatment	174v 176c	36 months	post-treatment
Pieralli, 2018 [23]	4-valent	RCT	Prospective	randomized	Age: <45 years disease-free and HPV-negative at 3 months after treatment	89v 89c	36 months	post-treatment

Note: v: Vaccination group; c: Control group. CIN: Cervical Intraepithelial Neoplasia; RCT: Randomised Clinical Trial.

Figure 1. Risk of bias" summary: Evidence Review Team members' judgements about each "Risk of bias" item for each included study. Created with Review Manager (RevMan) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014.

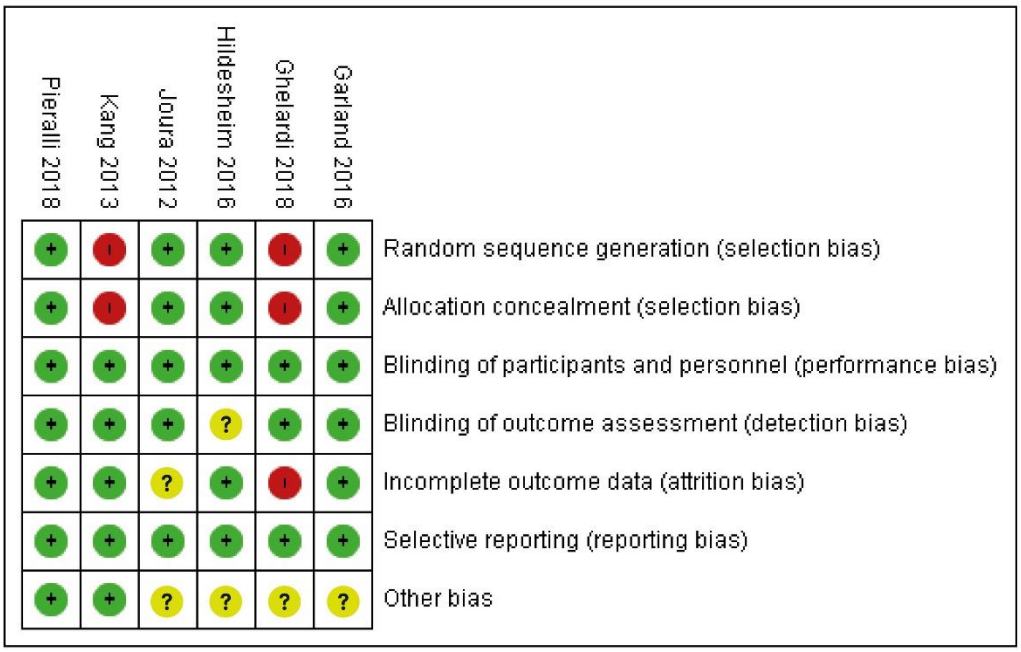


Figure 2. "Risk of bias" graph: Evidence Review Team members' judgements about each "Risk of bias" item presented as percentages across all included studies. Created with Review Manager (RevMan) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014.