European Journal of Gynaecological Oncology

Original Research

A prospective cohort study to identify biomarkers predicting the regression of grade 2 Cervical Intraepithelial Neoplasia. Study protocol

Annarosa Del Mistro[1,*], Helena Frayle[1], Silvia Gori[1], Alessio Pagan[2], Marika Soldà[3], Cesare Romagnolo[3], Egle Insacco[4], Licia Laurino[3], Mario Matteucci[4], Enrico Busato[2], Manuel Zorzi[5], Tiziano Maggino[3], on behalf of the CIN2 study Working Group[§]

[1] *Veneto Institute of Oncology IOV-IRCCS, 35128 Padova, Italy*

[2] *Local Health Unit Marca Trevigiana, 31100 Treviso, Italy*

[3] *Local Health Unit Serenissima, 30174 Venezia, Italy*

[4] *Azienda Ospedale Università, 35128 Padova, Italy*

[5] *Veneto Tumour Registry, Azienda Zero, 35131 Padova, Italy*

*Correspondence: annarosa.delmistro@iov.veneto.it (Annarosa Del Mistro)
[§]CIN2 study Working Group: Tiziano Maggino, Marika Soldà, Cesare Romagnolo, Licia Laurino, Pamela Zambenedetti, Alessio Pagan, Justyna Wojciechowska, Enrico Busato, Egle Insacco, Mario Matteucci, Maria Teresa Gervasi, Daria Minucci, Giuseppe Sordi, Marco Torrazzina, Annarosa Del Mistro, Helena Frayle, Silvia Gori, Rossana Trevisan, Manuel Zorzi.

DOI:10.31083/j.ejgo4205136

Submitted: 29 March 2021 Revised: 17 May 2021 Accepted: 19 May 2021 Published: 15 October 2021

Objective: The detection and treatment of high-grade cervical lesions prevent the development of invasive cervical cancer. Excisional procedures can pose a risk for subsequent pregnancies, thus conservative management of Cervical Intraepithelial Neoplasia grade 2 (CIN2) lesions should be adopted in young women. The aim of our study is to evaluate the ability of viral and cellular biomarkers in predicting regression/progression of CIN2. *Methods*: Women aged 25 to 45 years, participating to population-based organised cervical cancer screening programmes in the Veneto Region (Italy), diagnosed with a CIN2 lesion and fitting predefined inclusion/exclusion criteria, are invited to take part in a multicentre observational longitudinal cohort study with a follow-up of 24 months. Upon signing an informed consent, women are enrolled in the study and cervical cell samples collected. Treatment is delayed and subsequently performed in the case of lesion progression, or persistence for >12 months. HPV genotyping, $p16^{INK4A}$/ki67 expression and methylation status for L1 viral sequences and *FAM19A4/miR124-2* cellular genes are determined at baseline and during follow-up, and evaluated in relation to the clinical outcome. *Results*: The study, registered on ClinicalTrials.gov (ID: NCT04687267), is currently ongoing. Enrolment of women aged 25–45 years started in 2019, and will continue up to the end of 2021. *Discussion*: Since February 2020, the Veneto Region has been hit by the COVID-19 pandemic. The enrolment of women in the study was interrupted during an initial two-month lockdown, and slowed down during the subsequent months. The 12-month extension of the study period will partially counterbalance this delay.

Keywords

Cervical screening; CIN2; Conservative management; Regression; Progression; Biomarkers; HPV genotyping; Methylation

1. Introduction

Cervical cancer is causally associated with persistent infection of high-risk human papillomavirus (hrHPV) types [1]. Population-based organised cervical cancer screening and prophylactic anti-HPV vaccination are effective preventive strategies. Screening programmes can identify women at risk of developing cancer and prevent its development by detecting and treating grade 2 and 3 Cervical Intraepithelial Neoplasia (CIN2-3) carcinoma precursors. Vaccination prevents high-risk types HPV infections, a necessary cause for tumour formation.

Screening by pap test (i.e., cytological examination) has been in use for several decades and proved to be effective in reducing both the incidence and mortality of cervical cancer [2]. The results of large randomised clinical trials have demonstrated a higher efficacy of HPV testing (i.e., search for hrHPV DNA sequences) as primary screening for women older than 30 years [3]. Consequently, primary HPV testing (with clinically validated HPV DNA assays and triage of HPV-positive cases) is being progressively introduced in many Countries. Evidence-based recommendations, detailing protocols and quality assurance monitoring for screening, are issued and updated by international and national authorities. Besides indications on the target population and the protocols for primary testing and examinations to be performed on women with a positive result, indications on treatment and follow-up are also given [4]. In particular, excisional treatment is indicated for CIN2 and 3. These two categories of lesions, however, differ from each other in terms of clinical outcome and diagnostic concordance, with CIN2 having a higher spontaneous regression rate (40–60%, de-

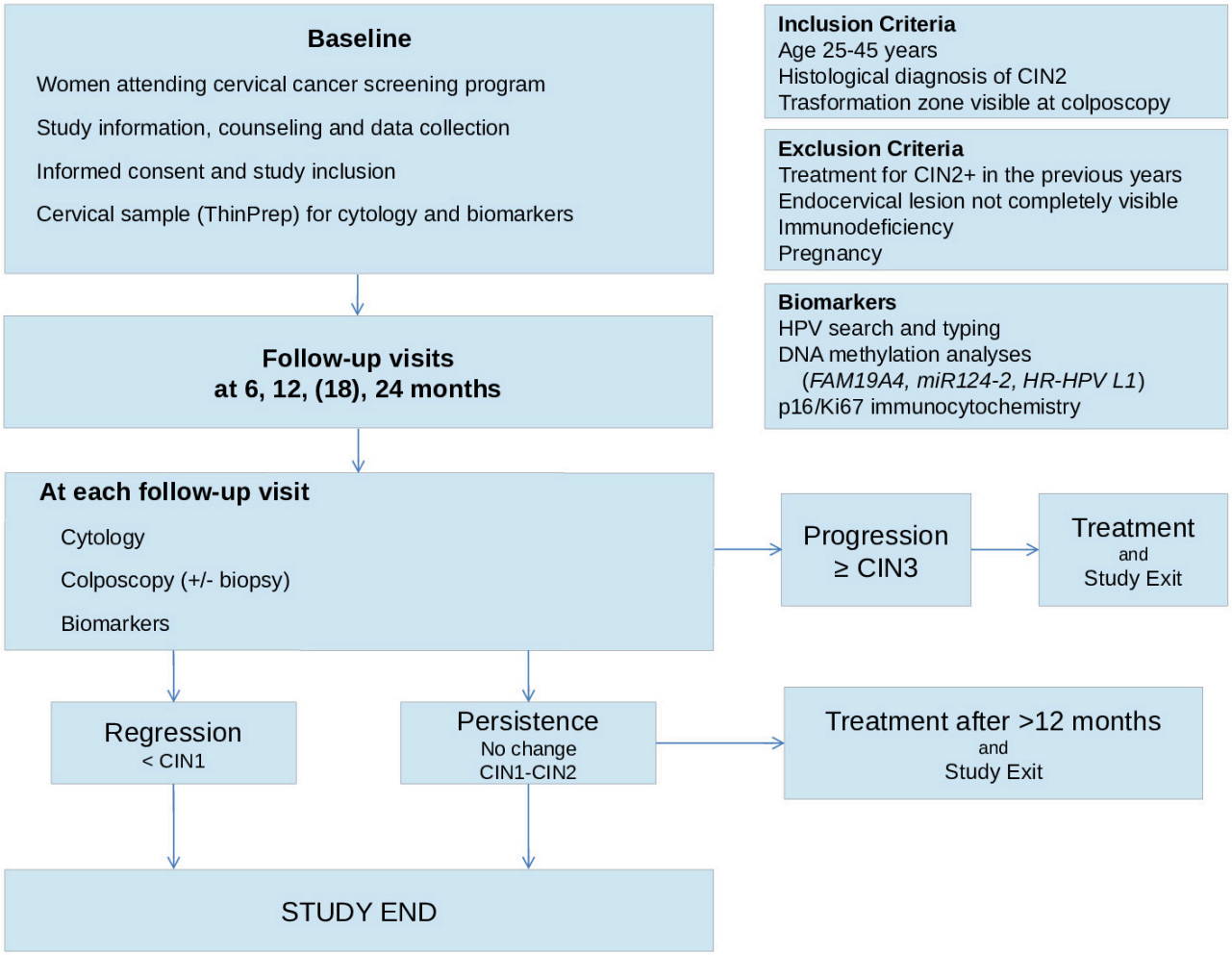

Fig. 1. Flowchart of the study protocol for women attending cervical cancer screening in the Veneto region (Italy). Inclusion and exclusion criteria, biomarkers under study, schedule of follow-up visits and analyses performed, and treatment decisions according to clinical outcome are reported.

pending on age) and a lower progression capacity (around 15%) than CIN3 (30–40% for both). Since the excision procedure can pose a risk for subsequent pregnancies, a conservative management for CIN2 lesions in young women has been proposed [5], and performed in selected cases, according to Skorstengaard M *et al.* [6] or in the absence of specific guidelines [7]. In order to provide guidelines and recommendations, several studies are ongoing to evaluate the clinical outcomes of untreated CIN2 under active surveillance [8–11].

The ability of biomarkers to predict the clinical outcome of CIN2, as well as women's acceptability of a conservative management [12] and its impact on the cervical screening organisation are important issues to explore. Based on the natural history of cervical HPV infection and of the association with lesion development, HPV genotyping [13], expression of $p16^{INK4A}$ and ki67 proteins [14], and the methylation status of cellular genes and viral sequences [15, 16] are promising biomarkers.

In Italy, organised cervical screening programs started in the late 1990s, and are being performed according to national recommendations, by call-recall invitation of all women aged 25–64 years-old. The programmes are implemented at a regional level, and each region is structured in Local Health Units (LHUs). Cytology-based screening was implemented with HPV-based testing for women aged 30–64 years after piloting phases in some regions. The Veneto region (North-East Italy) was part of the pilot phase with six LHUs from 2009 to 2015, when HPV testing was introduced in all the 21 LHUs.

A case-series of 111 women who had been diagnosed with a CIN2 in one of the study centres between 2011 and 2016 and who had not been treated immediately, showed a spontaneous regression in 69.4% cases (n = 77) during a 2-year follow up. Within the organised programme of four LHUs of the Veneto region, we are conducting a multicentre prospective study on the conservative management of CIN2, aimed at evaluating the predictive capacity of selected viral and cellular biomarkers and the impact on women and on cervical screening programmes.

2. Methods

2.1 Study design

This is a prospective multicentre study with a 24 month follow up, involving Gynaecologic Units within four screening programmes of the Veneto region (North-East Italy): Mestre, Padova, Treviso and Verona. The study was approved at the end of 2018, formally started on 15 April 2019 and, after receiving authorisation of a 12-month extension period, will end on 14 April 2022.

At diagnosis of CIN2, eligible women are invited to participate in the study. After enrolment (baseline visit), a cervical sample is collected from consenting women. Follow up visits are planned at 6, 12 and 24 months. An optional additional visit may be performed at 18 months. The timing of follow-up visits, colposcopy, cytology (liquid-based or conventional) and HPV testing is consistent with the routine post-treatment follow-up, while HPV genotyping, methylation and immunocytochemical analyses are specifically conducted in the study population. The procedures are summarised in the flowchart of Fig. 1.

Cervical samples are collected in PreservCyt solution (Thin Prep, Hologic, Bedford, MA, USA) and transported to the laboratory at room temperature. Three 1-mL sample aliquots are then taken and stored at –40 °C for subsequent molecular analyses. The residual sample is used for the immunocytochemistry analysis.

2.2 Inclusion and exclusion criteria

Women aged 25 to 45 years with a histological diagnosis of CIN2, and with both transformation zone and lesion fully visible at colposcopy, are eligible to participate in the study. Women with one or more of the following criteria are excluded from the study: pregnancy, previous treatment of a CIN2+ lesion, immunodeficiency, and presence of an endocervical lesion not completely visible at colposcopy. Eligible women are provided with information on the rationale and the procedures of the study and are invited to participate. Consenting women are requested to sign a written informed consent. Women unwilling to enter the study are treated according to the routine practice (lesion excision). Sociodemographic and clinical data of all women with a CIN2 diagnosis, as well as the exclusion criteria or the reason for refusal are collected and entered in a dedicated database. Three groups of women are hence defined: (1) not eligible, (2) eligible but refused participation, (3) eligible and participating in the study.

2.3 Study procedures

2.3.1 HPV DNA detection

Cervical samples are directly tested for HPV detection by the COBAS 4800 HPV assay on the automated cobas 4800 System (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. This is a real-time Polymerase Chain Reaction (PCR) assay for the detection of 14 high-risk types in a single analysis, that allow a concurrent partial genotyping for HPV16 and HPV18 and pooled detection of the other 12 high-risk types (HPV 31, 33, 35, 39, 45, 51, 52, 56, 56, 58, 59, 66 and 68), referred to as HR-HPV, according to the assay designation. Amplification of sequences of the cellular beta-globin gene serves as internal control for specimen quality.

2.3.2 DNA extraction

Total genomic DNA is extracted from one of the 1-mL aliquots using the QIAmp DNA Mini or Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA is quantified by the Qubit micro DNA assay Kit on a Qubit 2.0 fluorometer instrument (Thermo Fisher Scientific, Waltham, MA, USA).

2.3.3 HPV genotyping

HPV genotyping is performed by PCR with consensus MY09/MY11 primers (targeting the L1 region of most mucosal types; fragment length 450 bp), followed by restriction fragment length polymorphism (RFLP) analysis to define high- and low-risk specific types; positive (DNA from HPV16-positive SiHa cells or HPV18-positive HeLa cells) and negative (no DNA) controls are included, as already described [17]. DNA amplificability is evaluated by PCR with GH20/PC04 primers for the beta-globin gene (fragment length 268 bp).

2.3.4 Methylation analyses

Bisulfite conversion of DNA sequences is done using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions; 200 ng of genomic DNA in 50 μL is used, and modified DNA is eluted in 10 μL.

2.3.4.1 Detection of methylated host cellular DNA. The QIAsure Methylation test (Qiagen, Hilden, Germany), a multiplex methylation-specific real time PCR assay, is used for the analysis of the methylation status of the promoter regions of the *FAM19A4* and *miR124-2* genes, according to the manufacturer's instructions. An aliquot of 2.5 μL bisulfite-converted DNA from each sample is used as template for DNA methylation analysis and added to 17.5 μL QIAsure master mix reaction solution; the PCR is performed on the Rotorgene PCR-platform (Qiagen, Hilden, Germany). The housekeeping β-actin (*ACTB*) gene is used as a reference to monitor bisulfite conversion and sample quality, and DNA from HPV16-positive SiHa cells is used in each run as methylation positive control. The results of the QIAsure assay are expressed as Cycle threshold (Ct) values. According to the manufacturer's instructions, a test is considered valid when the Ct value for the housekeeping gene is $<$26.4. The dedicated software calculates the ΔCt value as the difference between the Ct value of the *FAM19A4* or *miR124-2* targets and the Ct value of the *ACTB* reference gene. This ΔCt is a relative quantitative value for the promoter methylation level of the *FAM19A4* or *miR124-2* genes. For normalisation, the ΔCt value of a calibrator is subtracted from the ΔCt of the *FAM19A4* or *miR124-2* targets, resulting in a $\Delta\Delta$Ct [18].

2.3.4.2 Detection of methylated viral sequences. The methylation status of high-risk HPV L1 viral sequences is investigated by pyrosequencing analysis after DNA bisulfite conversion and preliminary PCR reactions set up with primers previously established by Gillio Tos and collaborators [19]. Consensus primers for the L1 I regions of different types allow to investigate all high-risk HPV types by performing four PCR runs; HPV16, 31, 33, 35, 52, 58 and HPV39, 45, 51, 59 are amplified as groups, whereas dedicated primers are needed for HPV56 and HPV18; the amplified fragments are then analysed by type-specific probes. Synthetic DNA plasmids containing the complete genomes of HPV16 or HPV18 (Medical System, Genoa, Italy) are used as unmethylated controls. These synthetic HPV plasmids and DNA from HPV16-positive SiHa cells are also used as fully methylated controls after treatment with CpG (Cytosine plus Guanine) methylase (M. SssI, Zymo Research, Irvine, CA, USA), performed according to the manufacturer's instructions. Both CpG methylase-treated and -untreated HPV plasmids undergo two cycles of bisulfite modification with incubation time extended to 16 hours to achieve a complete conversion.

Methylation assays are performed by pyrosequencing onto a PyroMark Q24 MDx system (Qiagen, Hilden, Germany), as previously described [19]. Based on the correspondence of the sequence to that expected for each genotype and of the quantity of material available, the quality of the result at each position is classified by the PyroMark CpG mode software as "passed" (valid/acceptable), "check" (interpretation by operator needed) or "failed" (pyrogram not interpretable). For each sample, methylation at each CpG site is recorded as the proportion of methylated cytosines through the C/(C + T) ratio. When more than one CpG site is detected in one region (i.e., L1 I), the average methylation value of four sites is considered.

2.3.5 Immunocytochemistry analysis

The cytology slide for the $p16^{INK4A}$/ki67 immunocytochemistry analysis is prepared from the liquid-based cytology (LBC) specimen using a T2000 slide processor (Hologic, Bedford, MA, USA). The CINtec® Plus Kit (Roche mtm laboratories AG, Heidelberg, Germany) is used according to the manufacturer's instructions. An experienced cytotechnologist reviews all cervical cytology slides to detect the staining performance of the two markers. A slide is considered positive for $p16^{INK4A}$/ki67 immunocytochemistry if $\geq$1 cervical epithelial cell shows both a brown cytoplasmic ($p16^{INK4A}$) and a red nuclear (ki67) stain, irrespective of the morphology of the cells. Slides without a brown cytoplasmic stain and/or a red nuclear stain, and slides with an insufficient number of cells are considered negative and inadequate, respectively.

2.4 Study endpoints

2.4.1 Primary

The primary endpoint of the study will be the rate of spontaneous regression of CIN2 lesions. The rates of lesion regression will be calculated as the number of women with CIN1 or no dysplasia/total number of cases.

2.4.2 Secondary

The following secondary outcomes will be calculated:

- CIN2 clinical outcome by HPV genotype. The rate of CIN2 regression will be calculated in relation to a positivity for HPV16 vs a positivity for other high-risk types, as number of regressed HPV16-related CIN2 lesions/total number of HPV16-related CIN2 vs number of regressed non-HPV16-related CIN2 lesions/total number of non-HPV16-related CIN2 lesions.

- CIN2 clinical outcome by DNA methylation. The rate of CIN2 regression will be calculated in relation to the DNA methylation status of cellular and viral genes, as the number of regressed hypermethylated CIN2 lesions/total number of CIN2 lesions with valid result for each gene analysed.

- CIN2 clinical outcome by $p16^{INK4A}$/ki67 protein expression. The rate of CIN2 regression will be calculated in relation to the positivity for $p16^{INK4A}$/ki67 expression as the number of regressed $p16^{INK4A}$/ki67-positive CIN2 lesions/total number of CIN2 lesions with valid result for $p16^{INK4A}$/ki67 expression.

- Women willingness to accept CIN2 conservative management. The rate of eligible women consenting to participate to the study will be calculated as the proportion of enrolled women/eligible women.

- Impact of the conservative management on the screening program. The number of eligible women with regressive CIN2 lesions returning to regular screening as opposed to those needing treatment or prolonged surveillance will be calculated in order to predict the relative risk of avoided excisions.

2.5 Sample size calculation

The primary aim of the study is to quantify the proportion of CIN2 lesions that regress during follow up.

The study aims to recruit 322 participants in order to verify, at a significance level of 0.05 with a power of 85%, the null hypothesis that the proportion of spontaneous regression is inside the interval 0.70 ± 0.07 (where 0.07 is 10% of the expected proportion).

2.6 Statistical analysis

For descriptive findings, distribution of categorical variables will be presented as percentages, while those based on continuous variables as mean with standard deviation (SD). The proportion of regressive CIN2 (primary outcome) and the proportion of women accepting to delay the treatment (secondary outcome) will be estimated along with 95% Confidence Intervals approximated using the Wilson's score method.

The association between the primary outcome and the other categorical variables, including HPV genotype, genes methylation and $p16^{INK4A}$/ki67 positivity will be first evaluated with the chi-square test (χ^2) and the Fisher's exact test in case of small sample size. Additionally, a multivariate logistic regression will be used to estimate the primary outcome adjusting for factors associated with CIN2 regression in univariate analyses (i.e., with a p-value < 0.10).

Statistical significance will be set at 0.05 and analyses will be computed using SAS, version 9.4 statistical package (SAS Institute, Cary, NC, USA) and R-software environment, version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

2.7 Patient and public involvement

No patient advisors were involved in the development and design or conduct of this study. Study results will be communicated to the study participants via an information letter.

3. Results

The trial is registered on ClinicalTrials.gov (ID: NCT04687267). Study enrolment started in January 2019 and will continue up to the end of December 2021. The number of women being enrolled in the four participating centres differs due to the extent of the reference area and colposcopy centralisation. Moreover, rates of CIN2 detection differ depending on when HPV-based screening was introduced. In particular, two centres had previously participated in the HPV-based screening pilot phase while two were running the first round of HPV screening. As of 28 February 2021, a total of 230 women aged 25–45 years (mean and median age 33 years) have been included in the study, for a total of 457 cervical samples collected at baseline and during follow-up visits; samples are under investigation for the biomarkers analyses.

4. Discussion

The strengths of this study are the nesting into the population-based organised cervical cancer screening programmes, the collection of data regarding all the consecutive CIN2 cases recorded in the study centres, and the analysis of different biomarkers. On the other hand, a limitation is the difficulty in reaching the forecasted sample size as a consequence of the COVID-19 pandemic, due to a temporary suspension of invitations to cervical screening from March to May 2020, and to a reduced number of invitations thereafter, to comply with the regulations regarding SARS-CoV-2 prevention. These measures have negatively impacted on the study, mostly on the enrolment of new cases, and to a lesser extent on the timing of the follow-up visits. The 12-month extension of the study period will partially counterbalance this limitation. Another limitation is the lack of $p16^{INK4A}$ testing on the biopsies diagnosed as CIN2. Indeed, we are aware that the addition of $p16^{INK4A}$ staining improves the reliability of CIN2 diagnosis, but this is not included in the routine cervical screening protocols. As a consequence, a proportion of the enrolled women may have LSIL or even metaplasia.

5. Conclusions

Our ongoing prospective study will provide useful data to improve and personalize the management of women diagnosed with a CIN2 lesion at 25–45 years of age; a conservative approach can allow the spontaneous regression of the lesion, thus avoiding an unnecessary surgical treatment. The search of potential biomarkers will allow the implementation of the therapeutic protocols within organized cervical cancer screening programmes.

Author contributions

TM, ADM, MZ designed the research study. AP, MS, CR, EI, EB enrol and follow-up the patients. HF, SG, LL perform the biomarkers' analyses. ADM, MZ and MM analysed the data. ADM, HF, SG, MZ wrote the manuscript. All authors contributed to editorial changes in the manuscript. The other members of the CIN2 study Working Group contribute to the enrolment and follow-up of the patients or to the performance of the biomarkers' analyses. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval was obtained by the Ethics Committee of the province of Venice and San Camillo Hospital (Venice, Italy, ethics number 2017/79A). Additional approvals were obtained from the participating clinics. Eligible women informed on the rationale and procedures of the study, are invited to participate; consenting women are required to sign a written informed consent; those unwilling to take part in the study are treated according to the routine practice (lesion excision).

Acknowledgment

Not applicable.

Funding

This study has been funded by the Veneto Region (grant number RSFR-2017-00000523).

Conflict of interest

The authors declare no conflict of interest. TM is our Guest Board, given his role as Guest Board, had no involvement in the peer-review of this article and has no access to information regarding its peer-review.

References

[1] Bruni L, Albero G, Serrano B, Mena M, Gómez D, Muñoz J, *et al.* Human papillomavirus and related diseases in the world. 2019. Available at: hpvcentre.net/statistics/reports/XWX.pdf (Accessed: 9 April 2021).

[2] 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. Preventive Medicine. 2015; 75: 56–63.

[3] Ronco G, Dillner J, Elfström M, Tunesi S, Snijders PJ, Arbyn M, *et al.* Efficacy of HPV-based screening for preventing invasive cervical cancer: follow-up of European randomised controlled trials. Lancet. 2014; 383: 524–532.

[4] 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, Dillner J, Dillner L, Franceschi S, Patnick J, Ronco G, Segnan N, Suonio E, Törnberg S, von Karsa L (eds.) European guidelines for quality

assurance in cervical cancer screening (pp. 1–68). 2nd edn. Supplements. Luxembourg: Office for Official Publications of the European Union. 2015.
[5] Tainio K, Athanasiou A, Tikkinen KAO, Aaltonen R, Cárdenas J, Hernándes, *et al.* Clinical course of untreated cervical intraepithelial neoplasia grade 2 under active surveillance: systematic review and meta-analysis. British Medical Journal. 2018; 360: k499.
[6] Skorstengaard M, Lynge E, Suhr J, Napolitano G. Conservative management of women with cervical intraepithelial neoplasia grade 2 in Denmark: a cohort study. BJOG: An International Journal of Obstetrics & Gynaecology. 2020; 127: 729–736.
[7] Macdonald M, Smith JHF, Tidy JA, Palmer JE. Conservative management of CIN2: national audit of British society for colposcopy and cervical pathology members' opinion. Journal of Obstetrics and Gynaecology. 2018; 38: 388–394.
[8] Silver MI, Gage JC, Schiffman M, Fetterman B, Poitras NE, Lorey T, *et al.* Clinical outcomes after conservative management of cervical intraepithelial neoplasia grade 2 (CIN2) in women aged 21–39 years. Cancer Prevention Research. 2018; 11: 165–170.
[9] Kremer WW, Berkhof J, Bleeker MCG, Heideman DAM, van Trommel NE, van Baal MW, *et al.* Role of *FAM194A/miR124–2* methylation analysis in predicting regression or non-regression of CIN2/3 lesions: a protocol of an observational longitudinal cohort study. British Medical Journal Open. 2019; 9: e029017.
[10] Brun J, Letoffet D, Marty M, Griffier R, Ah-Kit X, Garrigue I. Factors predicting the spontaneous regression of cervical high-grade squamous intraepithelial lesions (HSIL/CIN2). Archives of Gynecology and Obstetrics. 2021; 303: 1065–1073.
[11] Louvanto K, Aro K, Nedjai B, Bützow R, Jakobsson M, Kalliala I, *et al.* Methylation in predicting progression of untreated high-grade cervical intraepithelial neoplasia. Clinical Infectious Diseases. 2020; 70: 2582–2590.
[12] Dodd RH, Cvejic E, Bell K, Black K, Bateson D, Smith MA, *et al.* Active surveillance as a management option for cervical intraepithelial neoplasia 2: an online experimental study. Gynecologic Oncology. 2021; 161: 179–187.
[13] Demarco M, Hyun N, Carter-Pokras O, Raine-Bennett TR, Cheung L, Chen X, *et al.* A study of type-specific HPV natural history and implications for contemporary cervical cancer screening programs. EClinicalMedicine. 2020; 22: 100293.
[14] Ordi J, Sagasta A, Munmany M, Rodríguez-Carunchio L, Torné A, del Pino M. Usefulness of p16/ki67 immunostaining in the triage of women referred to colposcopy. Cancer Cytopathology. 2014; 122: 227–235.
[15] von Knebel Doeberitz M, Prigge E. Role of DNA methylation in HPV associated lesions. Papillomavirus Research. 2019; 7: 180–183.
[16] Bonde J, Floore A, Ejegod D, Vink FJ, Hesselink A, van de Ven PM, *et al.* Methylation markers *FAM194A* and *miR124-2* as triage strategy for primary human papillomavirus screen positive women: a large European multicenter study. International Journal of Cancer. 2021; 148: 396–405.
[17] Del Mistro A, Matteucci M, Insacco EA, Onnis G, Da Re F, Baboci L, *et al.* Long-term clinical outcome after treatment for high-grade cervical lesions: a retrospective monoinstitutional cohort study. BioMed Research International. 2015; 2015: 984528.
[18] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods. 2001; 25: 402–408.
[19] Gillio-Tos A, Fiano V, Grasso C, Trevisan M, Gori S, Mongia A, *et al.* Assessment of viral methylation levels for high risk HPV types by newly designed consensus primers PCR and pyrosequencing. PLoS ONE. 2018; 13: e0194619.