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Preface

Ladies and Gentlemen, Dear Colleagues,

As Minister of Science, Education and Sports of the Republic of Croatia I am pleased to welcome all participants gathered for the International Workshop on Human Papillomaviruses and Consensus Recommendations for Cervical Cancer Prevention & Colposcopy Training.

It is a great honor to have experts and scientists from all over the world here in Croatia discuss an exceptionally important health risk factor, at this distinguished event.

Human Papilloma Virus (HPV) is known to play a significant part in the etiology of cervical cancer, which is the second most frequent malignancy in women worldwide. The mortality rate is growing, especially in younger women, so it is extremely necessary to carry out cervical cancer prevention programmes, as it is obvious that systematic and well-organized cervical cancer screening programmes can greatly reduce incidence and mortality rates from the disease.

The World Health Organization recommendation from 2006 bears witness to the importance of this issue, as it suggests that cervical screening should be presented with organized programmes. Additionally, the European Union Council declared in its recommendation from 2003 that all EU member-states should conduct organized cervical cancer screening programmes.

The development of science and technology, particularly HPV DNA testing, HPV RNA testing, HPV vaccination as well as the use of a selection of different other molecular markers, etc., allow you, as leading experts in the field, to make best possible Consensus Recommendations for Cervical Cancer Prevention.

This important scientific event will feature the latest advances concerning Human Papillomaviruses, cervical cancer screening and prevention through many remarkable lectures. It is also important to mention that the special issue of Collegium Anthropologicum, published on this occasion, includes interesting themes related to the most recent research findings, different aspects and experiences in the field. This will certainly be a considerable contribution in defining further guidelines in cervical cancer prevention in Croatia and abroad.

We are fully aware of how significant your scientific achievements are and how huge an impact your decisions will have in this very important field, therefore in improving the quality of life of this and future generations.

I sincerely wish you a memorable, inspirational and very successful gathering, in hopes that all of us will greatly benefit from this event.

Professor Dragan Primorac, MD, PhD
Minister of Science, Education and Sports
of the Republic of Croatia

Introduction

Cancer is the second major cause of death in Croatia, every fourth of our citizens dies of it. From 1978 to 2004, the incidence of cancer has increased by 58%, while deaths from cancer from 1978 to 2005 have increased by 59%. This means that there are about 20,000 new cancer cases per year in our country with 4,4 million inhabitants. Meanwhile, the cancer mortality rates in Croatia are higher than those seen in the countries of the European Union or in countries that have implemented cancer prevention and early detection programmes. Among Croatian women, breast cancer is the most common cancer overall. Cancer of the cervix is the second one in women, aged 25–49 years.

The burden of cervical cancer in Croatia is considerable as we have about 350 (332 in year 2004) incident cases and about 100 deaths from cervical cancer yearly. Although, invasive cervical cancer incidence is only the 8th most common cancer among Croatian women, the important distinction with cervical cancer is that it is almost entirely preventable. Effective, organised cervical screening programmes have been shown to prevent about 80% of cervical cancers and we need to work toward the implementation of these programmes in our country. In addition, we now have vaccines against the two most common types of HPV, the virus that can cause cervical cancer, and the implementation of vaccination programmes for pre-adolescents before the start of sexual activities carries with it the promise of even further reduction in cervical cancer rate.

Cervical cancer screening is available in Croatia but this is not being offered within a national, organised programme that effectively screens all Croatian women. Following the publication of the European Council's recommendations for cancer screening of December 2003, a working group of the Croatian Ministry of Health and Social Welfare was convened and in 2005, recommended a national programme for early detection of cancer. This represents a basic document for the implementation of organised screening programmes for cervical, breast and colorectal cancer in Croatia. Under the direction of the Croatian Ministry of Heath and Social Welfare, a programme for the early detection of breast cancer started in Croatia in 2006 enrolling about 560,000 women. Now, the Croatian Ministry of Heath and Social Welfare is planning a pilot project for cervical cancer screening programme and this should start before the end of the year.

In this context the Croatian Ministry of Health and Social Welfare supports the editing of this special issue of Collegium Aiitropologicum, which is dedicated to the International Workshop on Human Papillomaviruses and Consensus Recommendations for Cervical Cancer Prevention that will be held in Dubrovnik – Cavtat, Croatia from 18 to 21 April, 2007.

Professor Neven Ljubičić, MD, PhD
Minister of Health and Social Welfare
of the Republic of Croatia

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Editorial

Editorial

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Cervical cancer still remains an important public health issue in Europe where it is the 7th most common cause of cancer deaths in women[1]. Each year in Western Europe, 13,000 women develop cervical cancer and 6,000 women die from this disease, while the situation in Eastern Europe is much worse with approximately 31,000 women developing cervical cancer and about 17,000 dieing every year[1]. The differences within Europe are largely due to the absence of effective cervical screening in Eastern Europe and the implementation of properly organised cervical screening programmes would inevitably have a major impact on this disease.

Cervical cancer screening based on the Pap smear can reduce cervical cancer rates by 80% and it is the only method that has been proven to do this. However, cervical screening will only achieve these high rates of prevention if implemented within programmes that achieve high levels of population coverage together with extensive quality assurance procedures to monitor performance at all levels and promptly rectify failures when they occur. Without these elements, you essentially revert to opportunistic screening which has been demonstrated to be less effective and to promote health inequalities by over-screening the wealthy and well-educated while under-screening lower socioeconomic groups and minorities.

This has been recognised by many international, European and national institutions including:

1) The World Health Organisation's recommendation[2] from 2006 stating that cervical screening should only be offered in organised, rather than opportunistic, programmes in which screening is centrally managed, achieves high population coverage particularly among the women at highest risk and include appropriate quality control procedures.

2) The Council of the European Union which stated in it recommendation of December 2003[3] that all EU Members States should implement organised cervical cancer screening programmes.

3) The new European Guidelines for Quality Control in Cervical Cancer Screening[4] that specifically note cervical cancer screening should not be offered opportunistically and the publication of these guidelines will raise serious ethical concerns for the many European countries that still rely on opportunistic screening.

The last 20 years has seen an explosion of new technologies for cervical cancer prevention. These include the liquid-based cytology methods together with an array of new technologies that stem from the discovery that cervical cancer is caused by certain types of the Human papillomavirus (HPV) such as HPV DNA testing, HPV RNA testing, HPV vaccination and the use of a selection of other molecular markers. Many of these new technologies will have an impact on cervical cancer prevention and it is extremely important for those who are responsible for national cervical screening programmes to carefully consider benefits and drawbacks that all these technologies may be able to offer when deployed within comprehensive organised cervical cancer prevention programmes that effectively integrate the technologies that are appropriate for the country in question. Further, it is clear that the field will continue to evolve and it would therefore be advantageous to establish these prevention programmes on the basis that they are evolving processes that continuously evaluate new developments and integrate them as appropriate.

It was the recognition of these issues that led to the International Workshop on Human Papillomavirues and Consensus Recommendations for Cervical Cancer Prevention and to the publication of this Special Issue of the Collegium Antropologicum. Both are intended to bring Croatian public health officials, medical specialists and academic experts together with their counterparts from around the world for a free and open exchange of the latest scientific results on cervical cancer prevention. This information can then serve as the basis for the development of Croatian recommendations for cervical cancer prevention and the subsequent implementation of national programmes for cervical cancer prevention.

Acknowledgement

The publication of this Special Issue of the Collegium Antropologicum in a relatively short period of time would not be possible without the enthusiastic contribution of all the authors and reviewers. The Invited Editors are grateful to Ivan Sabol, Nina Milutin-Gašperov and Josip Nemet for their devoted technical assistance, editing of Croatian and English, respectively.

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Professional paper

The Burden of Cervical Cancer in South-East Europe at the Beginning of the 21st Century

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A B S T R A C T

The situation of cervical cancer prevention in South-East Europe is hardly documented, in spite of the fact that it encloses the most affected countries of Europe. We estimated the number of cases of cervical cancer, the number of deaths from this malignancy and the corresponding rates for 11 countries located in South-East Europe, in the period 2002–2004. Each year, approximately 9,000 women develop cervical cancer and about 4,600 die from the disease in this subcontinent. The most affected country is Romania with almost 3,500 cases and more than 2,000 deaths per year. High world-age standardised mortality rates (>7.5 [expressed per 100,000 women-years]) are observed in 7 countries: FYROM (7.6), Moldova (7.8), Bulgaria (8.0), Bosnia & Herzegovina (8.0), Albania (9.8), Serbia & Montenegro (10.1) and Romania (13.0). A matter of concern is the increasing mortality rate, in younger women, in the countries with the highest burden of cervical cancer. Thus, appropriate cervical cancer prevention programmes should be set up without delay in this part of Europe.

***Key words**: cervical cancer, prevention, mortality, incidence, Europe, South-East Europe*

Introduction

This paper has been written as a contribution to a special issue of Collegium Anthropologicum edited at the occasion of the International Workshop on Human Papillomaviruses and Consensus Recommendations for Cervical Cancer Prevention, Dubrovnik-Cavtat (Croatia), 18–20 April, 2007. This workshop offers an excellent opportunity to attract the attention of clinicians, epidemiologists and health authorities on a public health problem which is responsible for considerable human suffering and loss of lives but which is highly preventable if preventive activities are well organized. In the present study, we evaluate the burden of cervical cancer in 11 countries in the South-Eastern part of Europe based on the most recent available data and on estimates of the incidence and the cause-specific mortality, computed by the International Agency for Research on Cancer, for the period 2002–2004.

Material and Methods

We describe the estimated number of cases of cervical cancer, the number of deaths from cervical cancer and the corresponding crude and age-standardised rates with the world standard population as reference for 11 countries located in the South-Eastern part of Europe (Albania, Bosnia & Herzegovina, Bulgaria, Croatia, Cyprus, Greece, FYROM [Former Yugoslavian Republic of Mace-

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donia], Moldova, Romania, Serbia & Montenegro and Slovenia) for the period 2002–2004. We identified countries as defined in 2004, therefore we did not separate Serbia and Montenegro. Registered data were available from the Cancer Registry of Slovenia for the year 2003[1]. Estimates for 2004 were available for two countries (Cyprus and Greece)[2]. For the other 8 countries, estimates were derived from GLOBOCAN 2002[3].

Methods used for estimation have been described previously[3,4]. Shortly: the most recent mortality rates were derived from the published vital statistics, stored at the World Health Organisation (WHO) Mortality Database (http://www.who.int/whosis/mort) for all countries except Cyprus and Bosnia & Herzegovina. Mortality for Bosnia & Herzegovina was estimated by averaging rates from neighbouring countries. The mortality in Cyprus was estimated from national incidence and pooled European survival data. For Albania, reported mortality rates were multiplied with a correction factor to compensate for under-registration of deaths. Mortality from cancer of the cervix uteri were adjusted by reallocating deaths from uterus cancer not otherwise specified using age-specific rules. Incidence data were available from national cancer registries for Bulgaria, Croatia, Cyprus and Slovenia. The incidence for Serbia & Montenegro was computed by taking the average of the regional registries of Vojvodina and Central Serbia. For the other countries incidence was estimated from reported or estimated national mortality rates using incidence/mortality (I/M) ratios computed by Poisson regression using representative regions where both incidence and mortality data were available.

Number of cases and deaths were computed by multiplying the most recent age-specific rates with the corresponding population size for 2002 or 2004, derived from the World Population Prospects published by the United Nations Population Division (http://esa.un.org/unpp/). For Slovenia, the mid-year population of 2003, published in Cancer Incidence in Slovenia 2003 was used[1].

Results

The number of cases and deaths and the corresponding rates by country are shown in Table 1. The standardised rates, ranked by increasing mortality are displayed in a bar graph (Figure 1). The geographical distribution of the standardised mortality is mapped in Figure 2. In total, each year, approximately 9,000 women in South-Eastern Europe developed cervical cancer and about 4,600 died from the disease. A low to moderately high age-standardised mortality was observed in the North-West and the South-East margin of the region: Slovenia ($4.1/10^5$), Croatia ($5.0/10^5$), Greece ($2.1/10^5$) and Cyprus ($5.6/10^5$). However, in the core of the region, high to very high mortality rates were noted varying between $7.8/10^5$ (Moldova) and $13.0/10^5$ (Romania). The standardised incidence rate varied from $7.2/10^5$ in Greece to more than $20.0/10^5$ in Albania, Romania and Serbia & Montenegro. In general the crude incidence and mortality correlated well ($r=0.88$). The parameter 1-M/I, a surrogate index for survival, varied between 39% in Albania and 66% in Serbia & Montenegro. However, the correlation coefficient and the survival surrogate index are rather artificial indicators since incidence often was derived from mortality.

Discussion

South-East Europe is a region of major contrasts. It contains the 2 countries with the highest burden for the

TABLE 1
INCIDENCE OF AND MORTALITY FROM CERVICAL CANCER IN 11 SOUTH-EAST EUROPEAN COUNTRIES: NUMBER OF CASES AND DEATHS (IN MULTIPLES OF HUNDRED), CRUDE AND WORLD-AGE STANDARDISED RATE (W-ASR) (PER 100,000 WOMEN-YEARS [WY]

Country	Incidence			Mortality			Year	Source
	Cases (x 100)	Crude Rate (/10^5 WY)	W-ASR (/10^5 WY)	Deaths (x 100)	Crude Rate (/10^5 WY)	W-ASR (/10^5 WY)		
Albania	3.9	25.1	25.2	1.5	9.4	9.8	2002	2
Bosnia & Herzegovina	5.5	26.6	21.3	2.3	11.1	8.0	2002	2
Bulgaria	9.8	24.4	18.7	5.1	12.6	8.0	2002	2
Croatia	4.3	18.0	13.3	2.1	8.7	5.0	2002	2
faautoCyprus	0.5	13.2	12.5	0.3	6.1	5.6	2004	1
Greece	4.8	8.9	7.2	2.1	3.9	2.1	2004	1
FYROM	1.7	16.4	13.9	1.0	9.7	7.6	2002	2
Moldova	4.8	21.4	18.0	2.2	9.9	7.8	2002	2
Romania	34.5	30.3	23.9	20.9	18.4	13.0	2002	2
Serbia & Montenegro	18.2	34.4	27.3	8.2	15.5	10.1	2002	2
Slovenia	2.1	20.4	18.5	0.8	7.9	4.1	2003	3

The estimates are derived from a recent study of the burden of cervical cancer in member states of the European Economic Area for 2004[2], the Cancer Registry of Slovenia for 2003[1] and from GLOBOCAN 2002[3] for the other countries, figures are adjusted for mortality from not otherwise specified uterine cancer

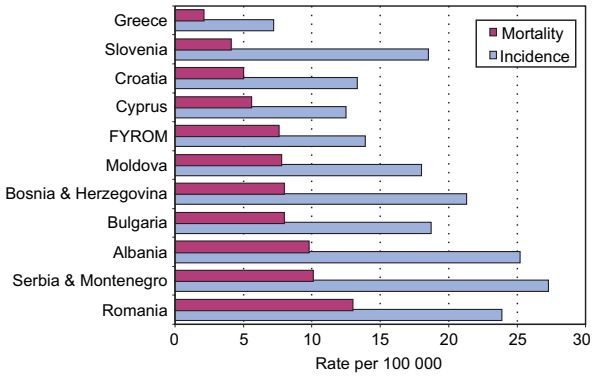

Fig. 1. Burden of cervical cancer: world age-standardised incidence and mortality in 11 countries of South-East Europe, estimates for 2002/2004 (Source: Globocan 2002[3], Cancer Registry of Slovenia 2003[1], Arbyn 2007[2]).

whole of Europe: Romania and Serbia & Montenegro. It contains, together with the Baltic countries, in Northern Europe, the 7 most affected states of the European continent with standardised rates of mortality from cervical cancer reaching 8/100,000 or higher (Romania, Serbia & Montenegro, Lithuania, Albania, Bosnia & Herzegovina, Bulgaria, Latvia)[2,5]. Otherwise, South-East Europe contains countries where the burden is low, for instance in Greece, where the standardised incidence is 3 to 4 times and the mortality 5 to 6 times lower than in Serbia & Montenegro or Romania.

The current cervical cancer incidence and mortality reflects exposure of successive generations to the main risk factor (infection of the cervix with oncogenic human papillomavirus [HPV] types) and the impact of cytological screening for HPV induced cervical lesions and treatment of those lesions. A generally observed phenomenon in industrialised countries is that women born after 1940 are at higher risk compared to older cohorts due to changed sexual behaviour (and hence increased HPV transmission) since the 1960s[6–8]. Increased frequency of smoking and use of oral anti-conception might have enhanced this cohort effect[6]. In the Nordic and some West- European countries, incidence of and mortality from cervical cancer dropped substantially subsequent to cytological screening[7,9–11]. Little information is available on the screening situation in South-East Europe but it is expected that, in general, the coverage and quality are moderate to poor. Only in Slovenia, a formally organised cervical cancer screening programme is in place since 2003[12]. The current particularly high burden of cervical cancer in Romania and other South-East European countries can be explained most plausibly as an effect of elevated HPV transmission, over the past decades, not counteracted by screening. On the other hand the low incidence in Greece might be due to the rather low background risk, also observed in other Mediterranean countries such as Spain and Italy[13]. It is expected that ongoing studies assessing the prevalence and the geographical distribution of HPV types throughout Europe, conducted in the framework of the introduction of HPV vaccination, will clarify this hypothesis.

The data estimated in this study should be considered with caution, since their reliability is determined by the quality and completeness of cancer and death registration and further by the appropriateness of external data used to model unavailable data. In particular, the propor-

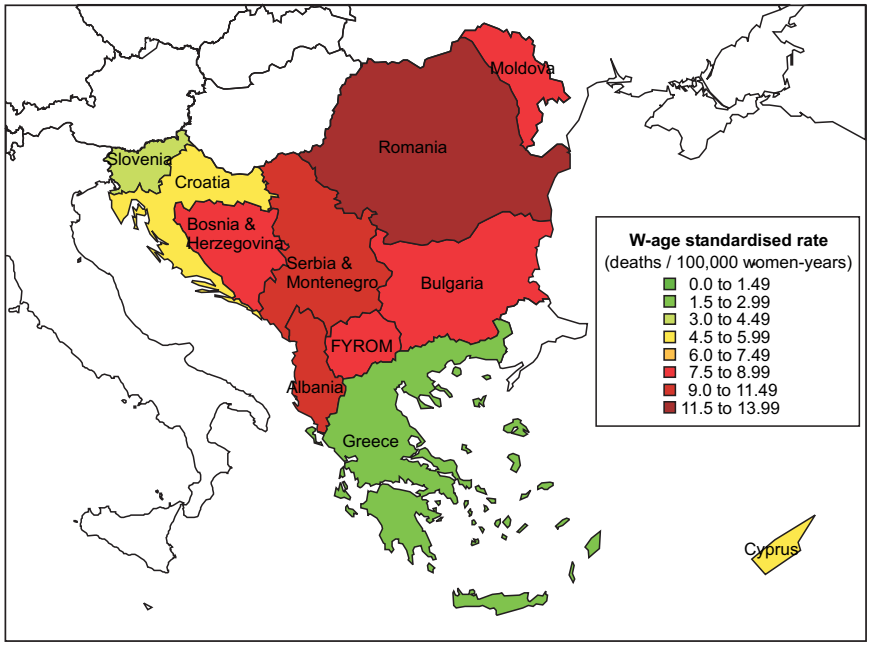

Fig. 2. Geographical distribution of the world age-standardised mortality (W-ASMR) from cervical cancer in 11 countries of South-East Europe, estimates for 2002/2004 (Source: Globocan 2002[3], Cancer Registry of Slovenia 2003[1], Arbyn 2007[2]).

tion of deaths from uterine cancer without specification of the exact topographic origin compromises the accuracy of the cause of death certification. We are currently conducting detailed trend studies, within the framework of the EU Network for Information on Cancer, where we look for the best possible solutions for the death cause certification problem and try to disentangle the dynamics of cervical cancer in all European countries. Preliminary results indicate that the mortality from uterus cancer, in age groups younger than 45 years – where nearly all deaths are caused by cervix uteri cancer, is falling in Greece, Croatia and Slovenia but continues to rise or remains stable at a high level in Bulgaria, Moldova, Romania and Serbia & Montenegro.

Conclusion

We have highlighted the elevated burden of cervical cancer in some South-East European countries – in particular in Romania. The present study should motivate public health authorities to set-up well-organised cervical cancer prevention programmes without delay as recommended by the European Council[14]. It is hoped that the pending publication of the new European Guidelines for Quality Assurance in Cervical Cancer Screening will contribute in establishing this goal[15]. It is particularly challenging for public health experts to define, in the future, how prophylactic HPV vaccination besides screening will contribute in tackling this highly preventable disease.

Acknowledgements

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BREME RAKA VRATA MATERNICE U JUGOISTOČNOJ EUROPI NA POČETKU 21. STOLJEĆA

SAŽETAK

Stanje prevencije raka vrata maternice u jugoistočnoj Europi je slabo zabilježeno, unatoč činjenici da ovaj rak pogađa najviše te države u Europi. Procijenili smo broj slučajeva raka vrata maternice, smrtnost od ove bolesti te odgovarajuće odnose između njih za 11 zemalja jugoistočne Europe, u razdoblju od 2002. do 2004. g. Svake godine na ovom potkontinentu oboli od raka vrata maternice približno 9.000 žena, a oko 4.600 ih umre od ove bolesti. Najviše slučajeva bilježi Rumunjska, gotovo 3.500 te više od 2.000 smrtnih slučajeva godišnje. Visoka godišnja stopa smrtnosti u svijetu (>7,5 [brojevi se odnose na 100,000 žena godišnje]) je zabilježena u 7 zemalja: Bivša Jugoslavenska Republika Makedonija (7,6), Moldavija (7,8), Bugarska (8,0), Bosna i Hercegovina (8,0), Albanija (9,8), Srbija i Crna gora (10,1) te Rumunjska (13,0). Pitanje od važnosti je porast stope smrtnosti kod mlađih žena u zemljama s visokom stopom obola od raka vrata maternice. Stoga bi, u ovom dijelu Europe, bez odgode trebali biti uspostavljeni odgovarajući programi prevencije raka vrata maternice.

Coll. Antropol. **31** (2007) Suppl. 2: 11–16
Review

The Future of Cervical Cancer Prevention in Europe

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A B S T R A C T

Cervical cancer remains a significant source of disease and death in Europe. However, we now have the means to prevent virtually every case of cervical cancer through comprehensive, population-based, organised cervical cancer prevention programmes that effectively integrate cervical screening with the new technologies and vaccines that are now available. Given the potential health benefits of these programmes in reducing disease incidence and mortality, their establishment is now an ethical imperative for all European countries.

Key words: *Cervical cancer, screening, vaccination, prevention*

Introduction

Cervical cancer is the second most common cancer in women worldwide and is the most common female cancer in the Caribbean, East / Central / Southern Africa and in South-Central Asia. Globally, an estimated 471,000 women develop cervical cancer and 233,000 die from it every year (estimate for 2000)[1]. The disease primarily affects younger women with the majority of cases appearing between the ages of 30 and 50[2], an age when many are actively involved in their careers, caring for their families or both and the impact on society as a whole is therefore greatly increased.

Cervical cancer still remains an important public health issue in Europe where it is the 7th most common cause of cancer deaths in women[1]. Each year in Western Europe, 13,000 women develop cervical cancer and 6,000 women die from this disease, while the situation in Eastern Europe is much worse with approximately 31,000 women developing cervical cancer and about 17,000 dieing every year[3]. This difference is largely due to the absence of effective cervical screening in Eastern Europe and the implementation of properly organised prevention programmes would inevitably decrease the burden of this disease in these countries.

Cervical Cancer Screening

Squamous cervical cancer is particularly amenable to screening as it has a long pre-clinical phase and identifiable precursor lesions that, if detected early, can be treated with high efficacy using simple outpatient procedures. Indeed, effective organised cervical cancer screening programmes have been proven to reduce cervical cancer incidence and mortality by more than 80%[4]. However, recent studies indicate that screening has limitations. Data available from organised screening programmes show that the initial declines in disease incidence seen following the establishment of screening have now levelled-off, indicating that the maximum effect of Pap smear-based screening has been reached in these countries[4]. Further, a meta-analysis of studies unaffected by verification bias has shown that the pooled sensitivity of the Pap smear was 77% (95% CI: 58% to 97%) when using low-grade squamous intra-epithelial lesions (LSIL) as the threshold to detect histologically confirmed cervical intraepithelial neoplasia of grade 2 or worse (CIN2+)[5].

Given these data, an enormous amount of research effort has gone into the evaluation of new technologies such as liquid-based cytology and HPV testing, together with HPV vaccination that offer the potential to make

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further progress in the battle against this disease. However, it must be remembered that achieving reductions in the incidence and mortality of cervical cancer is entirely dependent upon the effective operation of the entire programme including primary prevention strategies such as vaccination, screening, diagnosis and treatement of pre-invasive or invasive disease. For these reasons, new techologies with the potential to be deployed must also be studied within the programme so the overall effect, including all the benefits and drawbacks, can be properly evaluated. Further, they should be evaluated within randomised controlled trials in order to obtain un-biased etimates of their effects.

The Human Papillomavirus and Cervical Cancer

There is now an overwhelming body of evidence demonstrating that persistent infection with certain types of the Human papillomavirus (HPV) is the primary risk factor for the development of cervical cancer and its precursor lesions[6,7]. More than 100 different HPV types have been identified with approximately 40 of these infecting the anogenital epithelium that have been classified as either low-risk (LR) or high-risk (HR) for the development of cervical cancer based upon their identification in cervical tumour samples[8]. A recent analysis of 11 studies has designated 15 anogenital HPV types as HR (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), with a further 3 types designated as probably HR (26, 53 and 66)[9] although some of these designations have been disputed by others[10].

This body of evidence is strong, consistent across different populations, and conclusively demonstrates that HPV is a necessary (although not a sufficient) cause of cervical cancer[7,11]. On the basis of these data, it is logical to conclude that HPV testing could be a useful cervical cancer screening tool[12–18] and that vaccination against HPV could be used for primary prevention of cervical cancer.

HPV Testing for Primary Cervical Cancer Screening

For primary screening, a number of research studies (Table 1) have demonstrated that, compared to the Pap smear, HPV testing has a higher sensitivity and higher negative predictive value (NPV) for the detection of prevalent cervical cancer precursors, albeit with a lower specificity and lower positive predictive value (PPV)[19–24].

However, it is important to note, these studies used a cross-sectional design with double testing (cytology + HPV testing) of all women and short-term follow-up by colposcopy with biopsy for those having one or more positive screening tests. While this is appropriate to assess the relative sensitivity of each test to detect prevalent high-grade cervical intra-epithelial lesions (CIN2+), the design is subject to verification bias and it does not account for the long duration of pre-cancerous stages and the consequent increase in sensitivity that accompanies repeated Pap smear testing (i.e. programme sensitivity). Further, the high regressive potential of CIN2+ lesions means that its increased detection may be accompanied by the diagnosis and treatement of non-progressive lesions and the cross-sectional results therfore cannot be used to study the impact of different screening strategies on the incidence of invasive cancer[25,26]. As such, most jurisdictions have regarded these studies as insufficient to merit the introduction of HPV testing for primary screening, while an international expert group convened by the International Agency for Research on Cancer (IARC) has concluded that HPV testing has at least the same cross-sectional sensitivity as the Pap smear and that it could be used within organised screening programmes, either alone or in combination with the Pap smear, once rigorous evaluation of effectiveness and efficacy have been completed[4]. Such studies are underway in Europe where a number of researchers have established large-scale randomised controlled trials (RCTs) of HPV testing for primary screening which follow-up women for at least one screening round, and the Finnish Cancer Registry has initiated a randomised public health implementation evaluation of HPV screening[27–32] (Table 2).

Importantly, some of these RCTs have moved to the evaluation of HPV testing as a single primary screening test followed by cytology for the triage of women having a positive test on the basis that:

- screening would be undertaken with the test having higher sensitivity and triage undertaken with the test having higher specificity, in compliance with accepted principles of screening, and as is already the case with syphilis and HIV screening,
- it would maximise specificity and PPV while achieving 95–100% of the sensitivity and NPV of the combined HPV/cytology primary test. This would provide the same level of safety but with improved cost-effectiveness by minimising the number of women with false positive results that need to be followed-up,
- it would allow 85–90% of women to return immediately to routine recall without incurring the cost of cytology, which would be reserved only for the triage of the remaining 10–15% of women with a positive HPV test,
- the high-volume testing of screening samples would be undertaken with a non-subjective test that can be automated, while the subjective, labour-intensive test would be restricted to high-risk samples only, so they could be screened more intensively because of the reduced number that need to be processed.

The interim results of these trials confirm the earlier studies with HPV testing (either alone or in combination with cytology) having a higher sensitivity and lower specificity than cytology alone for the detection of CIN2+. However, those studies reporting the performance of HPV testing as a single primary screening test with cytology triage indicate that this HPV screening algorithm

TABLE 1
SENSITIVITY AND SPECIFICITY OF HPV TESTING COMPARED TO CERVICAL CYTOLOGY FOR THE DETECTION OF CIN2+ (USING ≥ASC-US AS THE REFERRAL THRESHOLD)

Study	Description	HPV		Pap Smear (≥ASC-US)		HPV/Pap Smear (≥ASC-US)	
		Sen	Spec	Sen	Spec	Sen	Spec
Cuzick et al.[19]	United Kingdom: n = 1,703 Conventional Pap Smear, HC2, ♀ ≥ 35 years	95	95	79	99	NA	NA
Schiffman et al.[20]	Costa Rica: n = 1,119 Conventional Pap Smear, HC2, ♀ ≥ 18 years	88	89	78	94	NA	NA
Ratnam et al.[21]	Canada: n = 2,098, 69% HC1/31% HC2, ♀ 18–69 years (adjusted for verification bias)	85* (68)	58 (91)	56 (40)	62 (92)	97* (76)	39 (86)
Clavel et al.[22]	France: n = 5,651 LBC, HC2, ♀ ≥ 15 years	100	86	88	93	NA	NA
Petry et al.[23]	Germany: n = 8,468 Conventional Pap Smear, HC2, ♀ ≥ 30 years	98	96	44	98	100	94
Cuzick et al.[24]	United Kingdom: n = 10,358 Conventional Pap Smear, HC2, ♀ ≥ 30 years	97	93	77	96	–	–
	HC2 using an elevated threshold (≥ 2pg) for a positive result	96	94	–	–	100	94

ASC-US — Atypical Squamous Cells – Undetermined Significance, CIN – Cervical Intraepithelial Neoplasia, Sen — Sensitivity, Spec — Specificity; HC2 — Hybrid Capture 2 HPV test and HC1 — Hybrid Capture 1 HPV test (Digene Inc. Gaithersburg, MA), LBC — Liquid Based Cytology, * Sensitivity of HC1 was suboptimal and would have contributed to the difference seen between HPV testing alone and the combination of HPV testing with Pap

TABLE 2
THE EUROPEAN RANDOMISED CONTROLLED TRIALS

Study	Country	Total Recruitment	Age Range (years)	HPV Test	Cytology	Main Study Outcomes
Finnish Randomised Public Health Trial[27]	Finland	200,000	25–65	HC2	Conventional Pap smear	Cumulative incidence of CIN2, CIN3 and cancer after initial screening
Swedescreen[36]	Sweden	12,527	32–38	PCR (GP5+/6+ primers)	Conventional Pap smear	Comparative prevalence of histologically confirmed CIN2+ at the exit screen
POBASCAM[29,30]	The Netherlands	44,102	30–60	PCR (GP5+/6+ primers)	Conventional Pap smear	Proportion of histologically confirmed CIN3+ found at any time during the trial from recruitment to exit screen
ARTISTIC[31]	United Kingdom	25,000	20–64	HC2	LBC	Comparative prevalence of histologically confirmed CIN3+ at the exit screen
NTCC[32]	Italy	95,000	25–60	HC2	LBC or conventional Pap smear	Comparative detection of histologically confirmed CIN2+ from the recruitment screen up to and including the exit screen

CIN – Cervical Intraepithelial Neoplasia, HC2 — Hybrid Capture 2 HPV test, PCR – Polymerose Chain Reaction, LBC – Liquid Based Cytology

also has a higher sensitivity than cytology alone but now with a specificity that is at least equivalent to cytology. In the Finnish trial, HPV testing with cytology triage detected 1.45 times as much CIN2+ compared to cytology alone, while the specificities were not significantly different at 98.9% (95% CI: 98.6–99.2) and 99.3 (95% CI: 99.0–99.5), respectively[27]. Similar results were reported by Bulkmans et. al in the preliminary prospective results from the POBASCAM trial in which indicate that HPV testing followed by cytology triage compared to cytology alone can be more sensitive (92.9 vs 64.3% respectively; p=0.065) and more specific (96.8 vs 95.1% respectively; p= 0.05)[29]. While the differences in sensitivity, NPV (99.96 vs 99.78% respectively; p=0.098) and PPV (14.6

vs 7.3% respectively; p=0.085) are not significant, it must be remembered that these are only the preliminary results on 2,810 women from a total of over 44,000 women that were recruited to the trial.

Taken together, these results indicate that HPV testing, if used as a primary screening test followed by cytology for the triage of women testing HPV positive, has the potential to provide improved sensitivity for the detection of clinically relevant disease without decreasing specificity or otherwise adversely affecting the efficacy of screening programmes.

Vaccination Against HPV for Primary Prevention of Cervical Cancer

A number of studies have now been conducted on the two first generation prophylactic HPV vaccines[33–38]. Both vaccines target HR-HPV types 16 & 18 (which are together responsible for over 70% of cervical cancers worldwide), while one (GARDASIL®, Merck and Co.) also includes types 6 and 11 which are non-oncogenic but still responsible for a substantial proportion of lower-grade cervical disease and the other (CERVARIX™, GlaxoSmithKline) has demonstrated to provide a degree of cross-reaction to HPV types 31 & 45 (which are the next most common oncogenic HPV types after 16 & 18)[38]. The result of the phase IIb and III trials have shown these vaccines to be safe, well tolerated and highly immunogenic. Further, both vaccines have been shown to offer HPV naive women high levels of protection against HPV infection with the HPV types contained in the vaccine as well as their resulting cervical lesions[33–38]. However, the GARDASIL licensing submissions filed with both the US Food and Drug Administration and the European Medicines Agency included data to show there was no clear evidence of protection from disease caused by HPV types which subjects were DNA positive and/or seropositive for at the time of vaccination[39,40]. Further data were presented to show that in the general public where a proportion of women will have prior or current HPV infections, GARDASIL can be expected to reduce the overall rate of CIN2/3 or adinocarcinoma *in situ* caused by vaccine or non-vaccine types by 12.2%[39].

The results of these trials have now led to the licensure of one of the vaccines in many countries with the second expected to follow shortly and it is anticipated that these vaccines will play a very important role in the prevention of cervical cancer going forward. However, the availability of these vaccines raises several implementation issues that must be addressed if the vaccines are to achieve their full potential in Europe where cervical cancer screening is already widespread and substantial efforts are underway to ensure the uniform implementation of properly organised cervical cancer screening programmes. In Europe, the enormous potential of HPV vaccination must be considered together with its limitations which are chiefly 1) its high cost (currently around €450 per person in France), 2) that the first-generation vaccines do not protect against all HR HPV types, and 3) inability to protect women who are already infected with HPV types 16 and 18. In addition, an important question remains about whether HPV vaccination will provide any supplemental protection against the development of cervical cancer in women who have been exposed to HPV 16 or 18 but subsequently mounted their own effective immune response and cleared the virus. This is a particularly important question for public health programmes where the cost for the vaccine must be taken from finite healthcare budgets and which will therefore lead to a diminution in other services. Clearly, there is an ethical concern about the implementation of vaccination programmes for women who may derive little or no additional benefit over that provided by their own immune system when the money must come from other programmes with clear and proven health benefits.

Under these conditions, there is little dispute that the vaccination of girls before the commencement of sexual activities is not only a public health priority but an ethical imperative. However, the use of vaccination in older girls and women will yield diminishing public health returns as the proportion of women exposed to HPV 16 & 18 increases, and this needs to be balanced against the clearly established protective effect of cervical screening in these populations. Unfortunately, it is not possible to have a single formula and each country must undertake its own cost-benefit analysis to establish the appropriate balance depending on their national priorities, healthcare budgets and the status of existing resources such as screening programmes. But what is clear that all European countries now need to implement comprehensive cervical cancer prevention programmes which integrate cervical screening together with HPV vaccination as it is the combination of these that will offer the most effective long-term protection against cervical cancer. Further, these new measures of cervical cancer prevention must be offered within population-based organised prevention programmes to ensure that the protection is equitably available to all women in the population.

The Effective Integration of Technologies for the Prevention of Cervical Cancer

Although the Pap smear has been the mainstay of cervical cancer prevention for more than 50 years, it now must be recognised that the ongoing uptake of HPV vaccination against HPV 16 & 18 will have a progressively detrimental effect on cytology based screening. Evaluations of the prevalence of HPV 16 & 18 among women with abnormal cytology indicate that ASC-US, LSIL and HSIL rates could be reduced by as much as 30%, 36% and 55% respectively in a population that was fully vaccinated with a vaccine having 100% efficacy[41–43]. If HPV 6 and 11 are also included, ASC-US, LSIL and HSIL could eventually be reduced by a total of 40%, 46% and 60% respectively[41–43]. Here, it is important to note that the reductions are likely to be greater for HSIL than for ASCUS or LSIL. Therefore, vaccination will both reduce the overall prevalence of cytological abnormalities and

shift the balance to the lower grades which have a lower PPV. These characteristics mean that vaccination will inevitably lead to a substantial deterioration in the efficacy of cytology-based screening because:

1) A decrease in disease prevalence will produce a direct and simultaneous decrease in the PPV of screening. Even with current disease rates in adequately screened populations, the PPV of cervical cytology ranges from only 10 to 30% for the detection of CIN2+ in well screened populations[19–24] a situation that is only tolerated because of the seriousness of the disease that cervical screening prevents. However, reductions in disease rates subsequent to widespread vaccination, together with the shift to the lower-grades of cytological abnormalities, will further reduce the PPV of cytology-based screening programmes, eventually to a point where the stress, morbidity and costs involved in the follow-up of these false-positive women may no longer be either ethically or financially justifiable relative to the yield of true disease.

2) Reductions in disease rates would also mean that cytology screeners would have less exposure to cytological abnormalities during the course of their working day with two possible outcomes. First, regular exposure to cytological abnormalities is necessary for the maintenance of cytology screening skills. Therefore, reductions in disease rates could be accompanied by a simultaneous reduction in the cytology screeners' ability to recognise these abnormalities and a reduction in the sensitivity of the screening program. Second, a reduction in the number of true abnormalities could lead cytology screeners to compensate by over-classifying inflammatory changes or reactive atypias leading to further decreases in specificity and PPV of the screening programme.

Although these changes will only be seen with the progressive implementation of HPV vaccination and the gradual expansion of the vaccinated cohort, it is nonetheless essential for screening programmes to make plans for the changes that will be required to maintain proper levels of protection against cervical cancer. One way this could be achieved is by stratifying the population to be screened according to their risk and then applying cytology based screening only to the subpopulation of women that is at increased risk of having clinically relevant cervical disease, i.e. those women who are HPV positive. On this basis, the uptake of HPV vaccination will necessitate a shift to HPV testing for primary screening together with cytology for the triage of women with a positive result in order to maintain the efficacy of screening in an environment with a reduced prevalence of cervical disease.

Conclusions

In Europe, cervical cancer remains a significant source of disease and death although we now have the means to prevent virtually every case through comprehensive cervical cancer prevention programmes. However, if we are to achieve this goal, it is essential that these prevention programmes effectively integrate cervical screening together with the new technologies and vaccines to ensure that the optimal protection is afforded to all age groups. In addition, they must be population-based to ensure that the protection is equitably available to all women and they are run in the most cost-effective fashion. The science has been done and the tools are now available to effectively prevent cervical cancer in Europe. What we now need is the political will to make this a reality.

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BUDUĆNOST PREVENCIJE RAKA VRATA MATERNICE U EUROPI

S A Ž E T A K

Rak vrata maternice je i dalje značajan izvor bolesti i smrti u Europi. Međutim, danas postoji način prevencije gotovo svakog slučaja raka vrata maternice kroz opsežne organizirane programe prevencije, temeljene na populaciji, koji učinkovito spajaju probir za rak vrata maternice s novim metodama koje su danas dostupne. Pridajući zasluge u smislu mogućih zdravstvenih dobrobiti ovom programu u smanjenju stope pojavnosti i smrtnosti od ove bolesti, njegovo uspostavljanje je danas etički imperativ za sve europske zemlje.

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Professional paper

Cervical Cancer Screening Programme in Finland with an Example on Implementing Alternative Screening Methods

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A B S T R A C T

In Finland (population 5 million) the organised Pap screening programme for preventing cervical cancer has been in action already for 45 years. Women aged 30 to 64 are targeted (N 1.25 million) and the screening interval is five years. The programme invites women seven times in a lifetime; the attendance rate per one screening invitational round is 73%. The programme has affected markedly the cervical cancer rates in our country. During the decennia of its action there has been about 80% decrease in the age-adjusted cervical cancer incidence and mortality rates. The current age-standardised incidence rate is 4 and mortality rate 1 per 100,000 woman-years. In the current article we describe the organisational aspects of the programme; and pay attention to renovation of the programme taken place during the last decade when novel technological alternatives have been started to be used as the screening tests. By expanding the coverage and compliance of screening we still expect to increase the impact of the programme. Same time, efforts are needed to avoid overuse of services due to spontaneous screening, in order to decrease potential adverse effects and improve overall cost-effectiveness. A large-scale public health policy trial on Human papillomavirus (HPV) screening is on-going. Cross-sectional information available thus far suggests promising results. Follow-up of cancer rates after screening episodes are still required to evaluate optimal screening policies (e.g., screening intervals by age groups, and starting and stopping ages). We propose speeding up the use of modern technological alternatives in organised screening programmes.

***Key words**: Cervical cancer, incidence, mortality, organised screening, effectiveness, cytological screening, HPV-DNA screening*

Introduction

In Finland (population 5 million) organised cervical screening was introduced in the early 1960s; piloting first within the area of three municipalities in 1963 and extending within a few years time to most parts of the country. From the early 1970s onwards, the registered screening invitational coverage has been almost complete within the centrally targeted screening ages.

During 1955–1964 the incidence of invasive cervical cancer in Finland was at a level of 15 cases per 100,000 woman-years; age-adjusted to the world standard population, with a slight increasing trend within the period. Mortality from cervical cancer was around 7 cases per 100,000 woman-years, respectively. Subsequent to implementation of organised screening, there was a rapid decrease in the invasive cervical cancer incidence and mortality rates. Currently the age-adjusted rates are 4 and 1 per 100,000 woman-years (Figure 1). Most of the reduction has occurred in the incidence of squamous cell carcinomas, whereas the incidence rate of cervical adenocarcinoma has been quite stable over the decades[1,2].

Effectiveness of Conventional Screening

Screening effectiveness in Finland was demonstrated first by a very large-scale cohort follow-up study among women invited in the implementation phase of the programme[3,4]. The study followed subsequent cervical cancer rates, based on about 425,000 invitations in 1963–

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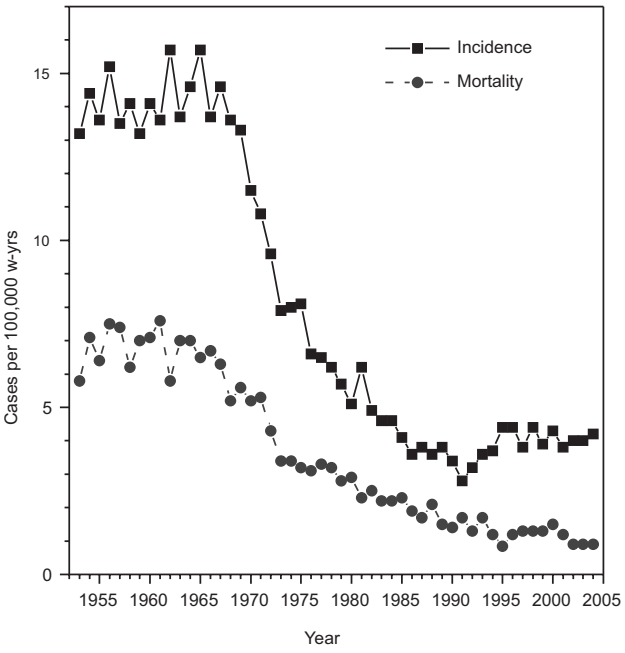

Fig. 1. Cervical cancer incidence and mortality rates in Finland in 1953–2004, adjusted for age to the World standard population (Finnish Cancer Registry).

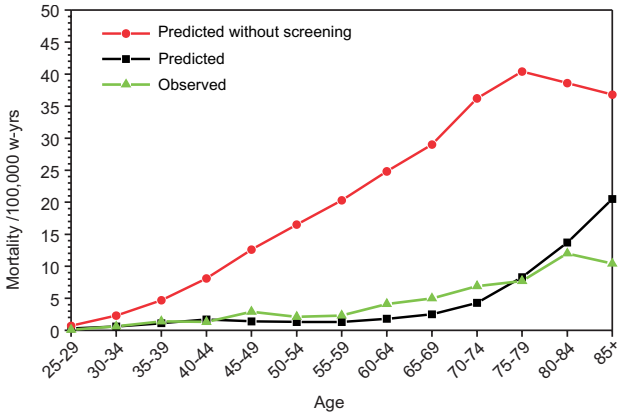

Fig. 2. Observed (Finnish Cancer Registry, 2007) and predicted (from Hristova & Hakama, 1997[6]) mortality rates from cervical cancer in Finland 1998–2002, by age.

1972. Most part of the women had been screened once during the study period. The efficacy estimate among screened was 80%. The attendance rate was 85% and the effectiveness of the programme was estimated at 60%. Among invited but not attended there was about 60% increase in the subsequent cervical cancers in comparison with the rates in the population before screening. It is likely that thereafter the effectiveness of the programme has increased, as more testing rounds have become available. However, there are mainly a number of ecological-level trend studies performed in the later years in our country (see IARC, 2005[4], for a recent synthesis on these). According to a cohort follow-up study among a

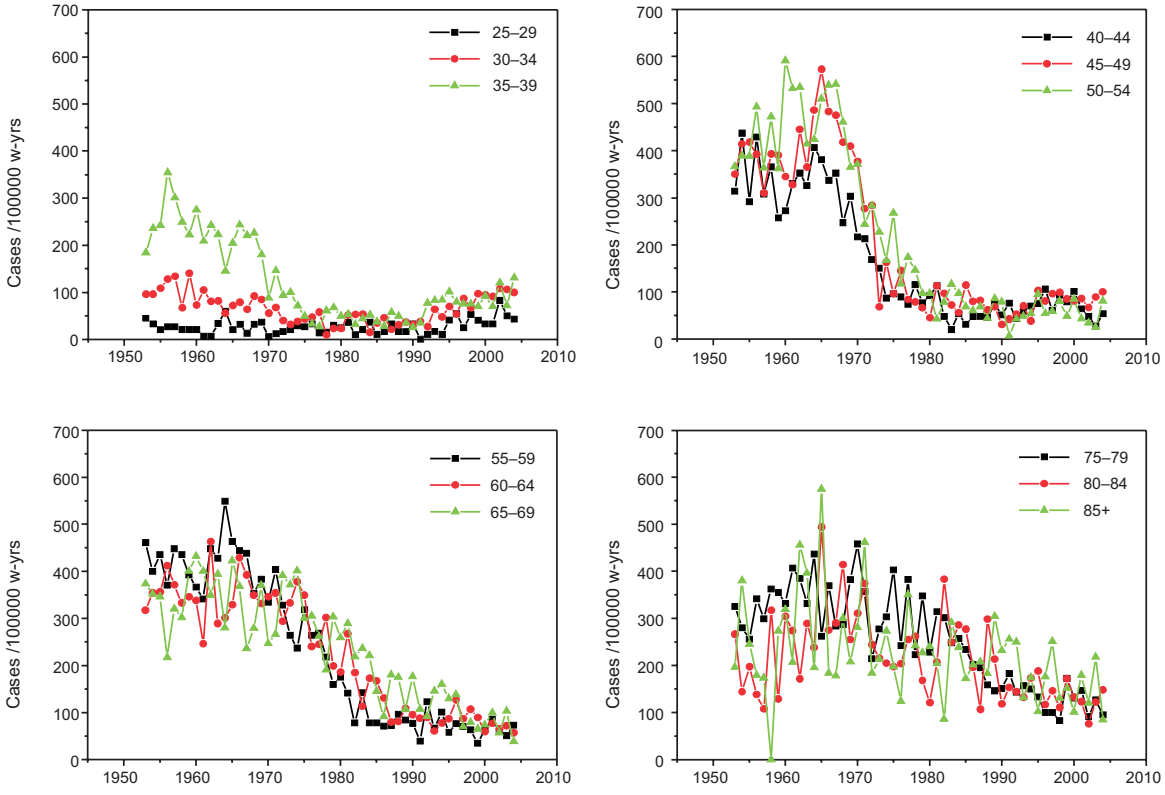

Fig. 3. Age-specific incidence rates of cervical cancer in Finland, 1953–2004 (Finnish Cancer Registry).

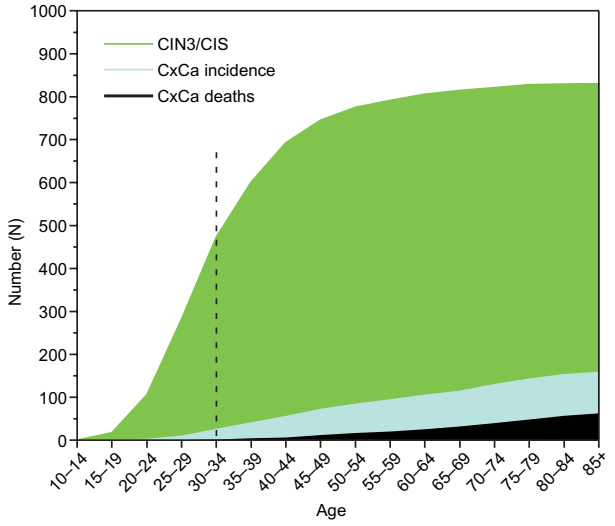

Fig. 4. Cumulative number of cases of severe dysplasia or carcinoma in situ (CIN3/CIS), cervical cancer incidence,0 and deaths from cervical cancer in Finland, annual averages in 1998 over age (Finnish Cancer Registry, 2007).

sample of women with a negative cytological result in the programme, the absolute rate of cervical cancer after screening negative is about 5 cases per 100,000 woman-years[5].

It is estimated based on the trends that each year almost 300 deaths are prevented due to Pap-screening; these deaths would have occurred at a broad set of ages from rather young (30 or 35 years) up to very old (more than 85 years) women (Figure 2)[6]. In the oldest age groups the incidence and mortality rates from cervical cancer are still expected to decrease due to ageing of female population screened previously in their life. There are still some 60 deaths per year, and the number of incidence cases is 160.

The incidence of cervical cancer has somewhat increased during the last decade in ages 25–39 (Figure 3) even though the number of cases in this age group is still small, below 30 cases annually (Figure 4). There are almost no deaths from this cancer type in ages below 50 years and the death rate among young women has not increased.

Growing Concerns on Opportunistic Screening

In the early days, opportunistic screening was not available in large-scale. Later on, use of such services has increased. There are no data to study the trends in the use of opportunistic screening. According to information from an annual population survey as available from late 1990s[7], any Pap smears (the estimate including also the opportunistic and diagnostic smears in addition to the programme smears) have been taken during a five-year period roughly for 93% of the women at target ages of the programme. The estimated coverage of any smears lifetime is 98%, respectively[8,9]. Trend studies[4] and a population-based case-control study using questionnaire-based self-reported information on the screening history (ever vs. never screened)[8] have suggested that the overall effect in decreasing cervical cancer incidence with spontaneous smears is up to 40%. In the latter study the effect among women participating in organised screening was about two-fold compared to those who had never participated (but given only spontaneous smears); spontaneous screening showed no additional impact among those subjected to organised screening.

One problem in the national screening policy is that there are apparently wide testing practices outside screening, also in rather young women – where almost all of the pre-cancerous lesions would regress naturally (see IARC, 2005[4] for estimates of regression probabilities). Figure 4 illustrates the numbers of CIN3/CIS incidence and treatments as available from cancer registry files: about 60% of these are diagnosed already in age before screening. More evaluation research, including also research on the potential adverse effects, on any screening is needed. There are yet no register-based data available on the spontaneous screening; such data is required in order to evaluate that activity and reduce the unnecessary actions. Recently, efforts have been started to include any smears in register-based evaluations.

Organisation of Cervical Cancer Screening

The screening activities have become an integral part of the health care system. Women in ages 30 to 60 years are invited with help of population registry, using a five-year interval when normal screening results. The two older age groups (55, 60) were added to the programme only in 1990s. There are nowadays thus seven invitations lifetime. Some municipalities invite also women in ages 25 and/or 65; women at a younger age than

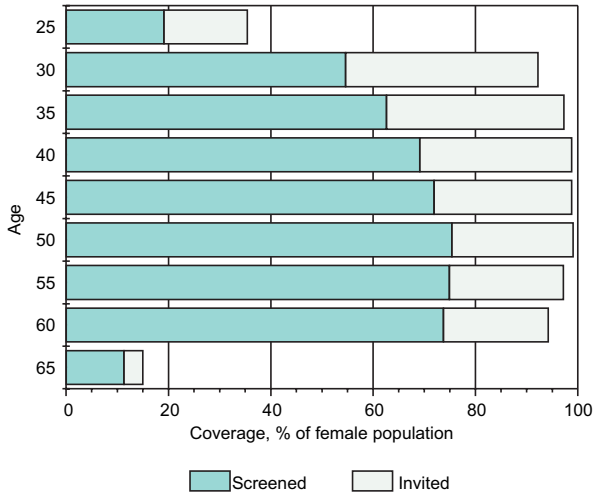

Fig. 5. Invitational and screening coverage within the Finnish cervical cancer screening programme in 2004 (Finnish Cancer Registry).

TABLE 1
CERVICAL CANCER SCREENING PROGRAMME IN FINLAND IN 2004: THE NUMBER AND PERCENTAGE DISTRIBUTION OF SCREENING TESTS IN THE ORGANISED SCREENING PROGRAMME, BY SCREENING TEST RESULTS, REFERRALS AND FINAL HISTOLOGICAL DIAGNOSIS (MASS SCREENING REGISTRY, 2007, PRELIMINARY INFORMATION BASED ON DATA FROM 437 MUNICIPALITIES OUT OF 444 IN THE WHOLE COUNTRY)

Screening test	Screened	Referred		Histological diagnosis									
				Cervix cancer		CIN3/CIS		CIN2		CIN1		Other or normal	
	N	N	%	N	%	N	%	N	%	N	%	N	%
Pap-test	170205	1290	0.8	18	0.01	178	0.1	192	0.1	175	0.1	727	0.4
I	158894	–	–	–	–	–	–	–	–	–	–	–	–
II	10022	132	0.1	1	0.0	4	0.0	6	0.0	10	0.01	111	0.07
III–V	1161	1158	0.7	17	0.01	174	0.1	186	0.1	165	0.1	616	0.4
Inadequate	128	–	–	–	–	–	–	–	–	–	–	–	–
HPV-test	14585	186	1.3	4	0.03	14	0.1	48	0.3	32	0.2	88	0.6
Negative	13433	7	0.05	–	–	1	0.01	–	–	1	0.01	5	0.03
Positive	1152	179	1.2	4	0.03	13	0.09	48	0.3	31	0.2	83	0.6
All	184790	1476	0.8	22	0.01	192	0.1	240	0.1	207	0.1	815	0.4

CIN – Cervical Intra-epithelial Neoplasia, CIS – Carcinoma *in situ*, Pap – Papanicolaou, HPV – Human papillomavirus, – magnitude nil, women with Papanicolaou group I or II or HPV test negative are not referred for further examinations unless repeated cytology or other results are suggestive of cancer or CIN, women with HPV test positive are referred for further examinations on the basis of cytology.

25 are not encouraged to be screened (http://www.kaypahoito.fi/). In the organised programme the present coverage of invitations is 95% of the target age (http://www.cancerregistry.fi/statistics). In one calendar year, there is about 250,000 invitations in the programme. The attendance rate is more than 70%.

Even though the attendance rate is satisfactory, close to 80%, in the older targeted ages, one main problem in the programme is the fact that among young targeted ages, particularly, in ages 25 to 35, the rate is very low, only slightly above 60%. This has lead to the situation that hardly half of the targeted population participates in those ages (Figure 5).

Attending organised screening for women is free of charge. Sample taking is done by trained nurses or midwives in the local primary health care centres or clinics. The sample quality is under continuous control done by the cytology laboratories, based on individual coding for sample-takers for a potential personal feed-back. The samples taken are VCE smears; i.e., samples from posterior vaginal fornix, cervix, and from endocervical canal are taken separately, using Ayre's spatula and endocervical brush, and are placed on the same slide. The samples are stained with modified Papanicolaou staining. The samples are screened by cytotechnicians, and the cytologist checks every abnormal smear and a proportion of normal smears. More recently, also the Human papillomavirus (HPV)-DNA samples are taken in contracted municipalities (see below).

There are some 15 cytology laboratories contracted by the municipalities to the programme. Confirmation and treatment is integrated into the normal health care routines. Treatment is provided for women with relatively mild lesions (CIN1) or with a more severe finding. All the data on the confirmation are included on the personal screening cards kept for every woman in the cytology laboratory; they are registered and the compliance and adequacy of treatment followed by the cytology laboratory and also by the mass screening registry.

The average referral rate is constantly about 1%, and the detection rate for an CIN+ (cervical intraepithelial neoplasia, or a worse histologically confirmed finding) about 0.3–0.5%. In addition, about 6% of the screened women get a re-invitation within a shorter interval, usually one year, based on borderline cytology (Table 1). There is still some variation in the detection rates and performance parameters which appear not to reflect directly the effectiveness aspects but, rather, some variation in the local cost-effectiveness[10].

Data collection infrastructures

Screening laboratories are responsible for recording screening visit and confirmation and treatment information within the programme. The invitational and screening data of the programme, including cytological and histological screening findings, are filed centrally at the Mass Screening Registry that it a subunit within the national Finnish Cancer Registry. This makes an efficient tool for evaluation and monitoring of the programme. The Finnish Cancer Registry provides complete data on cancer incidence and mortality, arranged individually with the help of the unique personal identifier. The mor-

tality records are obtained from the files of the Cause-of-Death Registry at the Statistics Finland. The cancer registry notifies also carcinoma *in situ* cases of the cervix uteri (CIS, including both squamous and adenocarcinoma *in situ* cases), as well as cases of severe intraepithelial neoplasia (dysplasia gravis, or CIN3 not specified in more detail).

Quality assurance

In the normal screening practice of the organised programme there is a number of quality control activities, such as control of sample quality that takes place mainly within the cytological laboratories within the programme[9]; and re-reading, consultation and training meetings; even though there have been no systematic publications on their results. Important for developing the screening specificity criteria, there are, normally, weekly sessions between cytology and pathology units/laboratories. Since 1999 a systematic re-reading programme of potentially false negative smears have been in action; including re-reading of these smears together with control slides both in a reference laboratory and in the original screening laboratory. This material is based on linkages between the screening and cancer registry files.

Novel Screening Methods

The main aim of the evaluation of alternative screening techniques is to assess screening effectiveness, i.e., comparing incidence of subsequent cervical cancers as the outcome and screen-detected pre-cancers as surrogates. Also performance e.g. in form of screen-detected findings will be monitored and compared. It is important to verify that, if the treatment rates would increase, it reflects, respectively, to better efficacy and effectiveness. Modifications on the screening policy need also to be considered, e.g. the need of lifetime number of tests in the programme. For the time being, approximately 860,000 women have been allocated to automation-assisted cytology, HPV DNA testing, or to conventional cytology within the organised screening programme[11,12,13]. In the HPV-DNA screening arm, run within a restricted area, the plan is to invite about 100,000 women in 2003–2008. In numerical terms, almost 10% of the whole national target population will be subjected to HPV screening. Follow-up results on subsequent cervical cancers will become available during 2007– 2015.

First reports on screening detection rates are available[12,13]. Screening detection rates as well as specificity estimates in automation-assisted screening are very similar to conventional screening. Based on early results from HPV screening, the detection rate of mild pre-cancerous lesions was in excess in the HPV screening protocol[12]; see also Table 1 on the routine statistics on 2004. CIN3+ detection rates were about the same as in conventional screening. There is a cytological triage protocol after a positive primary HPV test. Noteworthy, when considering the referral to colposcopy, based on the cytology triage, the cross-sectional specificity and positive predictive value estimates were closely resembling those of the conventional screening.

Discussion

Historically, the overall incidence of invasive cervical cancer, as well as that of *in situ* carcinoma of the cervix uteri, has drastically decreased in those ages subject to organised screening activities. Organised screening is effective. There is no similar decrease in the CIN detection frequencies, however. On the contrary, the detection rates of CIN grades 2 and 3 have even slightly increased[2], indicating that the biologic background risk has been likely increased. Also invasive cervical cancer incidence has increased in ages 25–39 years, even though based on a small number of cases registered annually. This still warrants improvements in the rather poor attendance rate in the programme in the above young targeted ages. Interventions testing written reminder or, preferably, reminding by phone, are required[14]. Self-sampling could also be tested in order to check whether one can increase compliance meaningfully.

On the other hand, based on the finding that a very large proportion of registered CIN3/CIS cases have been detected and treated outside the organised programme, e.g. in opportunistic screening, in parallel with improving the population-based coverage and access to the services, also decrease and stopping of unnecessary actions should take place.

Concluding Remarks

Following from the 45-year period of its action the cervical cancer screening programme in Finland has contributed to a large decrease in cervical cancer incidence and mortality rates. The purpose of cervical cancer screening is to prevent mortality and incidence from the disease. One can conclude that organised screening is effective in combating cervical cancers. With introducing modern screening technologies and more systematic quality control activities in the programme, and moderately expanding the coverage and as well as compliance – particularly, at the young target ages of the programme – we still aim to increase the effectiveness of the programme. Alternative methods in screening, such as automation-assisted screening and HPV testing, have shown promising cross-sectional findings. We propose speeding up the use of modern technological alternatives in organised screening programmes. Follow-up information of cervical cancers in still required, to acquire evidence for possible modifications on the screening policies.

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PROGRAM PROBIRA RAKA VRATA MATERNICE U FINSKOJ S PRIMJEROM UVOĐENJA ALTERNATIVNIH METODA PROBIRA

SAŽETAK

U Finskoj (5 milijuna stanovnika) je organizirani program Papa-testiranja u prevenciji raka vrata maternice u upotrebi već 45 godina. Testiranje obuhvaća žene u dobi od 30 do 64 godine (1,25 milijuna), a period između dva testiranja je pet godina. Prema programu, žene se poziva sedam puta tijekom života; odaziv jednoj rundi poziva na testiranje je 73%. Ovaj program je značajno utjecao na stopu pojave raka vrata maternice u našoj zemlji. Tijekom desetljeća njegove primjene zabilježen je pad od oko 80% u dobno-standardiziranim stopama pojavnosti i smrtnosti od raka vrata maternice. Trenutna stopa pojavnosti, obzirom na dob je 4, a stopa smrtnosti 1 na 100.000 žena godišnje. U ovom članku opisujemo organizacijske značajke programa; sva pažnja je usmjerena na obnovu programa tijekom zadnjeg desetljeća s novim, alternativnim metodama koje su se počele koristiti u sklopu testova probira. Povećanjem pokrivenosti i suglasnosti s programom, očekujemo povećanje utjecaja programa. Istovremeno, potreban je napor kako bi se izbjegla pretjerana upotreba službi zahvaljujući spontanom probiru, sa svrhom smanjenja mogućih štetnih učinaka te smanjenja troškova. Trenutno se pokušava uvesti pokrivanje troškova testiranja na HPV kroz zdravstveno osiguranje. Očekujemo povoljne rezultate. Praćenje stope raka nakon testova probira je potrebno i dalje kako bi se procijenila najbolja strategija probira (vremenski razmaci između testiranja po dobnim skupinama te početna i završna dob). Predlažemo brže uvođenje novih, modernih metoda u organiziranim programima probira.

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Review

Cervical Cancer Screening: A Slovenian Experience

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A B S T R A C T

In Slovenia, opportunistic screening was introduced in regular gynaecological practice in 1960. The proportion of population screened was unknown, as well as there were no standards for quality assurance and control. Despite great number of smears read, there were no major changes in invasive cervical cancer incidence in the period 1979 till 1993, but in 1994 the incidence rate started to increase again to reach its peak in 1997 (23,1/100.000, 241 new cases). Based on the experiences from the countries with effectively organised screening programmes, a decision was made in 1996 by the Minister of Health to nominate a group of experts to prepare a proposal for organised cervical cancer screening programme after testing the methodology in pilot study. In the pilot the central computerised information system (Screening Registry) was gradually established to register all smears from the whole country, to identify women who do not attend for screening to send them invitation for screening and to monitor screening activity and its quality. The aim of pilot was also to develop guidelines for quality assurance and control of all procedures involved in cervical cancer screening and treatment of intraepithelial lesions. In three years since the beginning of the national programme, nearly 70% of women in the target age group were registered with at least one smear. All other results are presented in regular programme reports. There is still place for further development of the programme, but the incidence of cervical cancer already started to decline especially among younger women, who attend for screening more often than those aged over 50.

Key words: *cervical cancer, screening, cervical smear*

Introduction

Cancer is one of the major public health problems as it is the most common cause of morbidity and mortality today, with more than 5.8 million new cases and more than 3.8 million of deaths each year in Europe[1]. It is projected that by 2020 there will be 3,4 million of new cancer cases and 2,1 million deaths. Much of this increase in absolute numbers derives from the ageing of populations. It represents a significant and growing burden on public health services today.

There is now sufficient understanding of the causes to prevent at least one third of all cancers. Information is also available that would permit the early detection and effective treatment of a further one third of all cancers worldwide. The overall goal of cancer control is to reduce the incidence and mortality of cancer and to improve the quality of life of cancer patients and their families. A well conceived national cancer control programme is the most effective instrument to bridge the gap between knowledge and practice and achieve this goal. Integrated into existing health systems and related services, this programme ensures systematic and equitable implementation of control strategies across the continuum of prevention, early detection, treatment and palliative care.

Screening for cancer consists of the identification of preclinical disease by a relatively simple test. The objective of screening is to reduce the risk of death, i.e. mortality from cancer subjected to screening. For cervical cancer the screening test is aimed at detection of preinvasive lesions. Therefore, reduction in the incidence of invasive disease is the objective of screening for cervical cancer and the indicator of the effect is the change of incidence between those subjected to screening.

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Screening for cervical cancer is fundamental to the provision of health care in Europe and, depending on each health system, performed by general practitioners, the gynaecologist or trained nurses. The dissemination of the cervical smears depends both on the professional interest and on the method of payment. The major increase in use was in those settings where smears were paid for through a public health insurance scheme.

Cervical Cancer Screening in Slovenia till 2002

In Slovenia, opportunistic screening was introduced in regular gynaecological practice in 1960, but in some regions already in 1955 and 1956. A preventive gynaecological exam (smear included) has been practised since then on a yearly basis and recommended to women by gynaecologic community and paid by the health insurance. The payment did not stimulate examinations of more women, as only attendances have been paid regardless on how many women the exams have been performed. Since 1998, a regular yearly gynaecological exam with a smear (endo- and ecto-cervical) and colposcopy has been paid from the obligatory health insurance and supposed to be the right of every woman from 20 years of age onwards. It was performed by gynaecologists working in primary reproductive health care, where changes have also been happening, because of introduction of registration with personal gynaecologists and in some places the accessibility to this service diminished.

Most of the smears were taken from young women, while older women and women from the lower socio-economic groups were frequently missed out altogether by the system. These women are at higher risk, but make less use of screening services, especially if attendance requires individual initiative (inequalities in risk and use of screening). In Slovenia it was found out that despite the formally equal accessibility, only 30% of women performed gynaecological examination yearly, less than half once in every three years, depending on the region of residence, and mostly those with better education. On the other hand, the number of smears examined in the laboratories (more than 300,000) could suffice to examine every women aged 20–64 at least every three years.

In Slovenia there is one of the oldest Cancer Registries in Europe so we can monitor the incidence of cervical cancer since 1950. The time trend of invasive cervical cancer is supposed to reflect the effectiveness of cervical screening in our country. The crude incidence rate of invasive cervical cancer increased from 22.5/100,000 in 1950 to 34/100,000 in 1962 and then decreased to 14/100,000 in 1979, when the incidence was the lowest[2]. Since then till 1993 there were no major changes (though nothing had changed in gynaecological recommendations) but in 1994 the incidence rate started to increase again. Furthermore, an increase of the invasive cancer incidence in the younger age groups (30–39) has been observed. In the period 1994–1998, the age specific incidence rate in the age groups 30–34 and 35–39 was nearly the same as in the period 1959–1963, at the start of the opportunistic screening. In 2000, the incidence rate was 20/100.000, one of the highest in Europe[3].

The descriptive epidemiologic analysis of cervical cancer in Slovenia revealed the inefficiency of opportunistic screening in Slovenia, where lots of resources have been wasted for a small effect on the female reproductive health. Even though the baseline risk of cervical cancer may have changed due to more liberal sexual behaviour of generations, born after the Second World War, the effective screening programme should cope at least with a part of this risk, resulting in better detection of precancerous lesions and not leading to such an increase of invasive cervical cancer.

Based on the experiences from the countries with effectively organised screening programmes[4], a decision was made in 1996 by the Ministry of Health and Health insurance Company to start a pilot study to gradually introduce organised cervical cancer screening. After initial preparations, the pilot started in 1998 in the central region of Slovenia, covering approximately 300,000 women, i.e., one third of the whole female population of Slovenia.

The objectives of this study were:

- to establish the central computerised information system (Screening Registry) linked to the Central Population Registry of Slovenia to register all smears and monitor screening activity and to identify women who do not attend for screening;
- to invite women aged 25–64, who supposedly had not been screened in the previous five years, as they have not been yet registered with a gynaecologist or did not have a smear registered in the study period and to estimate the proportion of women who attend screening as a result of personal invitation;
- to develop guidelines for quality assurance and control of all procedures involved in cervical cancer screening and treatment of intraepithelial lesion and of cervical cancer.

First, a uniform smear report form and skeleton of a computer database were constructed. From the Central Population Register, samples of women from the target population were regularly made and invited to pre-arranged gynaecologic exams. In the period 1998–2001, 28,804 invitations were sent to the samples of women mentioned. Personal invitations have resulted in nearly 50% participation rate in the group of women who do not regularly attend the opportunistic screening. All smear reports (in the electronic form) from all cytological laboratories in the region were gathered. A central database of the Screening Registry was thus created and then regularly updated. In the following years the reporting of smears from all cytological laboratories from the whole country was established, so since 2003 the register is covering the whole country and constant monitoring of the coverage and quality has been established. National guidelines for cytopathology and for management of women with abnormal smear have been published[5–7].

The legal basis for the programme was also established: the contents of the database is included in the law on health statistics[8], the special regulation for cytopathology laboratories then was published by the Ministry of Health[9], and laboratories then have been reviewed to evaluate whether they comply with these standards. With the ministry's recommendation on preventive examinations in primary reproductive health care where screening policy was introduced, the national programme started in 2003[10].

Organised Cervical Cancer Screening Programme in the Primary (Reproductive) Health Care

In 2003, the Ministry of Health supported the introduction of National Programme of Organized Cervical Cancer Screening. It has a name ZORA after Slovenian initials for organised cervical cancer screening programme. The central coordination office with the Screening Registry is at the Ljubljana Institute of Oncology.

The programme advocates the population-wide active cervical cancer screening based on quality-controlled procedures. The aim of this programme is to achieve that at least 70% of the female population aged 20–64 will have a smear taken in a the three-year interval.

Each woman between ages 20 and 64 is to be invited to perform a preventive gynaecological examination together with Papanicolau smear once in every three years (after two negative smears) either by her »personal« gynaecologist with whom she has already been registered or from the Screening Centre in case she has not been registered yet. Women aged 65 to 74 years are not invited but are offered screening when they attend gynaecologist for other reasons.

As it was decided that gynaecologists would invite women already registered with them, the initial stage included active involvement of gynaecologists in the primary health care, who had to review all their records and make lists of women to be invited. Different computer programmes in different practices hampered this but the difficulties should be overcome in the future. The coordination of the programme is assured through regional coordinators and national coordinator, nominated by the Minister of Health.

Regardless of the ability to pay, the universal access to ZORA programme is assured by including the ZORA expenditure into the Slovenia's compulsory Health Insurance Fund. Thus, the organized cervical cancer screening programme is based on all fundamental principles of equity, solidarity and participation.

The results show that in some regions the goal of 70% of women having a smear in the last three years, has already been achieved and preliminary data from the Cancer Registry do not show an upward trend of cervical cancer any more. The invitations resulted also in greater percentage of women being registered with their gynaecologists, which is currently about 80% in the target age group. Annual reviews on the cervical cancer screening programme ZORA is published regularly and available on the programme's web site also (http://www.onko-i.si/zora/)[11].

Conclusions

Systematic screening is a public health intervention often performed in primary health care. For its sustainability it should receive political support and supporting legislation. An advantage is the funding system for public health services separate and independent from the cure and care budget, but adequate resources are needed for management of women with abnormal smears. But in any case the health insurance systems should incorporate funds for screening that is evidence based as this means lower costs for treatment of advanced disease. The key for success of such a programme is organisation, existence of national standards for quality assurance and control and constant monitoring of short- and long-term indicators.

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PROBIR RAKA VRATA MATERNICE – SLOVENSKO ISKUSTVO

S A Ž E T A K

Oportunistički probir je uveden 1960. g. u ginekološku praksu u Sloveniji. Udio testirane populacije je bio nepoznat, a nije bilo niti standarda za osiguranje kvalitete i kontrole. Unatoč velikom broju testiranih obrisaka vrata maternice nije bilo većih promjena u pojavnosti invazivnog raka vrata maternice u periodu od 1979. do 1993. g., no od 1994. g. stopa pojavnosti je počela ponovo rasti kako bi dosegla vrhunac u 1997. g. (23,1/100.000, 241 novi slučaj). Zahvaljujući iskustvu zemalja s učinkovito organiziranim programima probira, 1996. g. Ministarstvo zdravstva je donijelo odluku o imenovanju skupine stručnjaka koja će pripremiti prijedlog organiziranog programa probira raka vrata maternice nakon testiranja metoda u sklopu pilot-studije. U pilot-studiji je uspostavljen središnji kompjuterski informatički sistem (Registar za probir) koji prikuplja podatke o uzorcima iz cijele zemlje, identificira žene koje nisu pristupile probiru, šalje im poziv za testiranje te bilježi aktivnost i kvalitetu probira. Cilj pilot-studije je također bio razvoj vodiča za osiguranje kvalitete te kontrola svih procedura uključenih u probir raka vrata maternice i liječenje intraepitelnih lezija. U tri godine provođenja nacionalnog programa gotovo 70% žena ciljane dobne skupine je pristupilo probiru barem jednom. Ostali rezultati su predočeni u klasičnim izvješćima o programu. Međutim, još uvijek ima mjesta budućem razvoju programa, iako je stopa pojavnosti raka vrata maternice počela padati, pogotovo među mlađim ženama, koje dolaze na probir mnogo češće nego one u dobi iznad 50 godina.

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Professional paper

Cervical Cancer Screening in the Czech Republic

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A B S T R A C T

Cytological diagnosis of atypical cells of cervix uteri by the Papanicolaou method was introduced in the Czech Republic (CR) very early – in 1947. The first data on the incidence of cervical cancer in CR are available from 1960 when the rate was 32.3 cases/10^5 women. In 1966 the Czech National Health Law was passed that guaranteed women a yearly preventive examination by a gynaecologist including screening for cervical carcinoma that would be covered by the compulsory health insurance. Notwithstanding high frequency of screening visits and the fact that all women are eligible, the incidence of CC has not changed in the last 34 years. The reasons for this include the coverage of Czech women, which is estimated to be low (50% at the most), and that none of the cytology laboratories are accredited for screening, there are no national registries for any aspect of screening and there are no mechanisms for evaluation of the screening process. As a result, it is likely that the majority of cervical screening activity that is undertaken is ineffective and the implementation of an organised and quality controlled screening programme, in compliance with the recommendations of many European Institutions, is urgently required to ensure that Czech women are properly protected against this disease and that scarce healthcare resources are used in the most cost-effective manner.

Key words: *cervix, cancer, screening*

Introduction

Cervical cancer prevention in the Czech Republic (CR) had a very auspicious start. Cytological diagnosis of atypical cells of cervix uteri by the Papanicolaou (Pap) method was introduced very early – in 1947 – and by 1954, Herold and Luksch had already published the manual, »Cytodiagnostics of cancer of female genitals«, in the Czech language[1]. In 1960, cytological consultation centres were established and these resulted in a drop in incidence 32.3 cases/10^5 women to 27.2 cases/10^5 women by 1965. Then, in 1966 the Czech National Health Law was passed which guaranteed women a yearly preventive examination by a gynaecologist, which included a Pap test and the incidence of cervical cancer decreased again, from 28.8 in 1966 to 21.9 in 1983. A further decline in incidence from 21.9 to 20.7 in the period of 1984–90 can be attributed to the establishment of a system of Centres of Gynaecology–Oncology Prevention. However, since 1990, no further decreases in the incidence of cervical cancer have been observed (Figure 1)[2].

Guidelines

In 1999, a committee of experts including gynaecologists, pathologists, cytologists and virologists prepared the first Guidelines for the management of patients with lesions of cervix uteri, which also included suggestions for algorithms for primary screening. However, these guidelines were never introduced into routine clinical practice. Subsequently, the Ministry of Health has on three occasion's convened committees for screening for cervical carcinoma with the first two of these cancelled shortly after their establishment. Then, in July 2004, the third committee published recommendations that included a 1-year screening interval with classical cytology as the primary screening test and HPV detection for the triage of borderline findings up to 4% of the amount of Pap smears for each laboratory. The recommended age range is 25–60 and the insurance companies should send invitation to women. If woman does not respond, the invitation should be repeated after 2 years. The basic requirement for cytological laboratory to be able to apply

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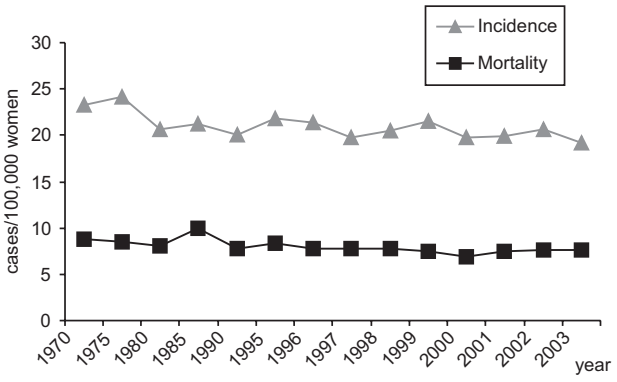

Fig. 1. Incidence and mortality of cervical cancer in the Czech Republic[2].

for accreditation is a minimum amount of 15,000 cervical smears per year. Originally, the start of the screening was expected in July 2006, but it has not yet begun.

The Current Situation

In 2003, there were 1,007 new cases of cervical carcinoma and 398 women died from this disease in the CR[2]. The highest incidence of cervical cancer occurs in the 45–49-age category (Table 1). In the age category of 15–34 years the death rate is low – 3.3%, but it is very high in the category of 35–59 – 38.4%. These numbers are smaller in the age category of 60–74 – 28.9% as well as in the category of 75+ years of age – 29.4%.

The Czech National Health Law from 1966 is still valid and it is the basis for screening in CR today where all women (no age is specified) are entitled to a free preventive gynaecological examination once per year. This preventive visit includes basic colposcopy and a Pap test. All gynaecologists can perform basic colposcopy in their office and it is paid for by the compulsory health insurance (€ 1.7). Only those specialists who are certified perform expert colposcopy and it is more expensive (€ 10). To become certified, a gynaecologist has to prove that he diagnoses ≥50 high-grade lesions annually and he has to pass an exam in one of two accredited colposcopy centres in CR. Altogether, there are about 2,000 private gynaecologists in the CR who provide this service.

For the cytological analyses of cervical smears in the CR, there are about 50 laboratories but only three of these process ≥50,000 slides per year as recommended by the European guidelines[3] and only about half of them that process ≥15,000 slides per year as required by the new recommendations of the screening committee of the Ministry of Health of the CR[4]. For the evaluation of cytological slides the 2001 Bethesda system[5] is used. Mortality and incidence data are available from the National cancer registry which was established in 1976 and since 1991 is a member of the International Association of Cancer Registries (IACR).

In the CR, there are 5,2 million women in total and 2.9 million women in the screening age (25–65 years of age). The Institute of statistics and health information reported more than 2.9 million preventive visits to gynaecologists in 2005 but as the identification number of the women is not recorded, these data are of limited value because it is impossible to distinguish repeat visits from new visits or in any way relate the number of visits to the number of women in the target population. Indeed, there are no data sources concerning screening coverage, but it is estimated to be 30–50%.

TABLE 1
INCIDENCE AND MORTALITY FOR CERVICAL CANCER (C53) BY AGE GROUPS IN THE CZECH REPUBLIC IN 2003[2]

	Total	Age group														
		15–19	20–24	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–79	80–84	85+
New cases	1007	1	7	38	70	105	110	124	115	132	73	62	60	55	34	21
Incidence*	19.2	0.3	1.9	8.5	19.2	30.8	34.9	33.2	28.7	34.8	25.8	27.0	25.4	26.9	26.2	30.3
Dead	398	–	–	2	11	12	17	43	43	38	48	35	32	49	44	24
Mortality*	7.6	–	–	0.4	3	3.5	5.4	11.5	11.5	10	17	15.2	13.5	24	34	34.6

*per 100,000 women/year

TABLE 2
WOMEN WITH AND WITHOUT GYNAECOLOGICAL EXAMINATION ACCORDING TO THE TUMOR (C53) STAGE (2002)

Stage	Number	Mean age [years]	Colposcopy/cytology examinatin in the last 3 years	No examination	
				>5 years	>10 years
IB-1	105	44.7	59 (56.2%)	21 (20%)	2 (1.9%)
IB-2	42	44.1	20 (42.9%)	14 (33.3%)	4 (9.5%)
IIB	91	54.5	31 (34%)	35 (38.4%)	14 (15.4%)
IIIB-IV	14	74	1 (7%)	6 (43%)	6 (43%)

IB-1 to IV – The FIGO (International Federation of Gynaecology and Obstetrics) system for clinical staging of cancer of the cervix

Data from the Clinic of Obstetrics and Gynaecology in Motol also show that 44.0% of women diagnosed with invasive cervical cancer had visited a gynaecologist for colposcopy/cytology within the 3 year, and 60.0% had visited within the 5 years before being diagnosed with cancer (Table 2). These data clearly indicate that the diagnostic process is failing a large proportion of the Czech women who are attending for screening.

Conclusion

Screening cervical carcinoma in the Czech Republic has been opportunistic since 1966. Despite a very high frequency of screening visits and a historically wide age range, the incidence of cervical cancer has not changed in the last 34 years. The coverage of Czech women by the screening is not known exactly but it is estimated to be 50% at the most. Further, for those who are attending for screening, the high percentage of women with IB-2 cervical cancer, who have been examined by cytology and colposcopy within the last 3 years, suggests that the screening and diagnostic processes are inadequate.

It has been clearly established that organised cervical cancer screening programmes with quality control at all levels of the process offer the most cost-effective protection against cervical cancer and can prevent up to 80% of cervical cancers in the populations they serve. Because of these facts, the Council of the European Union, the European Commission, the International Agency for Research on Cancer, the European Parliament Cervical Cancer Interest Group, together with a number of other prestigious organisations has recommended that all European countries should implement organised cervical cancer screening programmes. The data noted above clearly indicate that the Czech Republic is not fulfilling its obligations to the women of this country and the implantation of these programmes must now be a priority.

Acknowledgements

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PROBIR RAKA VRATA MATERNICE U REPUBLICI ČEŠKOJ

SAŽETAK

Citološka dijagnoza atipičnih stanica vrata maternice metodom Papanicolaou-a je uvedena već 1947. g. u Republici Češkoj. Prvi podaci o pojavnosti raka vrata maternice u Republici Češkoj su dostupni od 1960. g. kada je stopa pojavnosti iznosila 32,3 slučaja/10^5 žena. 1966. g. Češko nacionalno zdravstveno pravo je donijelo jamstvo ženama da će godišnji preventivni ginekološki pregled uključujući probir raka vrata maternice biti pokriven obaveznim zdravstvenim osiguranjem. Unatoč visokom odazivu na probir i činjenici da su sve žene podobne, stopa pojavnosti raka vrata maternice se nije mijenjala u posljednje 34 godine. Razlozi za to uključuju nisku pokrivenost žena u Republici Češkoj (najviše 50%), činjenicu da niti jedan citološki laboratorij nije ovlašten za probir, nepostojanje nacionalnih registara za bilo koji aspekt programa te nepostojanje načina procjene procesa probira. Kao rezultat se može očekivati da će većina aktivnosti probira raka vrata maternice biti neučinkovita te je uvođenje organiziranog programa probira s kontrolom kvalitete, zajedno s preporukama mnogih Europskih institucija, hitno potrebno kako bi se ženama u Republici Češkoj osigurala pravilna zaštita od ove bolesti te da oskudni izvori zdravstvenog osiguranja budu upotrijebljeni na najučinkovitiji način.

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Professional paper

Cervical Cancer Screening in Serbia

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A B S T R A C T

Cervical cancer is the second most common female malignancy in Serbia, after breast cancer, with 1089 new registered cases and an age-standardized incidence rate of 27.2 per 100,000 women in 2002. It is the fourth leading cause of cancer death with 452 deaths and an age-standardized death rate of 7.2 per 100,000 women. Compared with other European countries, the incidence of cervical cancer in Central Serbia is the highest. Regional differences in incidence are pronounced in Serbia with the lowest age-standardized incidence rate (16.6 per 100,000 women) registered in the Mačvanski region and the highest in eastern Serbia and the region of Belgrade where the rates are double at 32.5–38.1 per 100,000 women. Cervical cancer prevention in Serbia has relied on opportunistic screening that is characterized by high coverage in younger and low coverage in middle-aged and older women. Screening of selected groups of women employed in large companies is performed annually by many regional hospitals but this approach has little effect on morbidity and mortality. Recently, the Ministry of Health nominated an Expert Group to develop and implement a national cervical cancer screening program. A number of pilot projects have been undertaken with the results used for development of a national programme for cervical cancer screening. This is expected to be finalized in 2007, and launched over a 3-years period in order to cover all women aged 25–64 in entire Serbia.

Key words: *cervical cancer, screening, Serbia*

Introduction

The aim of the present study was evaluate the burden of cervical cancer in Serbia and present the status of cervical cancer prevention. The sources used to develop this report were official the Statistical Office of Serbia and the Cancer Registry of Serbia, as well as the results of the studies and projects related to cervical cancer screening conducted in Serbia during the period of 2002–2007.

Global Situation

Cervical cancer is the second most common malignancy in women worldwide, with about 490,000 newly registered cases every year. There are large differences in the incidence rate which varies from 2.0 per 100,000 women in Syria to 87.3 per 100,000 women in Haiti (age standardized rates). The highest rates are observed in Eastern and Southern Africa, Melanesia, Caribbean and Central America; the lowest rates are observed in Eastern and Western Asia, Australia, Northern America, Northern and Western Europe[1].

Several factors contribute to regional differences in cervical cancer rates and their trends. Persistent infections with the Human papillomavirus (HPV) are a necessary but not sufficient cause of cervical cancer. HPV is primarily transmitted by sexual contanct but differences in sexual behavior cannot entirely account for the geographic variation in cervical cancer. The most important factor is the availability of screening. In many developed countries where screening has been in place for a number of years, a decline in cervical cancer incidence and mortality has been observed over the last 30 years.

Cervical Cancer – a Major Health Problem in Serbia

From 1973–1982, the European extremes in cervical cancer incidence were the German Democratic Republic with an incidence of 33.2 and Spain with the one of 4.1[2]. At that time, the incidence in regions of Central Serbia where the cancer registries were functioning properly was 14.7 to 18.2 per 100,000[3].

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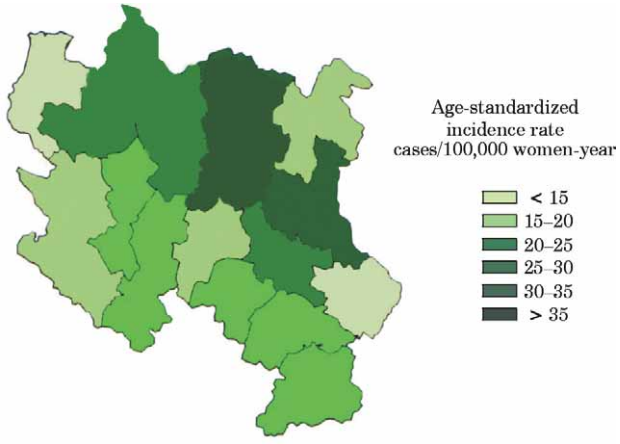

Fig. 1. Age-standardized cervical cancer incidence rates in Serbia, by regions, (per 100,000 women); average 27.3/100,000 women-year. Source: Cancer Registry of Serbia, 2002[6].

In 1995, the cancer registry in Central Serbia was reorganized with all new cases from Serbia's 16 regions registered centrally and the average age-standardized incidence rate for 1999 was 20.8/100,000 while the average standardized incidence rate for the European Union was 9.9[4]. Today, according to the data of the Cancer Registry of Central Serbia, cervical cancer is the second most common female malignancy after breast cancer with 1,089 new cases per year and the age-standardized incidence rate of 27.2 per 100,000 women in the year 2002[5]. However, there are also significant differences in cervical cancer incidence between the regions of Central Serbia (Figure 1). In 2002, the lowest age-standardized incidence rate (16.6 per 100,000 women) was registered in the Mačvanski region. The highest rates, more than twice higher, were registered in eastern Serbia, near the border with Romania, and in the region of Belgrade (32.5–38.1 per 100,000 women)[5].

When compared with data from European countries, the incidence of cervical cancer in Central Serbia is the highest in Europe. Similar high rates are observed in neighboring countries such as Romania, Albania and Bosnia & Herzegovina (Figure 2).

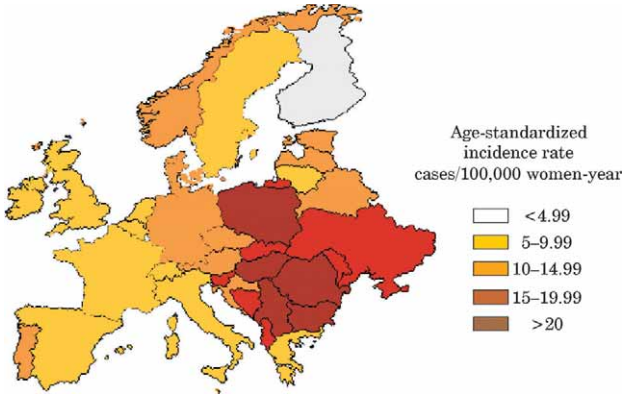

Fig. 2. Age-standardized cervical cancer incidence rates in European countries (per 100,000 women). Source: Ferlay J et al. Globocan 2002[1].

TABLE 1
COUNTRIES WITH HIGHEST CERVICAL CANCER MORTALITY RATES IN EUROPE (AGE-STANDARIZED MORTALITY RATES PER 100,000 WOMEN IN 2002)

Country	Age standardized mortality rate
Romania	13.0
Albania	9.8
Lithuania	9.0
Bulgaria	8.0
Bosnia & Herzegovina	8.0
Poland	7.8
Moldova	7.8
Macedonia	7.6
Latvia	7.4
Serbia	7.2
Hungary	6.7
Estonia	6.6
Russian Federation	6.5
Ukraine	6.4
Slovakia	6.1

Source: Ferlay J et al. Globocan 2002 and Statistical Office of Serbia[1]

In 2002, according to the data of the Statistical Office of Serbia, cervical cancer was the fourth leading cause of cancer death in females in Serbia with 452 deaths and the age-standardized death rate of 7.2 per 100,000 women[5]. As for mortality, Romania is the country with the highest mortality rate in Europe, while Serbian rate is significantly lower (Table 1). However, cervical cancer mortality depends on incidence and survival rates. As both Serbia and Romania have similar high incidence rates, difference in mortality could be explained by better availability and efficacy of cervical cancer treatment services in Serbia.

Age Distribution of Cervical Cancer in Serbia

The risk for cervical cancer increases with age. In Central Serbia very few cases are diagnosed before the age of 25. After that age, the incidence increases and reaches maximum in the 45 to 49 and 50 to 54 age groups[6] (Table 2).

Potential Target Population for Cervical Cancer Screening

Decisions on the target age group and frequency of screening are usually made at the national level, on the basis of local incidence and prevalence of cancer, HIV

TABLE 2
AGE DISTRIBUTION OF CERVICAL CANCER CASES IN CENTRAL SERBIA

Age group	No of cases	% of all cases	Age specific rate
0–14	0	0	0
15–19	1	0.1	0.6
20–24	1	0.1	0.6
25–29	23	2.3	12.4
30–34	40	4	22.7
35–39	75	7.5	42.9
40–44	108	10.8	56.7
45–49	162	16.1	75.3
50–54	173	17.2	77.9
55–59	105	10.5	66.3
60–64	89	8.9	55.4
65–69	93	9.3	52.2
70–74	77	7.7	46.5
75+	57	5.7	29.5

Source: Cancer Registry of Central Serbia 2003[7]

prevalence, availability of resources and infrastructure. WHO recommends that new programmes should start by screening women aged 30 years and more, and include women 25 to 29 only when the higher risk groups have been covered. Screening should not include women under 25 and for women over 65, screening is not necessary if they have had two previous negative smears[7].

The incidence of cervical cancer is high in all age groups from 35 to 69. The upper age limit of the target group should be set at least at the age of 64 so that there would not be many cases missed by screening in older age groups. On the other hand, lower age limit should be set at 30 so that as many lesions as possible would be discovered in precancerous lesions.

There is about 1,5 million women in the age group 30 to 59 and about 1,8 million women in the age group 30 to 64 in Serbia. With a three year interval, it would mean 0,5 or 0,6 million to be screened yearly.

Cervical Cancer Control Programme

A comprehensive, centalized screening programme for cervical cancer has never been implemented in Serbia and cervical cancer prevention has relied on opportunistic screening. This type of screening has been characterized by high coverage in younger and low coverage in middle-aged and older women. Screening of selected groups of women employed in large companies is performed annually by many regional hospitals. This approach, however, has had little effect on morbidity and mortality.

In spite of some efforts to initiate screening during the period between 1990 and 1999, the difficult situation in the country did not enable more organized approach. Besides the other effects on health service, the economic crisis left significant the consequences to general health:

- Economic resources for health were lowered.
- Investments for health were decreased (from € 150 per person in 1997 to less than € 50 in 1999).
- Prevention became inadequate.
- Overall mortality and morbidity increased.

In this situation, disease prevention through mass screening in central Serbia did not seem to be the primary problem and cervical cancer control in Serbia had unsatisfactory levels of effectiveness. This was primarily due to the absence of national strategy for cancer prevention, a lack of programme funds for cancer prevention or early detection, as well as an insufficient public and political awareness of the importance of cancer prevention. Primary prevention programs did exist but were carried out without well-defined methodology or objectives and without appropriate effectiveness analysis. As a consequence, a high proportion of cervical cancers were diagnosed when already well-advanced and metastatic, leading to a low probability of cure and high mortality rates.

Actions Against Cervical Cancer – Preparing the Foundation for Organised Cervical Cancer Screening

Beginning in the year 2000, the basic strategies applied to achieve the cervical cancer control in Serbia were focused on cancer prevention and early detection strategies. The main risk factors on the national level were defined and monitored. The efforts were made to improve the system for cancer registration. This could not be achieved without introduction of unique information system to help in overcoming communication problems on the local, regional and national level. To achieve these goals it was mandatory to increase the support of government, as well.

Becoming aware of the increasing incidence and mortality of cervical cancer in Serbia and the importance of early detection and treatment of this disease, the Ministry of Health nominated an Expert Group for the prevention and early detection of cervical cancer in 2003. The aim was to develop and implement national screening programme. It was expected that the systematic screening of non-symptomatic women would increase the proportion of localized tumors, but also the need of treatment resources for these cancers.

As a result, several projects have been completed to study the psycho-social aspects of cervical screening in this region and to improve the local screening infrastructure:

- A study conducted by the Institute of International Social Affairs in 2002 showed that an educational campaign on the importance of screening can increase participation by more than 60%[8].
- A survey sponsored by the Alliance for Cervical Cancer Prevention (ACCP) and conducted by Programme for

Appropriate Technology in Helath (PATH) in 2003–4, explored women's knowledge and perceived barriers towards cervical screening, so adequate campaigns can be designed.

The research was focused on urban women residing in the capital city and one major regional city. The study was comprised of qualitative (phase I) and quantitative (phase II) research. The main determining factors were the age and education of the women. The first phase has been realized through focus group discussions (62 women participated, recruited by network sampling) and in-depth interviews (conducted with 22 women)[9]. Data deriving from the qualitative study were used to develop a questionnaire on women's understandings and knowledge of cervical cancer. The survey was distributed to 800 women from a selected number of community health services[10].

The population sampled showed a broad lack of knowledge about the necessity of screening and shared attitudinal barriers with women in other regions. Results revealed that most of women do not regularly visit a gynaecologist, do not understand the purpose of Pap smear (even if they regularly have it), think that absence of symptoms means that they are healthy and do not need Pap smear, do not know the procedure of Pap smear, are often embarrassed about being examined (this was independent of the gynecologist's sex), believe that little can be done to prevent cancer and an unwillingness to talk about the illness. Education and economic status were not highly related to knowledge about cervical screening[10].

Thematic analysis identified that the interplay of social and personal barriers influenced women's poor presentation for screening. It found that the socio-economic situation prevented women from focusing on their health and it identified a number of problems with the delivery of health services in the region. Inadequate public health education, lack of patient-friendly health services, socio-cultural health beliefs, gender roles, and personal difficulties were the most salient barriers to screening[9].

The study findings suggest how, within the context of opportunistic screening, patient education maybe employed. The introduction of compulsory cervical cancer screening, suggested by some participants, may be a possible approach[9]. The success of public awareness campaigns elsewhere suggests that a media-centered approach could also have good results in Serbia. The lack of media attention noted in the study focus groups supports this conclusion.

In general, study findings was used to inform and facilitate the change in government's policy regarding cervical screening and to develop the public health campaign targeting cervical cancer.

- Clinical Center of Serbia was one of the partners in European Consortium for Cervical Cancer Education (ECCCE) project granted by European Commission and conducted from 2002–2004. This project has lead to the foundation of The European Cervical Cancer Association (ECCA). The ECCA was founded by 15 different organisations from across Europe, which included cancer charities, cancer treatment centres, university teaching hospitals and health education organisations. One of them was Clinical Center of Serbia. The ECCA was established specifically to co-ordinate a Europe-wide public health education programme that would raise awareness of cervical cancer and how it can be prevented. For the general public in Serbia the project was developing an educational programme to provide women with the information they need to reduce their risk of cervical cancer. This project has adopted the materials developed by ECCCE and ECCA. These materials are approved by Serbian Ministry of Health and widely distributed in Serbia through the network of regional health centres.
- Project »Improving Preventive Health Services in Serbia« (IPHSS) has started in September 2004. Its goal was to reduce the mortality by improving preventive health services. To achieve it, this project, funded by European Union, managed by European Agency for Reconstruction and realised by EPOS Health Consultants, targets both health professionals and women. IPHSS edited guidelines for prevention of cervical carcinoma elaborated in line with EU recommendations and will distribute them in 25 regional Serbian health centres[11]. It also supports the establishment of a national School for cytology. In parallel, IPHSS wishes to contribute to the education of women on the prevention of cervical cancer.
- A Pilot Programme for Cervical Cancer Screening was implemented 2004 in Branicevo, a region in eastern Serbia with a particularly high incidence of cervical cancer[12]. This project, supported by the French government and implemented by WHO, was the first organized screening programme in Serbia.

Screening Implementation

»Branicevo project« was the first organized screening in Serbia. In 2001, the French donor, the WHO and health authorities of the Republic of Serbia jointly agreed that the high mortality and incidence of cervical cancer in Serbia presents a major public health problem that needs urgent intervention. The French donor approved a grant for implementation of a pilot project for organized screening of cervical cancer that would serve as a basis for developing a national programme. The project management was entrusted to WHO Regional Office for Europe – WHO Country Office in Serbia (at the time Serbia and Montenegro) and was planned for implementation through the national health system (public health institutes and health service).

The project went through the following phases:

- Design of social mobilisation, action plan and implementation of social mobilisation.
- Setting up the Central Cytology Laboratory and the quality assurance system for cervical screening.

TABLE 3
THE DISTRIBUTION OF CERVICAL SMEAR RESULTS IN THE SCREENED POPULATION OF BRANICEVO REGION

Result of PAP smear	PAP I	PAP II	PAP IIIa	PAP IIIb L-SIL	PAP IIIb H-SIL	PAP IV	PAP V	Unsatisfacotry	Total
Number of women examined	554	11057	520	340	94	26	4	168	12,763
%	4.34	86.63	4.08	2.66	0.74	0.20	0.03	1.32	100.00

PAP – Papanicolaou, L-SIL – low grade squamous intraepithelial lesion, H-SIL – high grade squamous intraepithelial lesion

- Developing the Information Technology (IT) for screening.
- Training in software application and continuous on-site IT support.
- Regular weekly quality assurance visits to cytological laboratory.
- Setting up monitoring and evaluation system.
- Drafting of national strategy – process support.

Screening invitations were sent to 22,300 women aged 30–49 years in the Branicevo district and 12,763 women were screened. Sixty three high-grade squamous intraepithelial lesion (H-SIL) and 6 invasive cancers were detected in the screened population with the estimated cervical cancer incidence of 47.01 per 100,000 and 49.36 H-SIL lesions per 100,000 women (Table 3).

The specific project objectives were achieved:

- 60 % of target population (women aged 30–49 years of age) in the Branicevo district were screened for cervical cancer with cytological methods.
- Local Health experts were trained in methods of cytological screening and a system of quality assurance was set up.
- Bethesda system of cytological classification was successfully introduced into the practice.
- 16 % of all slides were re-screened through quality assurance system, all slides with positive findings at first screening and 10 % of slides assessed as negative at first screening.
- 95 % of matching between 1st and 2nd screening was identified.
- 1.32 % of slides were inadequate for cytology and had to be repeated.
- All women identified with pre-malignant lesions through the screening project were referred for full diagnostic procedure and required treatment.
- Effects of the pilot project on mortality for cervical cancer screening in the district will be monitored but will be visible only after a longer time period.

Conclusions

The results and experiences from the Branicevo pilot project have been used for development of a National programme for cervical cancer screening, which is expected to be finalized in 2007, and launched over a 3-years period in order to cover all Serbian women aged 25–64.

The development of national strategy is essential for the sustainability of the effects of the project in the pilot district and in Serbia in total. The Ministry of Health of Serbia had set up a National Committee for Prevention of Cervical Cancer, in July 2006. WHO will continue to provide technical support to the National Committee in 2007 (through its regular programme) until a National Programme for Cervical Cancer Screening and action plan for nation wide implementation over a 3-years period are fully developed.

In addition to supporting the development of the National Programme, the WHO also supported the National Cancer Registry so they were able to update the national cancer data-base. It is expected that in 2007, the National Cancer Registry will be able to start to monitor pre-malignant lesions of the cervix (from the pilot district) and when a national programme starts to be implemented to monitor pre-malignant cervical lesions nation wide. The Institute of Public Health (IPH) has been specifically supported to strengthen the Cancer Registry Department and to prepare and print Annual Cancer Registries for 2002 and 2003 (2004 and 2005 were prepared as well and will be published with internal resources of the IPH of Serbia in 2007).

At the end of 2006 the process of development of a National Programme for Cytological Screening of Cervical Cancer and preparing of a national action plan is fully under way. However, the operationalisation of the Programme and its implementation will depend on integration of this new public health programme into the financing mechanisms of the Serbian health system (combination of the national health insurance for individual services and Ministry of Health budget for the support to the public health component – social mobilisation, organisation of programme, programme monitoring and implementation).

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PROBIR RAKA VRATA MATERNICE U SRBIJI

SAŽETAK

Rak vrata maternice zauzima drugo mjesto među bolestima žena u Srbiji, odmah iza raka dojke, sa 1.089 registriranih novih slučajeva te dobno-standardiziranom stopom pojavnosti od 27,2 na 100.000 žena u 2002. g. Na četvrtom je mjestu po smrtnosti sa 452 smrtna slučaja te dobno-standardiziranom stopom smrtnosti od 7,2 na 100.000 žena. U usporedbi s ostalim europskim zemljama pojavnost raka vrata maternice u Centralnoj Srbiji je najviša. Regionalne razlike u pojavnosti ovog raka su jako izražene u Srbiji, sa najnižom dobno-standardiziranom stopom pojavnosti (16,6 na 100.000 žena) zabilježenom u području Mačvanski, a najvišom u istočnoj Srbiji te području Beograda gdje su stope pojavnosti dvostruko više (32,5–38,1 na 100.000 žena). Prevencija raka vrata maternice u Srbiji se temelji na oportunističkom probiru koji je karakteriziran visokom pokrivenosti mladih te niskom pokrivenosti starijih i žena srednjih godina. Probir odabranih skupina žena zaposlenih u velikim tvrtkama se provodi u mnogim područnim bolnicama jednom godišnje, međutim ovaj pristup ima mali utjecaj na bolest i smrtnost. Nedavno je Ministarstvo zdravstva imenovalo Grupu stručnjaka koja bi razvila i provela program probira raka vrata maternice na nacionalnoj razini. Provodi se velik broj pilot-projekata rezultati kojih se koriste za razvoj nacionalnog programa probira za rak vrata maternice. Očekuje se da će to biti dovršeno u 2007. g. te, kroz razdoblje od 3 godine, pokretanje programa probira koji bi pokrivao sve žene Srbije u dobi između 25 i 64 godine.

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Professional paper

Cervical Cancer in Croatia: State of the Art and Possibilities for Prevention

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A B S T R A C T

In Croatia, there are about 355 incident cases and about 100 deaths from cervical cancer every year. The aim of this study is to present the trends of cervical cancer incidence and mortality and to propose preventive strategies for cervical cancer in Croatia. Age-standardised and age-specific cervical cancer incidence rates were calculated for the period 1985–2004. For cervical cancer mortality data, the WHO Mortality Database was used. After an early decrease of cervical cancer incidence and mortality following the introduction of opportunistic screening in Croatia, no further decrease has been observed since the 1990s. An increase in incidence over the last 20 years was observed in the age-groups 40–44 and 45–49 years. To reduce cervical cancer rates, an organised cervical cancer screening programme is essential. In addition, HPV vaccination should be introduced in the school vaccination programme to achieve further reductions in cervical cancer incidence in the future.

Key words: *cervical cancer, prevention, Croatia*

Introduction

Cervical cancer is the second most common female cancer worldwide with about 493,000 incident cases per year, and it is the most common female cancer in Africa, South America and Asia[1]. There are about 273,000 cervical cancer deaths in the world yearly, 85% of which take place in developing countries[1]. In Europe, there are about 50,000 new cases, and about 25,000 deaths yearly[2]. The majority of incident cases occur between ages 30 and 50. The highest incidence of cervical cancer in Europe is observed in Eastern European countries. As a consequence of cervical cancer screening by Papanicolaou (Pap) smear, cervical cancer incidence and mortality rates have been decreasing over the last three decades in most of the European countries. In countries with organised cervical cancer screening programmes, cervical cancer is usually the 10th most common female cancer[1–3].

The Croatian population as assessed by the 2001 census was 4.4 million[4]. The annual cancer incidence is about 20,000 cases and cervical cancer is the 8th most common female cancer in this country. There are on average 355 new cases, and about 100 deaths each year[5,6]. Croatia has a lower cervical cancer incidence than most Central and Eastern European countries, but it is still much higher than in countries with organised cervical cancer screening programmes[1]. Relative 5-year survival of cervical cancer patients in Croatia diagnosed in the period 1994–1998 was 74%[7].

A possibility for primary prevention of cervical cancer is vaccination against the Human papillomavirus (HPV) that has been identified as a necessary cause of cervical cancer[8]. About 60% of cervical cancer cases are attributed to HPV type 16, and additional 10–20% to HPV type 18 infection[9–12]. The currently available HPV vaccines that have recently been approved in the US and Europe, protect against the oncogenic HPV 16 and HPV 18 (quadrivalent HPV [types 6, 11, 16, 18] recombinant vaccine GARDASIL®, Merck and Co.). Because the genital types of HPV are primarily transmitted by sexual contact, the optimal age for vaccination of girls is before the onset of sexual activity. Vaccination of males has not been considered cost-effective[13–15]. The report on GARDASIL® efficiency provided to the US Food and Drug Administration and the European Medicines Agency, states that the vaccine can reduce HPV 16 or 18 related cervical intraepithelial neoplasia grade 2 and 3 (CIN 2/3) or adenocarsinoma *in situ* (AIS) by 39.0% (95% CI: 23.3–51.7), while reducing treatment rates for any CIN by 16.5% (95% CI 2.9–28.2)[13,14].

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The current HPV vaccines will not prevent all oncogenic HPV infections and therefore cannot replace the traditional cervical cancer screening. However, screening methods and intervals may be modified to account for changes in population risk for the development of this disease[15–18].

Historically, cervical cancer screening by Pap smear, when followed by adequate therapy, has been proven to substantially reduce cervical cancer incidence and mortality. Following the introduction of such effective programmes, cervical cancer incidence and mortality has been reduced by more than 80% particularly in British Columbia and the Nordic countries[19]. The Pap smear was introduced in the mid 1940s, and entered wider use in the late 1950s. Organised national cancer screening programmes are currently available only in 8 EU countries (Finland, Denmark, Iceland, Italy, Norway, Sweden, Slovenia and UK). The European guidelines specify that cervical cancer screening programmes should target the age-group 25–64 years[20–22]. The International Agency for Research on Cancer (IARC) also recommends the introduction of HPV-DNA screening within organised programmes in such a way that the efficacy and effectiveness can be evaluated[9].

Croatia does not have an organised national cervical screening programme. Following the recommendations for cervical cancer screening in Europe, a Working Group of the Croatian Ministry of Health and Welfare has proposed a national cervical cancer screening programme, although this has not yet been introduced[23]. The proposed programme comprises screening of women aged 25–64 years every three years by Pap smear in the first phase. In the second phase of the programme, in addition to Pap smear, HPV test would be introduced for women aged 30–64, with five-year screening intervals[23].

The aim of this study was to present the trends of cervical cancer incidence and mortality between 1985 and 2004 in Croatia, and to propose preventive strategies for cervical cancer in Croatia.

Materials and Methods

The source of data on cervical cancer mortality in Croatia for the period 1985–2004 was the WHO mortality database[24]. Only the data for cervical cancer (ICD-10 code C53), without data on death associated to »Uterus not otherwise specified« (NOS) (ICD-10 code C55) are presented. However, the data on cervical cancer deaths are likely to be underestimates since there are over one hundred deaths per year from cancer of uterus NOS in Croatia and a proportion of these will be due to cervical cancer as well. For the data on cervical cancer incidence, we used the Croatian National Cancer Registry data[5]. To calculate the age-specific rates for the year 1985, we used the Croatian population census for 1981; for the period 1986–1995 we used the Croatian population census for 1991; and for the period 1996–2004, we used the Croatian population census for 2001[4,25,26]. Age-standardised rates of cancer incidence in Croatia for the period 1985–2004 were calculated by the direct standardization method, using the World standard population as a reference[27].

Results

The age-standardised cervical cancer incidence rates show a decreasing trend until the year 1991 but no further consistent decrease in cervical cancer incidence has been observed afterwards. The age-standardised rates of cervical cancer mortality remained at a low level during the entire period but no decrease was observed over the last decade (Figure 1).

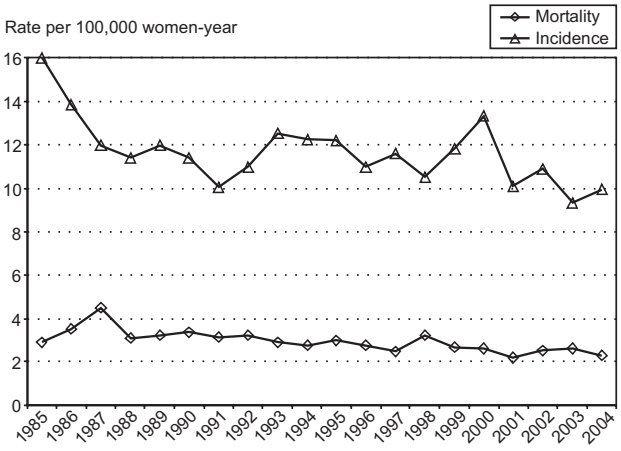

Fig. 1. World age-standardised rates (ASRW per 100,000 women-year) of cervical cancer incidence and mortality in Croatia, 1985–2004.

Table 1 shows the age-specific rates of cervical cancer in Croatia for the age-groups targeted by the proposed screening programme[23]. In the age groups between 25–39 years, a decreasing trend of cervical cancer incidence is observed. An increase of cervical cancer incidence over the last 20 years has been observed in the age-groups 40–44 and 45–49 years. In the age groups between 50 and 64 years, a decrease of cervical cancer incidence is observed, which is most prominent in the age group 60–64, being over 5-fold over the 20-year period.

Discussion

Opportunistic cervical cancer screening was introduced in Croatia in the 1960s and this was accompanied by decreasing cervical cancer incidence and mortality trends through to 1991. However, no substantial further decreases in cervical cancer incidence and mortality were observed subsequently (Figure 1). The largest decrease of cervical cancer incidence over time is observed in women between 25 and 39 years of age (Table 1). In addition to a higher awareness of the importance of preventive medical examinations in these women, this could be accounted for by opportunistic screening in scope of gynecological examinations for either pregnancy or con-

TABLE 1
AGE-SPECIFIC INCIDENCE RATES (PER 100,000 WOMAN-YEARS) OF CERVICAL CANCER IN CROATIA IN THE PERIOD 1985–2004

Year	Age-group							
	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64
1985	9.4	22.6	38.7	17.7	20.7	28.7	39.1	66.1
1986	10.6	14.4	17.5	22.5	36.8	31.7	36.4	40.0
1987	10.0	15.0	19.1	17.8	29.1	24.6	32.1	36.7
1988	7.1	18.8	14.2	17.8	16.9	31.7	25.9	35.4
1989	15.3	15.5	20.2	18.4	23.0	24.6	31.4	42.0
1990	10.6	13.8	24.6	26.7	16.1	16.8	25.3	26.9
1991	5.9	18.3	20.2	18.4	26.1	16.2	18.5	30.2
1992	5.3	14.4	17.5	27.2	23.8	17.5	25.3	28.2
1993	11.8	17.7	38.8	26.7	19.9	25.3	21.0	23.0
1994	9.4	17.7	27.3	26.7	29.1	22.0	20.3	31.5
1995	7.1	15.5	26.2	34.9	30.7	22.7	26.5	26.2
1996	6.2	10.9	21.4	32.4	24.2	20.5	30.6	24.8
1997	9.6	13.6	30.9	30.0	24.2	19.8	19.0	21.2
1998	6.2	11.5	20.2	28.2	25.4	19.1	25.6	25.5
1999	6.9	20.3	21.4	25.2	21.8	23.8	32.2	33.3
2000	11.0	21.7	29.0	29.4	39.9	23.8	22.3	23.3
2001	7.5	12.9	30.9	28.2	21.8	18.5	13.2	21.9
2002	4.8	16.9	25.8	26.4	23.0	29.0	16.5	24.1
2003	4.8	10.8	18.3	24.6	25.4	19.8	23.9	18.4
2004	5.5	9.5	22.7	21.6	34.5	22.4	28.1	12.0

traceptive use counselling. However, the lack of a decreasing trend in women between 40 and 49 years of age is more worrying, since this age-group comprises over a quarter of the incident cervical cancer cases in Croatia (Table 1). Meanwhile, the decrease of incidence in age-groups 50–64 probably reflects higher awareness of postmenopausal women about the importance of gynecological examinations (Table 1).

The available evidence indicates that opportunistic cervical cancer screening in Croatia has had an impact. The number of Pap smears taken yearly is still increasing and reached 433,671 in 2005. However, in the absence of an organised population-based programme it is difficult to assess the efficacy of this screening and it is clear that a large proportion of target population still remains un-screened or under-screened[23].

In spite of the decrease of cervical cancer incidence following its introduction, opportunistic screening is not an optimal method for the prevention of this disease as it tends to overscreen the wealthy and well-educated while under-screening the less affluent and minorities, thereby creating inequalities in the healthcare that is delivered to the population[20]. The only way to resolve this problem and to achieve further reductions in cervical cancer cases is through the introduction of an organised cervical cancer screening programme. Following the introduction of such programmes, cervical cancer incidence and mortality has been reduced by more than 80% particularly in British Columbia and the Nordic countries (Finland, Denmark, Iceland, Norway and Sweden)[19]. In addition, organised screening programmes are the only way that countries will be able to derive the maximum benefit from the new HPV vaccines that will soon be launched in Croatia.

Conclusion

There is now a very urgent need to move forward with the introduction of a comprehensive organised cervical cancer prevention programme in Croatia. Indeed, this is the only way that we will be able to further reduce cervical cancer rates while providing equitable, cost-effective protection to all the women of Croatia.

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RAK VRATA MATERNICE U HRVATSKOJ: POSTOJEĆE STANJE I MOGUĆNOSTI ZA PREVENCIJU

SAŽETAK

U Hrvatskoj godišnje oko 355 žena obolijeva, a oko 100 umire od raka vrata maternice. Cilj ovog rada bio je prikazati trendove incidencije i mortaliteta od raka vrata maternice u Hrvatskoj te predložiti strategije za prevenciju. Izračunate su dobno-standardizirane i dobno-specifične stope incidencije raka vrata maternice za razdoblje 1985–2004. Za podatke o mortalitetu od raka vrata maternice koristili smo bazu podataka Svjetske zdravstvene organizacije. Nakon ranog pada incidencije i mortaliteta od raka vrata maternice u Hrvatskoj koji je pratio uvođenje oportunističkog probira, daljnji pad se ne primjećuje nakon 1990-ih godina. Opažen je porast incidencije u zadnjih 20 godina u dobnim skupinama 40–44 i 45–49 godina. Da bi se smanjile stope incidencije i spriječile smrti od raka vrata maternice, potrebano je što prije uvođenje organiziranog programa probira. Da bi se postiglo daljnje smanjenje incidencije raka vrata maternice, potrebno je također uvođenje cjepiva protiv humanog papilomavirusa (HPV) u program obaveznog cijepljenja školske djece.

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Professional paper

Cervical Screening in the UK and Laboratory Quality Control in the Context of the 2007 European Guidelines

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A B S T R A C T

The first part of this article will provide an overview of cervical cancer screening in the UK during the years before, during and after the introduction of a highly successful centrally organised cervical screening programme in 1988: since then the incidence of invasive cervical cancer has fallen by more than 40%. Screening was introduced in a background of opportunistic screening with poor quality control during a period of time when risk of disease was increasing, which will be demonstrated by national registrations of carcinoma in situ as well as invasive cancer. The programme is still facing new challenges and has recently recorded falling screening coverage in younger women, the causes of which have yet to be established. Liquid-based cytology is in the process of being rolled out nationally but high-risk human papillomavirus testing has yet to be introduced into the National Health Service (NHS) programme. Lessons from our experience may be relevant to countries introducing and maintaining organised programmes elsewhere under similar circumstances. The second part of the article will consider laboratory quality control as practiced in the UK and as recommended in the second edition of the European Guidelines for Quality Assurance in Cervical Cancer Screening. These evidence-based guidelines provide recommendations for organising and monitoring quality control as well as for introducing new technology and standardising terminology, which are equally relevant for new and existing programmes. Invasive cancer audit may highlight areas where procedures could be improved in any programme but also can also demonstrate the effectiveness of screening.

Key words: *Organised cervical screening, quality control, invasive cervical cancer incidence, carcinoma in situ, cervical intraepithelial neoplasia, new technology*

Introduction

Across Europe in 2002 there were approximately 60,000 new cases and 30,000 deaths from invasive cervical cancer with a 5-fold variation in those rates[1]. The lowest were in countries such as the United Kingdom (UK) with organised programmes, which can maximise the positive and minimise the adverse effects, but there are many different models of care. The aim is to reach the population at risk and provide a high quality service that takes account of specificity as well as sensitivity of the test. Demographic differences and changes of risk with time must also be considered when comparing programmes in countries as diverse as the whole of Europe.

Although rates of mortality and incidence relate to screening coverage of the population at risk, no amount of screening will be successful without good quality control. The second edition of the *European Guidelines for Quality Assurance in Cervical Cancer Screening* provides a comprehensive overview of cervical cancer control in Europe and describes methodology for all aspects of quality control[1]. These guidelines are equally relevant to existing and new programmes with respect to target populations, intervals for screening, evidence-based indications for new technology, methodology for quality control, monitoring the effectiveness of screening and the importance of standardising terminology across a diverse and increasingly mobile group of nations.

Cervical screening has been successful in the UK since a centrally organised National Health Service Cervical Screening Programme (NHSCSP) was introduced

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in 1988, which can be demonstrated by national registrations of invasive and *in situ* carcinoma of the uterine cervix (www.statistics.gov.uk). Registrations prior to 1992 can be obtained from the Office for National Statistics on CD ROM[2]. The effects of changing risk of disease, screening coverage and quality control continue to influence the evolution of the programme and may provide lessons for other countries. The challenge of new technology affects new and existing programmes alike and should be introduced as and when it can be shown to improve sensitivity or specificity of the test. The experience of introducing liquid-based cytology (LBC) to the NHSCSP will be discussed as well as the role of high-risk human papillomavirus (hrHPV) testing, which is yet to be introduced nationally.

Screening in the UK Before and After Organised Screening Was Introduced

During the 1970s and 1980s general practitioners were paid a supplement to screen women aged 35–64, or earlier for women with three or more children. A relatively ineffective paper-based system of 5-yearly recall was in place but most screening was opportunistic, mainly in young women, and quality control was poor. Incidence per 100,000 total female population remained almost steady at 15.0 to 16.0 throughout the 1970s and 1980s when UK rates were the highest in Europe. However, there was a striking change in the pattern of disease during those decades[3]. Although overall numbers of cancers changed little during a 10-year period there was a nearly 3-fold increase in rates of deaths, invasive cancers, registrations of carcinoma *in situ* and high-grade cytological abnormalities in women born since the mid-1940s almost certainly because of the greater sexual freedom allowed by the availability of reliable contraception[4]. Screening coverage was poorly documented until 1990 but was lower in older (over age 40) than younger women. National registrations of carcinoma *in situ*, which include cervical intraepithelial neoplasia (CIN) grade 3 since 1983, provide evidence that many young women were effectively screened, albeit opportunistically: the number of registrations of carcinoma *in situ* had already overtaken those of invasive cancer as early as 1981 (Figure 1 and 2). Shortly before the introduction of organised screening in 1988 incidence of invasive cancer had started to rise, reaching a maximum 17.4 in 1988: the age distribution reflects the increased risk in younger women (Figure 3). It was recognised at the time that quality control was poor[5].

The organised programme aimed to improve all aspects of quality control as well as setting up a centralised register to invite all eligible women aged 20–64 at least every five years. Incidence fell steadily during the 1990s to a figure fluctuating between 8.0 and 9.0 since 2001. Mortality rates declined from 1988 in all birth cohorts (from 1922) screened at least once in the new programme but declined earlier in life for women first screened when they were younger (the latest in the study were born in 1952) in whom the death rate was substantially lower throughout life[6]. This evidence is supported by the observation that high-grade cervical intraepithelial neoplasia (CIN) is predominantly treated in young women: more than 80% of CIN3 was found in women aged less than 40 before, during and after the introduction of organised screening (Figure 4). There has been some concern about a recent decision in the UK no longer to invite women aged 20–24 for screening[7], which was based on results of a national audit[8] and is consistent with EU guidelines[1]. However, a recent study in Iceland showed the benefit of lowering the age limit to include women aged 20–24[9], which is in accordance with recommendations in the USA (www.ahrq.gov/clinic/3rduspstf/cervcan/cervcanrr). The NHSCSP is facing a new challenge since a fall in screening coverage has been recorded in young women, particularly those aged 25–29 in whom the highest prevalence of CIN3 is recorded. The reasons for the fall in coverage have yet to be established but the evidence suggests a need to encourage young women to be screened even though there is debate about the age of the first invitation.

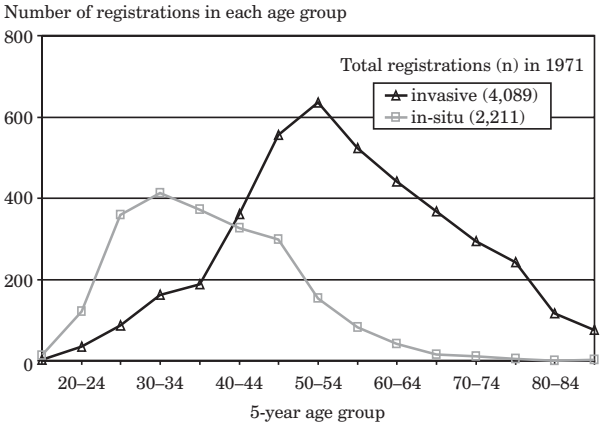

Fig. 1. Registration of invasive and in situ carcinoma of the uterine cervix in England & Wales (Source: Office for National Statistics, Cancer 1971–1997[2]).

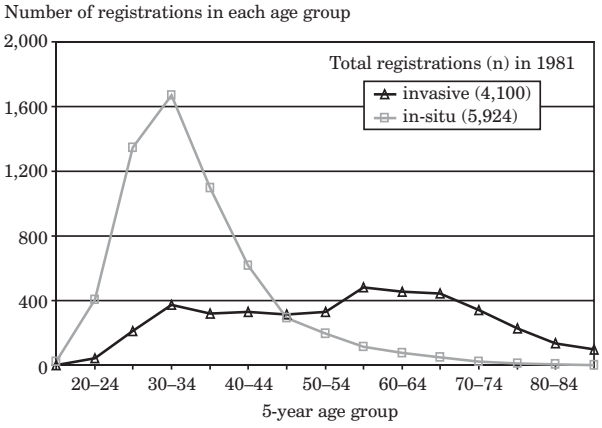

Fig. 2. Registration of invasive and in situ carcinoma of the uterine cervix in England & Wales in 1981 (Source: Office for National Statistics, Cancer 1971–1997[2]).

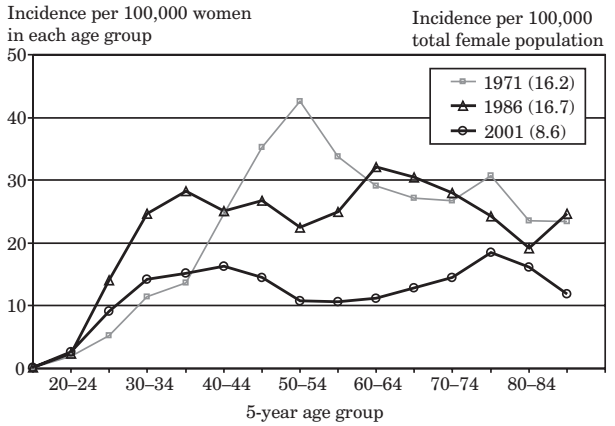

Fig 3. Incidence of invasive cervical cancer in England in 2001 (Source: www.statistics.gov.uk/statbase), and England & Wales in 1971 & 1986 (Source: Office for National Statistics, Cancer 1971–1997[2]).

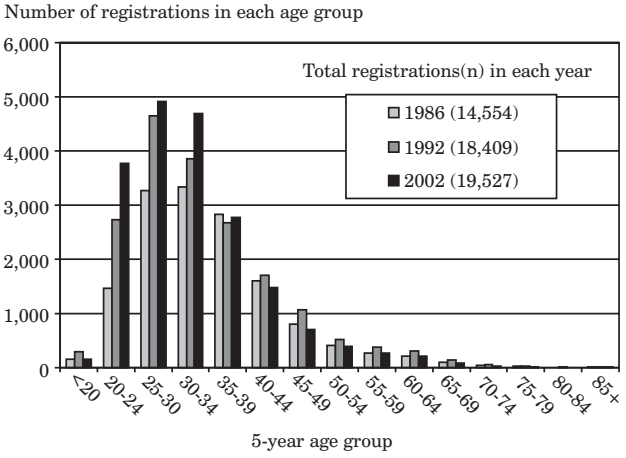

Fig. 4. Registrations of carcinoma in situ, England and Wales (1986) and England (1992 and 2002)[7].

Quality Control of all Aspects of the Screening Programme

The European guidelines emphasise the importance of quality control of all aspects of the screening programme[1]. Invitations for screening should be clear but informative, smear-takers should receive training and should be able to explain the test results to women; laboratories should have the infrastructure required for a high-quality service in terms of accommodation, equipment, adequate numbers and levels of non-medical and clerical staff, availability of training and update, information technology and medical supervision; colposcopy clinics should similarly be equipped, staffed, trained, supervised and monitored; and terminology should at least be translatable into nationally recognised systems. The NHSCSP also addresses all aspects of the programme, providing evidence-based guidelines and monitoring performance through regional quality assurance reference centres (QARCs). A centralised computer-based register of screening records is essential and ideally should include histology and colposcopy records and should be linked to regional and national cancer incidence and mortality registries.

Quality Control in the Laboratory

The mechanisms of laboratory quality control will be considered with respect to the interlinked functions of primary screening, final cytology reports and comparison with outcome. At each level accuracy depends on the subsequent as much as the previous step and the final outcome itself depends on the accuracy of the histology report, which should not be regarded as a »gold-standard« unless it is also subjected to audit and quality control.

Quality control of primary screening

Accurate primary screening is central to the cervical screening process and its quality control is essential. One of the strengths and weaknesses of cervical cytology screening is the ease with which the glass slide may be reviewed, often with the benefit of hindsight, and methods of quality control should recognise that no test will be perfect[10,11]. The most powerful methods of primary screening quality control involve re-screening the slides before the reports are issued, thus reducing false negative rates at the same time as monitoring individual and laboratory performance. Rapid re-screening of all negative and inadequate smears (or liquid-based slides) has been shown to be an effective method of minimising false negative results[12]. Rapid pre-screening may be even more effective[13] and both methods are recommended in the European guidelines. Both are preferable to proportional re-screening of 10% of negative slides or targeted re--screening of supposedly high-risk cases. Where rapid re-screening or pre-screening is in place, as in the UK, it may be possible to avoid targeted re-screening altogether. It should be recognised that either method may fail to detect the very abnormalities that are so easily missed: slides with small numbers of abnormal cells as well as pale and small cell dyskaryosis have been shown to be »at risk« for not being detected at primary screening of conventional smears[14,15] and small numbers of abnormal cells may equally well be missed in liquid-based cytology[16]. Sensitivity of primary screening for the individual and laboratory may be calculated against the final report after the second screen has taken place and should be calculated for high-grade cytology alone and for all abnormalities. It may be helpful to calculate the »detection rates« of the final report as part of a performance profile for each screener[17]. Performance profiles should be monitored confidentially and sensitively, expectations should not be too high, and procedures to deal with genuine poor performance should be agreed in advance.

Pathologists' and laboratory reporting rates

Apparent sensitivity of primary screening may be influenced by the accuracy of the final report. A pathologist with an inappropriately low threshold for reporting atypical or borderline changes may spuriously reduce the sen-

sitivity of the screener. Conversely it is possible for a pathologist to override a screener's opinion and report a genuine abnormality as reactive or benign. Pathologists and laboratory performance may be monitored by comparing reporting rates of high-grade and low-grade cytology results between observers and between laboratories, which can improve consistency[18]. Comparison of high-grade reporting rates is used for laboratory quality control in the NHSCSP and achievable ranges are set each year based on the 10th and 90th percentiles of national reporting rates (www.cancerscreening.nhs.uk/cervical/statistics). Rates are monitored internally by laboratory staff, externally by QARCs and are published nationally each year. While the low limit of high-grade cytology rates provides a surrogate for sensitivity, the high limit of low-grade cytology reflects specificity.

Positive predictive value

Positive predictive value (PPV) provides a convenient measure of accuracy of high-grade cytology reports and may be regarded as a surrogate for specificity. In the UK PPV is measured as the percentage of high-grade cytology results that are confirmed by at least CIN2 on biopsy (using cases with known colposcopy outcome as the denominator). Thus PPV may be influenced by the »moving target« of the histological distinction between CIN1 and CIN2 and by the sensitivity of detection of high-grade cytology: high PPVs may be seen with low detection rates of high-grade cytology. In the UK an achievable range of PPV is monitored rather than a lower limit only. Sensitivity of the test is more difficult to measure as women with negative cytology are seldom referred for colpsocopy. The rate at which referrals for persistent low-grade cytology or less are found to have at least CIN3 may be used as a surrogate for sensitivity and provides a useful balance for PPV (www.cancerscreening.nhs.uk/cervical/statistics).

Quality control of histology

Because of its central role in the screening process, cytology reporting has been subjected to far more intense quality control than histology reporting although the latter forms the basis for decisions about treatment and management of CIN and invasive cancer. This is stressed in the European guidelines as well as in the UK programme and the NHSCSP provides illustrated guidelines for reporting cervical biopsies[19]. It is more difficult to monitor reporting rates in histology because of the absence of a population baseline. Nevertheless, quality is greatly be improved by colposcopic biopsies being reported by specialist teams and slides being reviewed and presented along with the cytology at multidisciplinary meetings. Immunohistochemistry is more readily applicable to histology than cytology and difficult distinctions between low-grade and high-grade CIN may be augmented by Ki67 and $P^{16ink4a}$ staining[20]. CIN reflects a continuous spectrum of precancerous changes carrying an increasing risk of progression so there will always be inter-observer variation about the dividing lines between HPV infection and CIN1, between CIN1 and CIN2 and between CIN2 and CIN3. CIN3 has been shown to be the most robust of these diagnoses[21] and for this reason the EU guidelines are justified in maintaining the CIN classification for histological diagnosis although decisions for treatment are usually made at the level of CIN2.

Principles of laboratory quality control

Quality control should be a continuous process involving correlation of all parameters and taking account of the nature of the spectrum of CIN. It requires good record-keeping and communication – both personal and electronic – at all stages of the process. Communication is greatly improved by standardising terminology, which is naturally more difficult with so many different languages in use across Europe. The EU guidelines have solved this problem by recognising that all terminologies should be locally agreed and should at least be translatable into the Bethesda system (Figure 5), which is widely used throughout the world[22]. TBS is recommended for cytology, for which it was originally developed, and the CIN system is retained for histology.

Testing and Introducing New Technology

The European guidelines are being published at a time of greatest change since the Pap smear was introduced as a screening test more than 60 years ago. Laboratories, gynaecologists, politicians and women have been

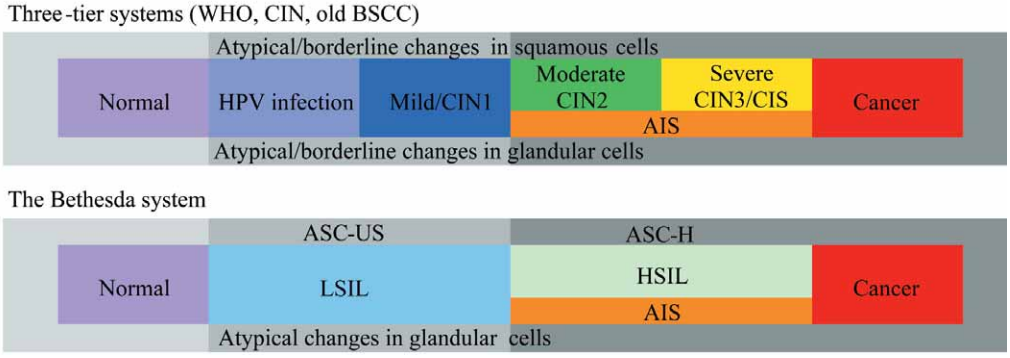

Fig. 5. Conceptual categorisation of cytological findings in a Pap smear of the uterine cervix[1].

placed under enormous pressure to introduce expensive new technology including LBC, hrHPV testing and automated screening. Technology is gradually converting a simple inexpensive test into a highly complex and much more costly multi-step process. Whether this new technology has saved any lives has yet to be determined[23]. LBC was introduced throughout the UK after implementation at pilot sites in Scotland, Wales and three screening centres in England. The report recommending its implementation made the guarded statement that »overall sensitivity was at least as good as, and may be better than, the Pap smear« [24]. The main advantage in the UK was the striking fall in the pilot sites in rates of inadequate tests, which were higher with Pap smears compared with elsewhere in the world. There are several caveats to this dramatic decline in rates of inadequate tests[25]. Firstly, as NICE pointed out, »there is no way of verifying that a sufficient number of cervical cells have been harvested by the smear taker«[24]. Secondly, all non-normal rates are higher in the UK, with 3–5 yearly screening than in places with annual screening because there are fewer negative tests. Thirdly, »quality indicator comments« about poor cellularity, lack of transformation zone sampling and inflammatory exudate, which are allowed with the Bethesda system[22], are not allowed in the UK. Furthermore, litigation is relatively common in the UK and cytologists are aware that inadequate smears inappropriately reported as negative have been sited as reasons for »false negative« cytology preceding invasive cancer[26]. A multi-centre study has been funded by the Health Technology Assessment programme to develop criteria for LBC adequacy appropriate for a 3–5 year programme. The European guidelines recognise that criteria may be different for organised programmes with restricted intervals and age ranges for screening and, while regarding LBC as an acceptable method of cell collection, have not recommended it in place of conventional cytology in the absence of a randomised clinical trial to demonstrate its superiority[1].

One of the main advantages of LBC is that it facilitates hrHPV testing using residual material in the vial. Some would say that hrHPV testing could replace cytology for primary screening[27] but this would probably only be feasible for women over 35 because of high prevalence of hrHPV in younger women. hrHPV testing is developing a firmer place in the triage of women with borderline or atypical cytology almost half of whom may not need investigation or frequent surveillance[28,29]. Similarly it is likely to have an important role in follow-up after treatment of high-grade CIN when the minority of women with persistent hrHPV could be monitored more closely, while the majority could be returned to routine screening. Essentially, hrHPV is the one development that has the potential to increase specificity of the test by reducing the number of women investigated for borderline and ASCUS smears.

HPV vaccination is the one new development that may fundamentally change cervical cancer screening and is the subject of a supplement in the European guidelines and as a separate publication[30]. Suffice it to say here that vaccination will not remove the necessity to screen the current generation of women, including women who are vaccinated in adult life. Falling rates of abnormal cytology in vaccinated women coupled with a false sense of security might present additional challenges to screening programmes. There will be even more need to maintain quality control and high sensitivity of the test – to the extent that hrHPV testing might be needed as a primary investigation. There is no doubt that vaccination will radically affect screening programmes worldwide but little is yet known of its likely uptake and even the duration of protection provided.

Invasive Cancer Audit and its Role in Quality Control

The ultimate audit of cervical screening lies in monitoring invasive cancer incidence and mortality but, as indicated above, these rates should be assessed in relation to levels of high-grade CIN or CIN3 successfully detected – both as a measure of effectiveness of screening and of risk of disease. Invasive cancer rates are likely to be low in local screening centres and are therefore inappropriate as a local guide of effectiveness. However, audit of mode of presentation, stage of cancer and screening history provides a valuable method of monitoring local, regional and national screening programmes. Screening histories identify areas where screening processes may be improved – not only with respect to screening and reporting the slides. There are multiple factors that may lead to cancers not being prevented in screened women: screening may have been intermittent or infrequent, abnormalities may have been missed on slides, samples may have been inadequate, low-grade abnormalities may have been under-interpreted or not followed up, high-grade abnormalities may have been reported but not investigated and treatment of CIN may have been incomplete or not followed up[31]. Isolated reasons or combinations of those reasons may be implicated in an individual case and bear witness to the effectiveness of the screening process itself if correctly and accurately conducted. In well-screened populations such as the UK it has proved to be easier to prevent invasive cancer in older age groups[8], presumably because there are more screening opportunities to detect a lesion that does not progress to invasion until later in life. Although cancers in young women are more difficult to prevent, and must have arisen in more aggressive lesions, they are frequently detected by positive cytology in asymptomatic women. These screen-detected cancers are almost invariably diagnosed at stage 1 (either 1A or 1B) and present a benefit of screening that provides a benefit in terms of survival that is not evident from incidence rates[32].

Conclusion

Experience of introducing a highly effective national screening programme in the UK, in a partially screened population during a period of increasing risk of disease,

may provide lessons to other countries introducing national programmes in the inevitable background of opportunistic screening. Auditing screening histories of the unfortunate few women who develop invasive cancer in well-screened populations may identify processes that could be improved. The latest European guidelines for quality assurance are being published as a time of enormous changes in technology and provide evidence-based advice to all of us, whether maintaining existing programmes or starting new ones, to help us navigate those changes in a cost-effective manner that carries cervical cancer control forward into the 21st century.

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PROBIR RAKA VRATA MATERNICE U UJEDINJENOM KRALJEVSTVU I LABORATORIJSKA KONTROLA KVALITETE U KONTEKSTU VODIČA EU 2007

SAŽETAK

Prvi dio članka će dati osvrt na probir raka vrata maternice u UK za godine prije, za vrijeme i nakon uvođenja vrlo uspješnog organiziranog programa probira raka vrata maternice 1988. g., od kada je stopa pojavnosti invazivnog raka vrata maternice pala za više od 40%. Organizirani probir je uveden u sjeni oportunističkog probira sa lošom kvalitetom kontrole u vremenu kada je rizik za bolest rastao, na što će ukazati nacionalni registri za rak vrata maternice *in situ* te za invazivni karcinom. Program još nailazi na nove izazove, a nedavno je zabilježio pad u pokrivenosti mlađih žena, uzrok čega još mora biti utvrđen. Tekućinska citologija je u procesu uspostave na nacionalnoj razini, ali testiranje na visokorizične humane papilomaviruse još mora biti uvedeno u program Nacionalnog zdravstvenog sustava (NHS – National Health Service). Lekcije iz našeg iskustva bi mogle biti od značaja zemljama koje uvode ili provode organizirane programe probira pod sličnim okolnostima. Drugi dio članka će se baviti laboratorijskom kontrolom kvalitete na način kako se provodi u UK te kako je to preporučeno u drugom izdanju »Europskog vodiča za osiguranje kvalitete u probiru raka vrata maternice«. Ovakvi vodiči pružaju preporuke za organiziranje i praćenje kontrole kvalitete, kao i za uvođenje novih metoda te standardiziranja terminologije, što je jednako važno za nove i postojeće programe. Ispitivanje invazivnog karcinoma bi moglo ukazati na područja gdje bi postupci mogli biti poboljšani bez obzira na vrstu programa, a ujedno i prikazati učinkovitost programa.

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Professional paper

Organisation of Cervical Cytology Screening in Croatia: Past, Present and Future

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A B S T R A C T

This presentation highlights strengths and weaknesses of cervical cytology screening in Croatia, with particular reference to the opportunistic screening, the use of conventional Papanicolaou (Pap) test and the analysis of some organizational, educational and performance issues that are associated with it. Its aim is to propose measures to improve the efficacy of cervical cytology screening in order to reduce cervical cancer mortality. Currently, in excess of 450,000 Pap tests/year are examined at 35 laboratories scattered throughout the country. All of these laboratories use standard operating procedures including internal and external quality control. They employ a total of 68 cytologists and 91 cytotechnologists. The sensitivity of cervical screening in Croatia is 90.0%, specificity 98.6%, positive predictive value 92.3%, negative predictive value 98.1% and overall diagnostic accuracy 97.2%. The high diagnostic accuracy of cervical cytology is attributed to the long-standing tradition of education and training of cytologists (postgraduate MSc course since 1967, independent residency since 1974) and cytotechnologists (since 1968). This tradition spanning more than half a century means that today in Croatia there is a developed network of cytology laboratories staffed by highly competent cytologists and trained cytotechnologists. The high accuracy of cancer detection through Pap tests provides strong evidence in support of cervical cytology screening remaining the basic method of prevention for cervical carcinoma. However, some modifications to the current situation are needed. These relate primarily to opportunistic screening. The current screening coverage rate is 68%, although there is capacity, which would allow for all women at risk, i.e. those aged 25–64, to be screened once in three years. The screening coverage relates mainly to those women visiting gynecological out patient clinics for unrelated conditions. A properly organized and controlled national screening programme should replace this. This should be accompanied by the introduction of alternative, highly sensitive methods of sample collection and preparation, such as are available through the introduction of new technologies, e.g. liquid based cytology.

***Key words**: Pap test, cervical carcinoma, cervical screening programme, Croatia*

Introduction

Cytological diagnosis of conventional cervicovaginal smear or Pap test is one of the most efficient screening tests known to date, which has been credited with the significant decline in the incidence and mortality of uterine carcinoma all over the world. In Croatia, it has been used as the main method of secondary prevention of uterine carcinoma for more than half a century now, being at the same time employed as a classic screening test for lesion detection and as a differential diagnosis method predicting histological diagnosis. The rate of cervical carcinoma in Croatia, directly resulting from the large-scale use of Pap test, reflects the indisputable value of the method, while also pointing to some shortcomings.

The current state of cervical cytology in Croatia is presented. In addition to its achievement, the aim was to identify the weaknesses, such as opportunistic screening,

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the use of conventional Pap test and analysis of the organizational, educational and performance issues with the aim to propose measures to upgrade the efficacy of cervical screening.

The History of Gynecological Cytology in Croatia

In Croatia, as in the rest of Europe, gynecologists were the first to perform microscopic analysis of cervical cytological specimens. The first laboratory headed by E. Baršić, a gynecologist, was established in 1953 at University Department of Gynecology, School of Medicine, University of Zagreb, now University Department of Gynecology and Obstetrics, Zagreb University Hospital Center. Jasna Ivić, MD was the first to devote exclusively to gynecological cytology since 1959. In 1968, she was appointed Head of the Laboratory of Cytology, which developed into Institute of Gynecological Cytology in 1984; Jasna Ivić organized cytology service in line with the principles adopted worldwide, and is most credited for the progress of cervical cytology in Croatia[1–3]. During the past fifty years, Institute of Gynecological Cytology in collaboration with other institutions, Department of Cytology and Clinical Genetics, University Department of Gynecology and Obstetrics, Merkur University Hospital in particular, has stimulated continuous development of gynecological cytology in the professional, scientific and educational aspects in Croatia, while promoting due recognition of the Croatian gynecological cytology abroad. At the beginning of the third millennium, these efforts have also been intensified at Department of Gynecological Cytology, University Department of Gynecology and Obstetrics, Rijeka University Hospital Center and Department of Clinical Cytology, Osijek University Hospital, which also became the leading laboratories in the field in Croatia.

The key events in the development of clinical cytology in Croatia are mentioned in Table 1, for which merit goes to many professionals[1], amongst others: Professor Ante Zimolo, Professor Erik Hauptmann, Professor Inga Črepinko, and already mentioned Professor Jasna Ivić.

Specialist Education of Cytologists and Cytotechnologists

The 1967–1974 period characterized by the establishment of organized education of cytotechnologists (continuing education course for cytotechnologists in 1968) and cytologists (Postgraduate Study in Medical Cytology in 1967 and respective residency in 1974, both subsequently renamed as Clinical Cytology). Both played a key role in the development of diagnostic as well as gynecologic cytology in Croatia.

The Postgraduate Study in Medical Cytology was established at the Zagreb University School of Medicine in 1967, with 31 courses attended by 397 postgraduate students held until 2006. The study underwent two revisions, first in 1996 with the introduction of professional studies, and second in 2005 as residency study.

In 1974, independent residency in Medical Cytology, subsequently renamed as Clinical Cytology, was introduced. Initially, residency took three years (pathology one year and cytology two years, with postgraduate study as a form of organized education). Twenty years later, it grew into four-year residency (pathology one year and cytology three years, also with two terms of postgraduate study), within the frame of general revision of residency *curricula* in Croatia. The introduction of independent residency resulted in appropriate education of these professionals, qualified to meet the very demanding challenges posed by the profession.

In the process of approaching the European Union, the phase of adjustment of education in clinical morphology professions of cytology, pathology and forensic medicine has begun, resulting in a new proposal of residency in all three until now independent professions.

The education of cytotechnologists is an important part of education in cytology. Professor Inga Črepinko organized the first course of additional education of medical technicians for cytology technicians. During the 1968–

TABLE 1
KEY POINTS IN THE DEVELOPMENT OF CLINICAL CYTOLOGY IN CROATIA[1]

Year	Event
1967	Postgraduate Study in Medical Cytology, later Clinical Cytology at the School of Medicine of the University of Zagreb
1968	Continuing education course for cytotechnologists at the Medical College of Zagreb
1970	Section of Cytology and Cytological Diagnosis established at the Croatian Medical Association (CMA), to be later renamed as the Croatian Society of Clinical Cytology of the CMA
1970	Later, continuing education courses for the cytologists, cytology technicians, gynecologists, etc., organized by the Croatian Society of Clinical Cytology of the CMA and Zagreb University School of Medicine
1974	Residency in Medical Cytology, later Clinical Cytology, as independent residency regulated by the Ministry of Health and Social Welfare of the Republic of Croatia
1981	Fifth grade qualification – laboratory technician at the Medical College, Zagreb
2000	Continuing education for cytotechnologists at the Ministry of Health and Social Welfare of the Republic of Croatia

1977 period, the education of cytotechnologists was performed in the form of six-month course upon completion of medical technician high school education at Medical College in Zagreb. One-year programme in the form of fifth grade education was offered from 1981 to 1992 at the same College; however, the status of thus trained professionals was not properly solved. The pursuit for continuing, complete and officially recognized education led to the development of two-year programme, initially delivered at the Medical College. The program was verified by the Medical College and the School of Medicine (at the time, College was part of the School of Medicine); however, it has not yet been performed in practice.

Since 2000, sixty cytotechnologists have received education at three courses organized by the Ministry of Health and Social Welfare of the Republic of Croatia, in collaboration with the Croatian Society of Clinical Cytology of the Croatian Medical Association (CMA), and held by Assist. Professor Željka Znidarčić, having thus at least in part mitigated the worrying shortage of these extremely important team members.

The one-year course consisted of 630 periods of practical and theoretical education in all fields of cytology diagnosis with 200 periods of education in particular cytology services. Adjustment to the Bologna Process has opened new options for cytotechnologists at the Medical College; harmonization of the new form and *curriculum* of their education has just been under way.

Current Organization, Medical Staff and Number of Cytological Analyses

Presentation of the current structure in terms of organization, staff profile and number of Pap tests performed is based on a survey performed by the Croatian Society of Clinical Cytology of the CMA in 2003 and 2005.

According to the above survey, gynecological cytology is performed at 35 of 48 (73%) organizational units. Twenty-seven of these are at state-owned health institutions (25 at hospitals and 2 at health centres) and eight in private offices (6 specialist practices and 2 at private polyclinics). Of the twenty-five hospital units, eight are organized as independent departments and seventeen are combined with other professions, i.e. fourteen within pathology, two at the University Departments of Gynecology and Obstetrics, and one at the Department of Laboratory Diagnosis. Eight cytology laboratories are predominantly engaged in gynecological cytology, whereas the remaining 27 laboratories are equally dealing with general diagnostic by cytology and gynecologic cytology.

Two private cytology laboratories were excluded from analysis because their heads failed to submit current data, and one hospital laboratory was excluded due to the very low proportion of Pap tests in their overall performance; thus, data on 32 units are presented (Figure 1).

Sixty-two professionals with university education (not including residents), i.e. 58 clinical cytologists, two pathologists, one anaesthesiologist and one graduated biologist, along with 91 cytotechnologists, and 43 technical, administrative and auxiliary staff members are employed in 32 cytology laboratories. The cytologists to cytotechnologist to other personnel ratio is 1:1.4:0.6. An ideal screening team would consist of one cytologist, two cytotechnologists and one laboratory technician; accordingly, the majority of cytology teams in Croatia are in part incomplete.

A total of 574,290 cervical smears were taken in 2005 in a whole country, of which 452,809 (79%) Pap tests were successfully performed. The proportion of Pap tests

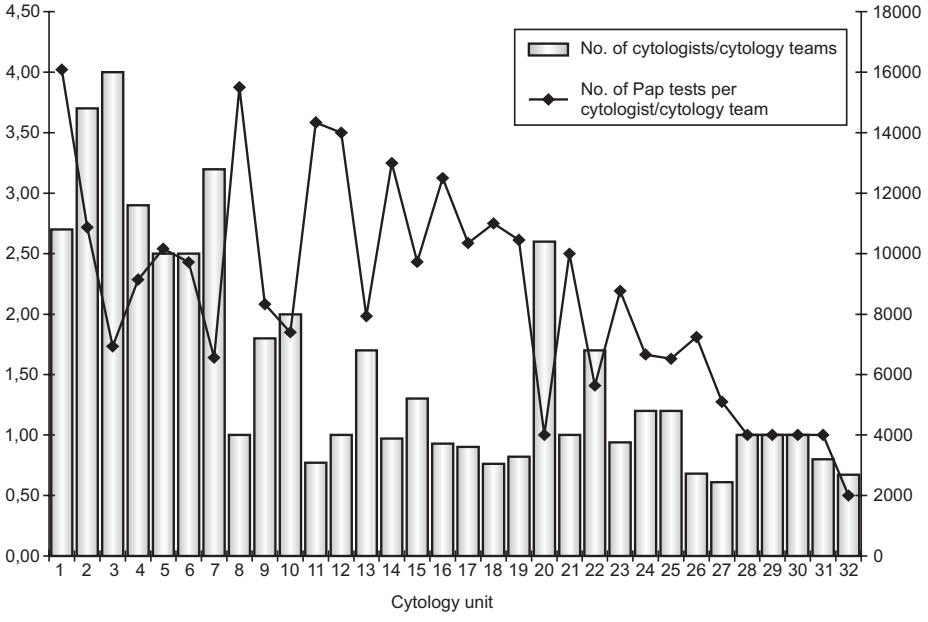

Fig. 1. Number of Pap tests per cytologists/cytology team at 32 cytology units in Croatia in 2005.

out of the total number of tests varied from 59% to 100% in different counties. Forty-nine cytologists and seventy-one cytotechnologists performed these Pap tests. Comparison of the number of Pap tests and number of cytologists and cytotechnologists engaged in their performance showed that one cytologist with the respective team have performed a mean of 9,241 Pap tests, whereas one cytotechnologist examined a mean of 6,378 Pap smears (approximately 28 *per* day!). The laboratories included in the analysis varied greatly according to the number of tests performed, Pap test in particular (Figure 1). The number of Pap tests *per* cytologists/cytology team ranged from 2000 to 16,000, and *per* cytotechnologist from 3300 to 10,000.

The highly uneven pattern of performance recorded in the cytology service across Croatia according to organizational unit and localization, number and profile of professional staff, and number of tests *per* team performed imposes the need of developing distinct legal regulations to standardize the mode of organization, location, staff structure and performance standards in the field of cytological diagnosis, consistent with the respective catchments population. Taking into account more than 30 years of residency in clinical cytology, it is not justifiable that cytology testing is performed by anyone but physicians-cytologists.

Overall, the high number of specialists in cytology and properly educated cytotechnologists, and the number of Pap tests performed (n=452,809) offer a capacity adequate to cover the female population at risk aged 25–64, if screened once in three years. According to the 2001 census, there were 1,178,052 women of these age groups in Croatia[4]; thus, screening per year should cover 392,684 women.

»Zagreb 2002« Uniform Classification of Uterine Cervix Cytological Findings in Croatia

In Croatia, a uniform classification named »Zagreb 2002«[5], a modification of »Zagreb 1990«[6] and »NCI Bethesda System 2001«[7], has been used in cytological analysis of cervical smears. Two groups of »satisfactory« and »unsatisfactory« (explaining the reason for the latter) are used on assessment of specimen adequacy. According to general classification, findings are categorized as »negative for intraepithelial or invasive lesion« (normal finding, changes with reactive and reparatory reactions, a finding suggesting certain risk) and »abnormal cells« (cellular changes that are morphologically consistent with intraepithelial or invasive malignant lesions).

Descriptive diagnosis includes the items »microorganisms« (listing the microorganisms that can be identified directly or based on the specific cytopathic effect), »other non-neoplastic findings«, and »abnormal cells« (squamous, glandular, of undetermined significance, and other malignant neoplasms). The group of »other non-neoplastic findings« that may be found with or without abnormal cells includes reactive cell changes, reparatory epithelium, spare cells, parakeratosis, dyskeratosis, hyperkeratosis, a post-hysterectomy finding of cylindrical cells, a finding of endometrial cells beyond the cycle or in menopause, and a conclusion that the cytohormonal status does not correspond to the patient's age and/or history.

Squamous lesions are divided into three groups, as follows: »atypical squamous cells« (ASC), »squamous intraepithelial lesion« (SIL), and »squamous cell carcinoma«. The ASC group is further subdivided into »undetermined significance« (ASC-US), »high-grade SIL cannot be excluded« (ASC-H), and »invasion cannot be excluded«. Considering SIL group, all three terms currently in use have been retained, with the addition of »invasion cannot be excluded«. This parallel terminology (*dysplasia* – CIS, cervical intraepithelial neoplasia – CIN and SIL) has been retained to avoid diagnostic-therapeutic identification of moderate *dysplasia* (CIN 2) with severe dysplasia and carcinoma *in situ* (CIN 3), thus leaving an opportunity for cytological and colposcopical follow- up of the lesion up to its regression or progression.

Glandular lesions are also classified into three groups, as follows: »atypical glandular cells« (three subgroups: probably reactive, probably intraepithelial, and probably invasive), »adenocarcinoma *in situ*« (AIS), and »adenocarcinoma«, with a note on its origin.

The groups of »abnormal cells of undetermined significance« and of »other malignant neoplasms« refer to abnormalities where differential cytological diagnosis cannot be established.

This uniform classification enables both internal and external quality control of the laboratory performance, along with appropriate reproducibility of cervical cytology relative to the terminology adopted in the world.

Methods of Quality Assurance in Cervical Cytology

All laboratories have been structured in line with the standard work protocols, to include internal and external quality control[8–11].

Internal quality control of laboratory performance includes the following:

a) control of material sampling,
b) control of technical processing (preparation) and smear staining,
c) primary screening using the following methodology[8,10]:
   - *selected pre-screening* – refers to pre-screening of cervical smears in patients with particular clinical entities such as abnormal bleeding, clinically suspect cervix, etc. In this case, all slides should be observed as having evidence for abnormality, i.e. they should be examined twice by a cytotechnologist and also by a cytologist if some abnormality has been demonstrated;

- *double screening* – it is a reliable method of internal control, ensuring continuous caution on test performance. It is used in all diagnostical specimens;
- *proportional re-screening* – so-called random screening of some 10% of negative specimens;
- *rapid screening* – it includes re-screening of all negative and inadequate smears by use of the known rapid screening technique, performed by cytotechnologists that have not previously analyzed the respective specimens[12];
- *previous cytology review* – it is performed when high-grade dyskaryosis/dysplasia is found in cytological specimen, and previous cytology was negative or has been inadequate for 5-years back, when carcinoma or high-grade CIN is histopathologically diagnosed following negative cytology, and when cytological finding is negative following previous cytological abnormalities;

d) slides and findings are stored for at least 10 years;

e) result analysis by daily cyto-histological correlation of findings; and

f) continuing education.

External quality control implies control of performance of a number of cytology laboratories by a commission consisting of the representative profession and national health authorities. The commission controls the work of a cytological laboratory and tests skill of the cytotechnologists and/or cytologists either by mail or by testing organized at a previously agreed location[13]. *Preparation exchange* includes several (e.g., four or five) laboratories, each submitting up to 10 slides sent from one to another laboratory for screening. Eventually, they all meet together to discuss their results. *Verification tests* make an alternative method of external quality control, where 10 slides are analyzed within 2 hours; all slides included in cervical carcinoma screening can be analyzed[8,10].

The Value of Opportunistic Screening by Conventional Pap Test

The efficacy of conventional Pap test in opportunistic screening is assessed on the basis of its detection and differential diagnostic value, and population coverage. In addition to accurate Pap test may also produce inaccurate diagnosis, i.e. false negative or false positive results. A false negative finding may be due to insufficient sampling, inappropriate laboratory processing, screening failure, or erroneous evaluation of the cells present in the respective smear. A false positive cytology finding is by definition a positive cytology finding in a woman free from cervical lesion. It also includes erroneous interpretation as well as so-called falsely false positive findings, referring to a positive cytology finding in a patient with cervical lesion but negative biopsy finding. The proportion of cytological screening error can be defined as the number of false negative findings in the total number of cytology smears analyzed over a period of observation, yielding a very low error rate, or as the number of false negative findings in the total number of women with cervical lesion, which yields a much greater error percentage. As about 95% of smears negative for CIN lesions, the value of cytological screening lies in the possibility of detecting those 5% of specimens that are abnormal; thus, the error proportion may be expressed also as the number of false negative findings out of the total number of women with cervical lesion. When thus expressed, the rate of false negative findings varies from nil to 29.7%[14–27].

The measures of cervical cytology appropriateness as a screening test are its sensitivity, specificity, predictive values, and diagnostic accuracy. In case of cervical cytology, sensitivity is the rate of positive cytology findings in a group of women with intraepithelial or invasive cervical lesion, ranging from 30 to 87%[28]. Specificity is the rate of negative cytology findings in a group of women free from intraepithelial or invasive cervical lesion, varying from 86 to 100%[28]. Sensitivity and specificity are not independent measures. An increase in sensitivity with a seemingly decreased rate of false negative findings may be accompanied by an increase in the number of false positive findings, and *vice versa*. The relationship between sensitivity and specificity is best illustrated by predictive values.

Positive predictive value (PPV) is a measure of probability that an individual with positive finding is ill. In cervical cytology, it is a useful measure of finding accuracy in case of lesions that require additional diagnostic procedures. On determining PPV, the time to histologic verification should be limited to a maximum of six months. In case of a longer period, the inconsistency between histological and cytological diagnoses resulting in a low PPV may be due to lesion regression rather than inaccurate cytological finding. Negative predictive value (NPV) is a measure of probability that an individual with negative finding is healthy.

In a study conducted in Croatia to estimate the reliability of conventional Pap smear in the screening for both low and high grade intraepithelial cervical lesions and early invasive cervical cancer[29,30], the rate of false negative findings was 10%, of which screening error accounted for 3.1% and sampling error for the rest of 6.9%. The rate of false positive findings was 1.4%, of which erroneous smear interpretation accounted for 0.6%, and SIL detected on follow-up and/or repeat biopsy for the rest of 0.8%, switching the categorization of these cases from »cytologically false positive« to »histologically false negative« due to error on collecting biopsy specimens. The sensitivity was 90.0%, specificity 98.6%, PPV 92.3%, NPV 98.1%, and overall diagnostic accuracy 97.2%.

The main objection to conventional cytology as a screening test refers to its low sensitivity. However, the rate of false negative findings can be considerably reduced by strictly following professional rules, primarily careful sampling and smear preparation, fixation and processing through thorough screening and professional interpretation, thus upgrading the test sensitivity.

TABLE 2
NUMBER OF WOMEN AGED 25–64 IN OSIJEK-BARANJA COUNTY (2001 CENSUS) AND NUMBER OF WOMEN COVERED BY CYTOLOGY SCREENING DURING A THREE-YEAR PERIOD (2003–2005)

Number of women	Age (years)								Total
	25–29	30–34	35–39	40–44	45–49	50–54	55–59	60–64	
Osijek-Baranja County	10,378	11,188	12,396	12,635	12,045	10,730	9,195	10,655	89,222
With Pap test	10,081	9,180	8,922	9,124	8,761	6,908	4,288	3,110	60,374
% of tested	97	82	72	72	75	64	47	29	68

Conventional Pap test is not only used as a classical screening test for lesion detection but also as a differential diagnostic method predicting histological diagnosis. It is of great importance because the type and severity of the lesion detected will dictate further diagnostic and therapeutic procedure. Differential cytology of squamous intraepithelial lesions, distinguishing three grades of dysplasia and carcinoma *in situ*, has a total sensitivity of 67%, specificity of 87%, PPV of 59%, NPV of 90%, and overall diagnostical accuracy of 75%; diagnostic accuracy increases with lesion severity and is directly proportional to reproducibility of each cytological diagnosis[29,31–33].

The small number of glandular intraepithelial lesions neoplasia relative to squamous ones, and the fact that their morphological properties have only been intensively investigated and characterized in the last two decades, have resulted in the lack of experience and attention in the search for these lesions in cytological smears. Consequentially, diagnostic accuracy in recognizing abnormal columnar cells in cytological smears, alone or in combination with a squamous component, is only 55%[33].

The Osijek-Baranja County was taken as a representative sample to assess the risk population coverage by opportunistic screening[4]. Pap smears collected at gynecology clinics and hospital departments in the County were analyzed at Department of Clinical Cytology, Osijek University Hospital in Osijek, Croatia. Dur- ing a three--year period (2003–2005), 104,062 conventional Pap smears, 77,692 (74.7%) of these primary cases and 26,370 (25.3%) duplicate cases, were examined. The number of primary cases corresponded to the total number of women examined. The target population of women aged 25–64, that should be examined once in three years, accounted for 60,374 (68%) Pap smears (Table 2).

Opportunistic screening covered 68% of the target population, i.e. those spontaneously visiting gynecological offices for reproductive age physiology/pathology, while the total number of tests performed should have included the entire population at risk. Study results explained the unfavourable pattern observed in the incidence and mortality of cervical carcinoma in Croatia as compared with other west and south European countries, pointing to the need of implementing the national programme of cancer prevention and early detection, which should include the entire population at risk[34].

Liquid-Based Cytology: Alternative Cytology Method of Higher Sensitivity

New techniques of cervical-endocervical cytology sampling and processing have been developed in the last few decades, with the introduction of liquid-based cytology (LBC) as one of the most important achievements in the field. In contrast to conventional Pap test, in LBC technique the specimen obtained from uterine cervix is not applied onto the slide but the instrument is immersed in a glass containing transport fixation liquid, where it is thoroughly vortexed for optimal utilization of the entire cell material[35]. Using conventional method, up to 80% of the specimen is lost with the discarded instrument[36–38]; it is considered to be one of the main reasons for false negative Pap tests (6–50%)[39–42] and false negative findings in women with HGSIL and invasive carcinoma (20–50%)[42–44].

Cell suspension in fixation liquid is stable at room temperature for up to several weeks[38,45], and is laboratory processed by use of semi-automated or automated commercial devices. Currently, a number of such devices are available, with those approved by the United States Food and Drug Administration (FDA) for cervical screening being best known: ThinPrep® processors (Cytic Corporation, Massachusetts, USA) and AutoCyte-Prep® system (ThiPath Imaging, Inc., North Carolina, USA). Excess blood and inflammatory exudate is mechanically removed from suspension specimen, and a small representative specimen is transferred and uniformly applied in thin layer onto the slide, in a circle of 13 mm (AutoCyte--Prep®) or 19 mm (ThinPrep®) in diameter.

Most authors point to the advantages of LBC over conventional smear, which include a reduced rate of inadequate samples[35,46–48] (by even up to 97%)[47] and of false negative findings[49–51], higher rate of abnormal cytology findings detected[52,53], and significantly shorter time (by up to 60%) needed for analysis of a specimen prepared by the new method[48]. Another advantage of the LBC method, also admitted by those who found no difference when evaluating these two methods of cervical cytology sample preparation[54], refers to the fact that the remaining cell suspension can be used for more sophisticated diagnostical methods such as Human papillomavirus (HPV) analysis (so- called reflex testing), thus upgrading the screening test sensitivity and specificity, in order to prevent unnecessary overtreatment of lesions that would otherwise undergo spontaneous regression. In addition, cell suspension can also be used for the diag-

nosis of other sexually transmitted diseases (*Chlamydia trachomatis*, gonorrhea, etc.), or molecular and cytogenetical methods can be employed, thus making »the impossible possible« in the future[55]. The more so, the rest of material can be used for additional preparations for education and external quality control.

The major hindrance to the introduction of LBC technique is the high cost of its utilization in organized screening actions. Yet, according to the report issued by the Sheffield School of Health and Related Research (ScHARR)[48], the initially high price of the instrumentation will pay off, primarily through a reduced number of inadequate specimens, avoiding unnecessary retesting, and centralization of material processing at only a few laboratories. Furthermore, the implementation of LBC requires additional training of cytologists and cytotechnologists. Cytological analysis of thick layers may frequently prove quite demanding, along with a lower specificity as compared with conventional Pap test[56].

Conclusion

The tradition of more than half a century, the network of cytology laboratories staffed with cytologists and properly trained cytotechnologists of enviable skill and competence, and more than 450,000 Pap tests performed *per* year provide strong basis for the success of cytological screening as the basic method of secondary prevention for cervical carcinoma in Croatia. Opportunistic screening in Croatia reflected in the substantial decrease of cervical cancer incidence from 26 to 15 per 100,000 women-years between 1970 and 1990 remaining almost constant till then[57]. However, some modifications appear to be necessary, which primarily refers to the substitution of opportunistic screening by a properly designed, organized and controlled national screening, as all preconditions for it have been fulfilled. In addition, alternative, high sensitivity methods of sample preparation should be introduced in the cytology service at the national level.

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CITOLOŠKI PROBIR RAKA VRATA MATERNICE U HRVATSKOJ – PROŠLOST, SADAŠNJOST I BUDUĆNOST

S A Ž E T A K

Prikazan je segment kliničke citologije u Hrvatskoj koji se bavi cervikalnom citologijom, kako bi se analizom organizacije, izobrazbe i učinjena rada, uz objektivnu vrijednost identificirale i slabe točke oportunističkog probira konvencionalnim Papa-testom, te predložile mjere za postizanje njegove najveće moguće učinkovitosti u cilju smanjena smrti od raka vrata maternice. U 35 laboratorija koji su ustrojeni u skladu sa standardnim protokolom rada uz kontinuiranu unutarnju i vanjsku kontrolu kvalitete, u kojima je uposleno 68 specijalista citologa i 91 educirani citoskriner, pregleda se na godinu preko 450.000 Papa testova. Iako je tim brojem moguće jednom u tri godine obuhvatiti sve žene u rizičnoj dobi od 25–64 godine, obuhvati se tek 68% onih koje se spontano javljaju ginekologu zbog fiziologije/patologije reproduktivne dobi. U Hrvatskoj je pouzdanost citološkog probira na zavidnoj razini, što se može pripisati dugoj tradiciji sustavne izobrazbe citologa (poslijediplomski studij od 1967. i samostalna specijalizacija od 1974.) i citoskrinera (od 1968.). Osjetljivost Papa-testa je 90,0%, specifičnost 98,6%, pozitivna prediktivna vrijednost 92,3%, negativna prediktivna vrijednost 98,1%, a ukupna dijagnostička točnost 97,2%. Glavna zamjerka se odnosi na nisku osjetljivost. Tradicija duža od pola stoljeća, razvijena mreža citoloških laboratorija sa zavidnim fondom specijalista citologa i educiranih citotehnologa, te broj pregledanih Papa-testova na godinu snažan su argument mišljenju da citološki probir u Hrvatskoj treba ostati temeljnom metodom sekundarne prevencije raka vrata maternice. Nužne su pritom određene promjene, u prvom redu zamjena oportunističkog probira dobro osmišljenim, organiziranim i kontroliranim nacionalnim probirom za koji su ispunjeni svi preduvjeti, te uvođenje alternativnih metoda pripreme uzorka više osjetljivosti.

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Review

HPV Technologies Advancing Public Health: Discussion of Recent Evidence

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A B S T R A C T

Effective primary and secondary cancer prevention programmes are key to improve public health. Cervical cancer is preventable if high quality screening programmes, diagnosis and treatment are offered to female populations at high coverage. Nevertheless, it continues to be a public health problem, and screening programmes need improvements. Human papillomavirus (HPV) has been firmely established as the necessary cause of virtually all cervical cancer cases. To date we count two clinically validated and approved HPV technologies, available to prevent cervical cancer, and other diseases caused by these carcinogenic viruses: Prophylactic vaccines for primary prevention, and HPV DNA tests for secondary prevention, to detect life threatening infections by carcinogenic HPV types, allowing timely diagnosis and clinical management of precancerous lesions. The new technologies will help improve the health of the public if made widely accessible. Similar to vaccination programmes, systematic and well organized cervical screening programmes, with high quality validated HPV tests, can save more lives than ever and improve women's health, in an effective manner.

***Key words**: Human papilomavirus, screening, vaccine, cervical cancer*

Introduction

Public health is the approach to medicine that is concerned with the health of the community as a whole: public health is community health. In this context, the mission of public health professionals is assuring conditions in which people can be healthy. This can be achieved through three key public health functions: 1) systematic assessment and accurate monitoring of the health of communities and populations at risk, to identify health problems and their causes; 2) assuring access to appropriate and effective interventions, including health care and disease prevention services, and evaluation of the cost-effectiveness of such services; and 3) formulation of public policies designed to solve identified global health problems in a sustainable manner. These functions can include the provision of personal health care (services at the clinic level, district or referral hospital) or be population-based such as immunization or screening programmes, and may also include legislation (guidelines, mandatory interventions) and economic incentives such as subsidies. Public health professionals are concerned with planning and implementation of activities that fulfil one of the three functions, leading to measurable outcomes and improvements in the health of the public, within reasonable time frame. Primary and secondary prevention programmes have a synergistic effect in improving health.

Cervical cancer is the second most common cancer in women worldwide with about half million new cases every year[1]. Cervical cancer is preventable and readily treatable, still it kills one of two women diagnosed with cancer, and over 250 thousand women die annually. Worldwide, survival rates vary between regions with good prognosis in some regions, e.g. 73% in the United States[2], and 63% showed in European registries[3], where high quality screening programmes have been implemented in large scale.

The Human papillomavirus in the Root of New Technologies for Cancer Prevention

The notion that papillomavirus infection underlies the development of cancer of the cervix in women was

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first described in 1976 by zur Hausen[4]. Since then, on the basis of a variety of scientific assessements, Human papillomaviruses (HPVs) have been firmely established as the major cause of virtually all cancers of the uterine cervix[5]. This breakthrough in knowedge of cancer etiology led to the development of innovative technologies, that can be used as tools to improve health care and strategies to accelerate cervical cancer prevention. For instance, screening to identify precancerous disease states, that can be successfully removed without sequelae, can save lives. Cervical screening programmes have been recommended to targeting women after the age of 25 in some countries, although the target age and screening intervals can be adapted to needs and resources of different countries.

HPVs are small DNA viruses wraped by a shell or viral capsid, composed of two structural proteins expressed late upon viral replication, known as L1 and L2. HPVs infect the stratified squamous epithelia of skin and mucous membranes, where they may cause benign lesions, some of which have the potential to progress to invasive cancer. Most infections are self-limited and asymptomatic, presumably because the host eventually mounts a successful immune response. There are co-factors that increase the risk for cancer development in infected subjects.

While there are over 100 different types of HPVs molecularly characterized[6] about 15 types have been evaluated as highly carcinogenic to humans, and two types among those have consistently been reported as most common in cervical cancer cases, HPV types 16 and 18[7]. The genome of these two common types have been isolated by molecular cloning in 1983[8] and their cloned DNAs served as basis for producing vaccines by biotechnolgy methodology, as well for molecular diagnostic tests as described bellow.

To date we count two prominent clinically validated and approved technologies available to prevent cervical cancer, and some other cancers caused by these carcinogenic viruses: 1) prophylactic vaccines against HPV the two most common carcinogenic types, and 2) molecular HPV DNA tests to detect life threatening infections by carcinogenic HPV types, and to allow timely diagnosis and clinical management of precancers. On one hand, prophylactic HPV vaccines can prevent the infections, and therefore the associated diseases that the infectious viruses can cause. Because they cannot influence the course of already established infections, vaccines may be most beneficial if administered before any infection occurs. On the other hand, HPV DNA tests can identify already existing infections and prompt to early clinical management and treatment, as appropriate, before the early HPV associated precancerous lesions can progresses to invasive cancer. These two available technologies are schematically illustrated in Figure 1, and will be discussed bellow. Understanding the successes and limitations of new technologies will help to make best use of them for improving the health of the public in general.

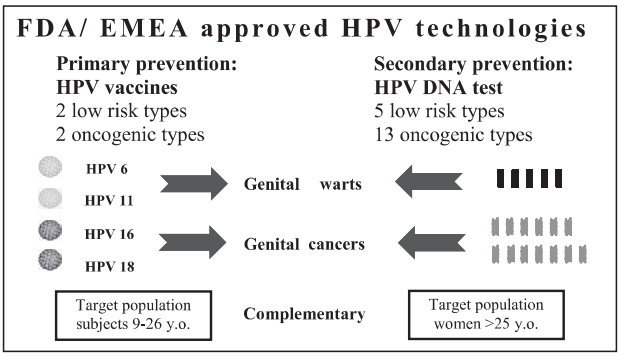

Fig. 1. FDA approved technologies for use in primary and secondary cancer prevention to date. Two health technologies have been so far approved by the US FDA and EMEA, as well as other regulatory authorities, for prevention of neoplasis. For Primary prevention, a prophylactic recombinant quadrivalent vaccine, based on proteinaceous Virus Like Particles against two low risk HPV types, 6 and 11, and two high risk HPV types 16 and 18, is available for administration to subjects 9–26 years of age. For secondary prevention a screening test available as two sets of reagents to detect 5 low risk HPV types, and 13 high risk types, for use in laboratory diagnosis of neoplasias at risk to progress to cancer, and recommended for routine use on women over 30 years. HPV – Human Papillomavirus, y.o. – years old, FDA – Food and Drug Administration, EMEA - European Agency for the Evaluation of Medicinal Products.

HPV Vaccine Technology for Primary Prevention

The vaccines under consideration here are recombinant protein vaccines comprising L1 proteins that self-assembly into particles similar to empty shells of the virus, and are therefore non-infectious and non-oncogenic[9].

The aim of vaccination against HPV is to induce immunity to neutralize HPV infections and later associated diseases and cancers. Clinical data originated in several studies in phase 1, phase 2 and phase 3 have been published[10,11.] Although the primary concern is reduction in cervical cancer cases and deaths, the impact of vaccination on surrogate markers and intermediate diseases can be assessed sooner, and may have implications for design and implementation of effective prevention programmes. This information will also be crucial for planning succesful health policy initiatives that involve both screening and vaccination.

Vaccines that have completed controlled efficacy studies have demonstrated high levels of efficacy against histologically characterized high grade dysplasias associated to the viral antigen types included in the vaccines, namely cervical intraepithelial neoplasias (CIN2-3) or worse, following administration of a three-dose regimen among women who had no evidence of previous infection with HPV[13].

The indications for the use of HPV vaccines in the EU are, so far, for prevention of HPV 16/18 related cervical

TABLE 1
SUMMARY OF STATISTICAL ANALYSES RESULTS FROM CLINICAL TRIALS TO MEASURE THE EFFICACY OF A QUADRIVALENT VACCINE IN PREVENTING CIN 2–3 ASSOCIATED TO HPV INFECTIONS IN THE TRIAL POPULATION OF FEMALES AGED 15–26 YEARS OLD

Source reference	Endpoint	Vaccine cases	Placebo cases	Efficacy
Package insert Gardasil™ (Merck & Co.)*	HPV 16/18 CIN2–3 or worse in Per Protocol efficacy analysis	0 (N=9,342)	53 (N=9,400)	100%
Package insert Gardasil™ (Merck & Co.)*	HPV 16/18 CIN2–3 or worse in the general trial population (MITT-3)	122 (N=9,831)	201 (N=9,896)	39%
Statistical review and evaluation Gardasil™ (Merck & Co.)**	any HPV type CIN2–3 or worse in the general trial population	287 (N=8,814)	328 (N=8,846)	12.2%

Clinical trials analysis to measure the efficacy of a quadrivalent vaccine in preventing CIN 2–3 associated to HPV infections in the trial population of females aged 15–26 years old. Three trial sub-populations considered for analysis are indicated here: the HPV type specific per-protocol-efficacy analysis, the type specific modified intention to treat analysis, and the modified intention to treat analysis as to efficacy against CIN2–3 associated to any HPV type. *Data available in the public domain, at package insert Gardasil label http://www.fda.gov/cber/label/hpvmer060806LB.pdf, and **Interim analysis data adapted from Dr. N. Miller, available at http://www.fda.gov/ohrms/dockets/ac/06/slides/2006–4222S-2.ppt[16]. HPV – Human Papillomavirus, CIN – Cervical Intraepithelial Neoplasia, MITT – Modified Intention-To-Treat.

cancinomas, high grade cervical dysplasias CIN2 and CIN3, high grade vulvar dysplasias, VIN2 and VIN3, as well as prevention of HPV 6/11 related genital warts (condyloma acuminata), and in children and adolescents 9 through 15 years of age, and women 16 through 26 years of age[14].

One pivotal vaccine trial included over 20.000 females 13–26 years old (median age of 20) enrolled in different geographical regions. The population for the efficacy studies included large proportion of women in Europe (44.1%), mostly from Nordic countries, 25.3% women in North America, 27% in Latin America and only 3.6% in Asia (available under Food and Drug Administration [FDA] and European Agency for the Evaluation of Medicinal Products [EMEA] websites[15–17]). It was noted that of the females in the trial population aged 13–26 years overall 12% had an abnormal baseline Pap test with squamous intraepithelial lesions. The majority of these were low grade SIL and atypical squamous cells of undetermined significance (ASC-US). In addition, 27% of these subjects had been previously exposed to one or more of the vaccine HPV types (sero+ and/or PCR+).

Different subpopulations among the randomized females enrolled in the studies were considered for analyses of efficacy (Table 1)[15–17]. Per-Protocol Efficacy (PPE) included subjects who received all 3 vaccinations, were seronegative to the appropriate HPV type(s) at day 1 and PCR-negative to the appropriate HPV type(s) day 1 through month 7, and generally did not deviate from protocol. Modified Intention-To-Treat (ITT-1) analysis included subjects who received all 3 vaccinations, were seronegative to the appropriate HPV type(s) at day 1 and PCR-negative to the appropriate HPV type(s) day 1 through month 7, and included general protocol violators. Modified ITT-3 included all subjects who received at least 1 vaccination, regardless of initial serology and PCR status.

Per Protocol HPV type-specific analyses indicated a very high level of efficacy in naïve subjects, while the efficacy for all HPV related disease on a population basis, especially if given to many females who already have an HPV infection, appear to be lower, as summarized in Table 1. For subjects naïve for the relevant vaccine HPV type(s), the measured vaccine efficacy against HPV 16 and/or 18 related CIN2/3 or worse was 100%, and for all randomized trial population, the vaccine efficacy was about 40%, due to the fact that about a quarter of women had evidence of previously been infected with HPV. Noteworthy, data was analysed in a HPV type specific manner. Hence, females naïve to the four vaccine HPV types are expected to benefit most from vaccination.

Furthermore, vaccinated subjects naïve to all four vaccine HPV types could still develop disease related to an HPV type not included in the vaccine. In one case scenario, vaccination of naive populations shows that approximately 50% reduction in cervical cancer mortality could be achieved by vaccination in over many years[12,13]. Combination of vaccination and screening strategies are likely to offer the most effective prevention to cervical cancer.

HPV DNA Test Technology for Secondary Prevention

An independent study conducted by the International Agency for Research on Cancer (IARC), and a independent Advisory Group concluded there is *sufficient evidence* that screening for cervical cancer by cytological examination of Pap smear cell samples does prevent death[18]. The experts, however, emphasized that in order to achieve this goal optimally, an organized programme with quality control of every key step of the entire process is a prerequisite. Tests for the presence of viral DNA in a sample of epithelial cells have been established as a step toward

TABLE 2
OVERVIEW OF RESULTS OF SOME EUROPEAN STUDIES COMPARING CYTOLOGY AND MOLECULAR METHODS DETECTION RATES FOR HIGH GRADE CERVICAL DISEASE

Source reference	Study site and size (N)	Any HR HPV type CIN2+ or worse Number of Cases		Clinical Sensitivity CIN2+ or worse Endpoint	
		HC2**	Cytology	HC2**	Cytology
Cuzick et al.[26]	United Kingdom (N=10,358, 30–60 years)	87	69	96.8%	76.9%
Petry et al.[27]	Germany Tuebingen/Hannover (N=8,967; 30–87 years)	52	22	97.5%	48.9%
Clavel et al.[28]	France (N=14,123)	199	120	98.1%	62%
Ronco et al.[29*]	Italy (N=33,364)	73 (16,706)	51 (16,658)	97.3%	74%
Cuzick et al.[24]	United Kingdom (N≥60.000)	513	283	96.1%	53%

*Randomized trial, **The HC2 assaw shows consistenly higher rates of disease detection in large studies, including randomized trials, HR HPV – High Risk Human Papillomavirus, CIN – Cervical Intraepithelial Neoplasia, HC2 – Hybrid capture 2 assay.

identifying potentially precancerous conditions. In this context, the IARC expert Group concluded that there is also *sufficient evidence* that the HPV test for women 25–65 years *can* reduce mortality from cervix cancer[18]. If high quality screening test is provided to the public it will likely have an immediate impact on disease burden, in contrast to prophylactic vaccination, because it is designed to identify and avert cases in the women who already have some precancer pathology and are at high risk of progression to invasive cancer.

It is important to understand the difference between analytical and clinical sensitivity in order to allow effective use of HPV technology for clinical diagnostics. While analytical sensitivity relates to the amount of analyte or genome equivalent or copy number of viral particles present in a given sample, the clinical sensitivity relates to the degree of agreement of a positive test result with a positive disease status. Generally a test with high analytical sensitivity, detecting down to 10 viral copies per sample, would give positive results to all infected individuals, irrespective if this is a transient subclinical infection or an infection associated to neoplasia. Tests that detect only higher levels of viral DNA, eg. more than 5000 copies per sample, give positive results that are more likely associated to neoplasias at risk to progressing to cancer, and so are clinically relevant[19].

New diagnostic assays must be validated using data regarding prediction of risk of cancer and CIN3 from large representative study populations. In addition to targeting the correct genotypes, HPV tests must have clinically validated viral load cut points, ie. analytical sensitivity[20]. Hybrid Capture 2 (HC2) is FDA-approved, CE-marked, clinically validated, and commercial HPV test available worldwide. The test is available with two sets of reagents, one set to detect five low-risk types HPV, and another reagents set to detect 13 high-risk types HPV[21]. The analytical detection level of HC2 HPV has been set at 1.0 HPV DNA pg/mL (5000 genomes/assay) based on multiple clinical trials over a long period of time with high grade cervical intraepithelial neoplasia (CIN 2+) as the disease endpoint[22].

Primary screening with combined cytology and HPV testing is already an accepted and approved option in North America. The HPV HC2 test is recommended in the United States as adjunct to cytology screening for women over the age 30, or for triage of inconclusive cytology results in women under 30-years old[23].

In the European guidelines to be released now, evidence for HPV testing is accepted for two clinical applications: triage of equivocal cytology (ASCUS), and follow-up of treated lesions to predict failure or success of the offered therapy[24]. HPV triage of LSIL is recommended for women over 30 years of age, where the specificity of HPV test is higher than in young women[25]. In Europe a high level of confidence on primary HPV screening from randomized trials is awaited to complement the guidelines. At present, there are five randomized trials under way in Europe, to assess the effectiveness of HC2 as primary screening test for public health programmes. Similarly to endpoints used in vaccine studies, screening studies considered detect prevalent CIN2–3 in long follow-up periods. In general interim studies results showed that HC2 clinical performance in the field, as measured by biopsy confirmed cervical histopathology, is consitently higher than cytology based methods (Table 2)[24,26–29]. A meta-analysis of the various studies conducted in Europe and in North America involving over 60.000 women over 35-years old, confirmed that HC2 performance in the field to detect women with cervical premaligant lesions, is higher than cytology. With cytology triage, the specificity improves to the level of repeated conventional cytology. The studies also showed that combining HC2 with cytology maximizes the clinical benefits of large cervical screening programmes[22].

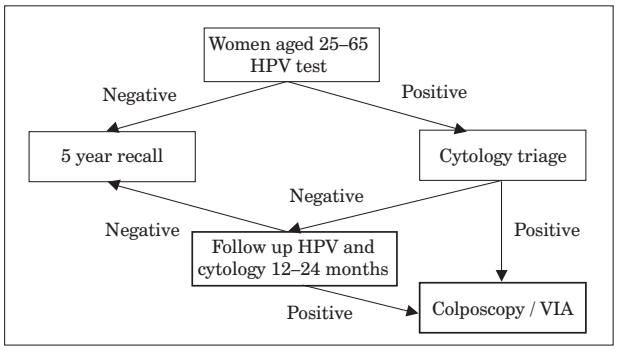

Fig. 2. Schematic representation of a possible algorithm for the use of HPV testing as the primary screening method followed by triage using cytology based methods, for women eligible for cervical cancer screening. The age group targeted for screening may vary in different countries and the interval considered for recall and follow-up,ay also be adapeted to national needs. Adapted from Cuzick et al. 2006[34]. HPV – Human Papillomavirus, VIA – Visual Inspection using 4% Acetic acid.

Another advantage of HPV test is that women with a negative result have an extremely low probability of having a CIN in the following 10 years. The longitudinal sensitivity to predict CIN3+ over a period of 10 years is 66% for HC2 whereas only 35.4% for baseline cytology defined as ASCUS+, while the positive predictive value of cytology remains higher than HC2[30–31]. Importantly, the positive predictive value of HPV test can be significantly increased by typing for HPV 16 and 18[32]. These observations indicate that HPV testing is safe and could be cost-effective allowing longer screening intervals, as opposed to methods such as Pap smears. In addition it may decrease significantly psychological anxienty associated with screening practice. Positive results for HPV 16 and 18 may warrant a shorter follow-up period.

A proposed new paradigm for cervical screening management is schematically illustrated in Figure 2. If resource constrains need to be respected, a HPV test would be a possible option because it is based on the higher sensitivity consistently demonstrated using HPV test, and high sensitivity could be achieved using cytology triage, for example[34].

Conclusion

To date, two HPV technologies reviewed and approved by regulatory authorities in North America and Europe for use in populations are at hand: HPV vaccination and HPV testing. Both technologies are derived from the cancer causing virus, and represent effective interventions to eliminate cervical cancer. Vaccines are tools for primary prevention strategies, i.e. to prevent the life treatening infection of establishing, and HPV tests are tools for secondary prevention strategies, i.e. prevent infections and neoplasias of progressing to invasive cancer.

There are some limitations that need to be acknowledged: vaccines to date target two out of fifteen carcinogenic types of HPV, and will likely prevent 70% of potential cancer cases in vaccinated subjects, so screening will need to continue in order to prevent those cases not protected by these vaccines. The vaccines are essentially prophylactic and have no effect on the course of already acquired infections, therefore screening needs to continue ensuring the population that previously acquired infections will be prevented of developing to cancer. Vaccination may have a lag of decades between the intervention and a reduction in cancer incidence at population level. Nevertheless, these limitations could be surmounted by vaccines that would be effective against most of the HPV carcinogenic types.

There are also limitations to HPV testing, as HC2 HPV test detects the 13 carcinogenic types identified, and has the potential to identify 95% of cases at an early stage to allow timely treatment. Cases caused by some HPV types not included in the test may not be detected. Notably, this can be overcome if the test is combined to cytology where is has demonstrated to be able to achieve 100% sensitivity. Negative HPV test results may warrant an assessment for appropriatness of sampling, such as cellular DNA content.

In an ideal public health service primary and secondary prevention strategies implemented in parallel will have a synergistic effect and solve the public health problem faster, than each strategy isolated. Noteworthy the key for success in vaccination programmes lies on the systematic and well organized approach to vaccinate the populations at high coverage, in addition to use quality products. Similarly, only screening programmes conducted with quality products, implemented in a systematic and well organized manner at high coverage, while targeting the female population at risk, can save lives and improve women's health, and will impact on the health of their families and communities.

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POMACI U JAVNOM ZDRAVSTVU ZAHVALJUJUĆI NOVIM METODAMA VEZANIM UZ HPV: RASPRAVA O NEDAVNIM DOKAZIMA

S A Ž E T A K

Učinkoviti primarni i sekundarni programi prevencije raka vrata maternice su ključni u poboljšanju javnog zdravstva. Rak vrata maternice je moguće spriječiti ukoliko se ženama ponude kontrolirani visoko-kvalitetni programi probira, dijagnostike i liječenja. Unatoč tomu, ovaj rak i dalje predstavlja javno-zdravstveni problem, a programe probira treba poboljšati. Humani papilomavirus (HPV) se smatra neophodnim uzročnikom gotovo svih slučajeva raka vrata maternice. Danas postoje dvije klinički potvrđene i odobrene metode vezane uz HPV, dostupne u prevenciji raka vrata maternice i drugih bolesti uzrokovanih ovim karcinogenim virusima: profilaktička cjepiva za primarnu prevenciju te HPV-DNK-testovi za sekundarnu prevenciju, detekciju infekcija karcinogenim tipovima HPV-a opasnih po život, što omogućuje pravovremenu dijagnozu te kliničko liječenje stadija prije raka. Nove metode će pomoći pomacima u javnom zdravstvu ukoliko budu široko dostupne. Slično programima cijepljenja, sustavno i dobro organizirani programi probira raka vrata maternice, sa visoko-kvalitetnim važećim HPV-testovima, mogu spasiti više života nego ikad te poboljšati zdravlje žena na vrlo učinkovit način.

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Professional paper

Quality Assurance of Human Papillomavirus Testing

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A B S T R A C T

The External Quality Assurance (EQA) in medical microbiology in the Czech Republic is well organized. It is coordinated by the Accreditation Department of the Centre of Epidemiology and Microbiology (AD-CEM) of the National Institute of Public Health in Prague. Since 1993 when the first samples were sent out the number of programmes and participating laboratories has been rapidly increasing. EQA for Human papillomavirus (HPV) has been available since 2000. As has been shown for other programmes, the EQA for HPV has proved to be useful, helping to improve the accuracy of analyses and contributing to the standardization of methods of HPV DNA testing. EQA for HPV has been well received by routine laboratories, demonstrated by a high number of these institutions voluntarily participating in EQA.

Key words: *external quality assurance (EQA), human papillomavirus (HPV)*

Introduction

Today, most diagnostic laboratories are capable of detecting a wide range of infectious agents. The In Vitro Diagnostic Industry is committed to bringing an even bigger variety of diagnostic assays on the market in the future. Even though the process of certification of *in vitro* diagnostics for infectious agents is obligatory, this is not always a guarantee that the assay is working properly in the end-user's laboratory. Especially for very sensitive molecular diagnostic assays, which often straddle the divide between research and routine, internal and external quality assurance is important. Several institutions worldwide provide panels of reference samples for the most commonly diagnosed infections. For Human papillomaviruses (HPV), the first internationally available panel of samples for quality assurance was offered by Instand (WHO Collaborating Centre for Quality Assurance and Standardization in Laboratory Medicine)[1] in 2004.

Long Tradition

The system of External Quality Assessment (EQA) in the Czech Republic was introduced in 1993. The first programme focused on sera for detection of the hepatitis B virus and strains of bacteria for identification. From 1993 till 2002 the number of provided surveys increased from 2 to 34 and the number of participating laboratories increased from 79 to 440 (Figures 1 and 2). In order to provide technical support for this programme, a coordinating centre for EQA in medical microbiology was established (the Accreditation Department of the Centre of Epidemiology and Microbiology (AD-CEM), National Institute of Public Health). Since then, AD-CEM has devel-

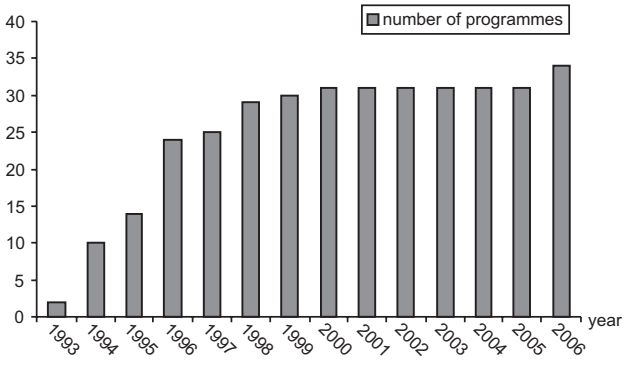

Fig. 1. Number of programmes provided by the Accreditation Department of the Centre of Epidemiology and Microbiology (AD-CEM) from 1993–2006[2].

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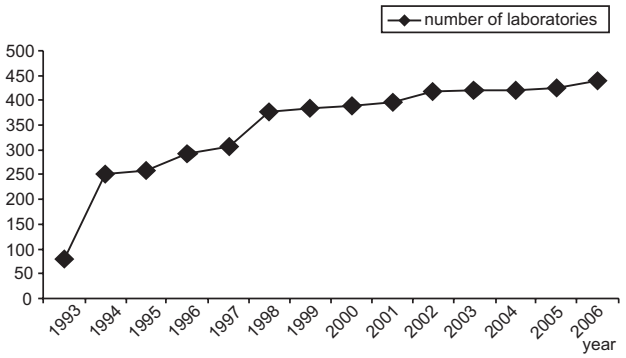

Fig. 2. Number of laboratories registered in the External Quality Assurance (EQA) in the Czech Republic from 1993–2006[2].

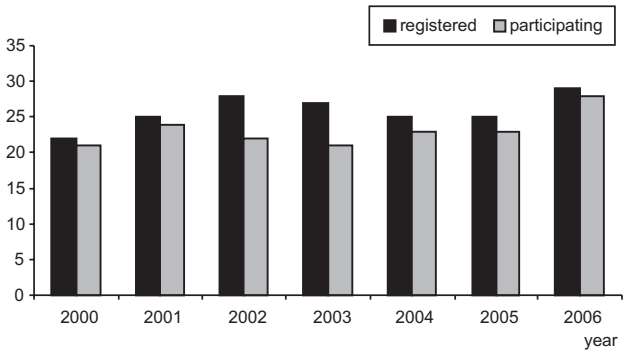

Fig. 3. Number of laboratories registered and participating in EQA for Human papillomavirus (HPV) testing in the Czech Republic from 2000–2006[2,3].

oped a wide network of national reference laboratories and some other highly specialized centres, which prepare samples for EQA[2].

AD-CEM distributes the coded samples together with all required protocols containing detailed instructions. Each programme has an individual deadline for receiving the results. AD-CEM sends the protocols with the results to the contractual laboratory for evaluation. Each participating laboratory in this system has a unique identification code and the evaluation of results is therefore anonymous. The certificate of participation is issued to all participants who registered for the programme in the particular year and those who successfully passed the EQA also obtain the Certificate of proper diagnostics.

After ten years of its existence, AD-CEM has achieved some very positive outcomes. First of all, the accuracy of analyses has increased and methods have become better standardized. In addition, significant variations have been detected between different diagnostic sets; as a result, those sets that have yielded unsatisfactory results have been eliminated.

Participation in the system of EQA is not compulsory, but most of the routine laboratories voluntarily take part since it allows them to compare the performance of a wide variety of detection techniques and diagnostic sets with many others laboratories. All results are published in the journal, »Zprávy CEM« (News of CEM), issued by the Centre of Epidemiology and Microbiology, National Institute of Public Health as well as on the websites of some of the contractual laboratories.

External Quality Assurance for HPV

In 1998, the National Reference Laboratory (NRL) for Papillomaviruses was established by the National Public Health Authority on the recommendation of the Working Group for Virology of the Society for Clinical Microbiology of the Czech Medical Association JEP to improve the interpretation of results and disseminate information concerning the clinical usefulness of HPV DNA detection among specialists.

Since 2000, 5 EQA sample series have been distributed annually to each of the participating laboratories (Figure 3). Since 2003, EQA has also been provided to laboratories using PCR-based diagnostic kits. EQA is carried out depending on the diagnostic kit used (i.e. depending on whether the kit detects both low risk [LR] and high risk [HR] HPV types or HR HPV types only). If the kit used detects both LR and HR HPV types, the participating laboratory is awarded a score of 2 points for each correct result and loses 1 point when failing to detect either LR or HR HPV types. When an incorrect result is reported for a sample with a borderline amount of virus for a detection limit of the given kit (provided the test is recommended for diagnostic use by the NRL), NRL requires correct interpretation of any equivocal result. If possible, NRL informs the participating laboratory about the mechanism likely to be responsible for the error (e.g. low sensitivity of the method, defective kit batch, improper performance of the test, sample contamination, etc.).

The samples for EQA are prepared to simulate the cervical smear samples taken to a transport medium. At the beginning of EQA, the Specimen Transport Medium (STM, Digene) was used. Later, when PCR-based commercially available sets appeared, samples prepared in the PreservCyt medium (Cytyc) for PCR-based methods were used. Recently, we finished a study whose results show the possibility of using STM medium for both non-amplification and amplification-based methods. In each sample, a background of 100,000 HPV negative cells is included. Each series contains an HPV negative, LR HPV positive and HR HPV positive sample and also a sample positive for both LR and HR HPV. Both HPV positive cell lines and cloned DNA is used for sample preparation. Different amounts of HPV positive cells and/or cloned DNA are used to simulate different viral load and verify the sensitivity of methods used in laboratories. To illustrate, we include a table showing the results of participating laboratories in 2001 and 2006 (Table 1 and 2)[3].

At the beginning of EQA for HPV, most of the laboratories used Hybrid Capture Tube test (HCT, Digene), which has been shown to have a limited sensitivity for

TABLE 1
RESULTS OF EQA FOR HPV IN 20013

Test	Laboratory number.	Sample No.1		Sample No.2		Sample No.3		Sample No.4		Sample No.5		Rating
		LR RLU/CO	HR RLU/CO	LR RLU/CO	HR RLU/CO	LR RLU/CO	HR RLU/CO	LR RLU/CO	HR RLU/CO	LR RLU/CO	HR RLU/CO	
HCT Digene	26	0.25	0.24	0.33*	0.25	0.25	0.26*	0.73*	0.42*	0.26	0.26*	2.0
HCT Digene	118	0.19	0.25	0.48*	0.26	0.25	0.58*	1.44	1.03	0.34	2.75	6.0
HCT Digene	211	0.58	0.26	0.72*	0.30	0.62	0.35*	1.38	0.64*	0.54	0.31*	2.0
HCT Digene	28	0.23	0.24	0.32*	0.20	0.22	0.34*	0.75*	0.40*	0.20	0.24*	0.0
HCT Digene	371	0.28	0.49	0.39*	0.32	0.25	0.35*	0.77*	0.63*	0.25	1.04	4.0
HCT Digene	406	0.89	1.28**	ND	ND	0.67	2.01	ND	ND	3.33**	0.72*	0.0±4.0
HCT Digene	18	3.78**	3.6**	0.53*	0.99	0.31	0.68*	3.84	1.41	0.29	0.26*	0.0
HCT Digene	192	0.37	0.30	0.45*	0.38	0.37	0.38*	1.07	0.64*	0.43	0.43*	2.0
HC2 Digene	165	0.14	0.17	1.18	0.17	0.19	0.87*	7.02	2.60	0.16	16.89	8–9.0
HC2 Digene	373	0.93	0.19	2.44	0.20	1.14**	1.47	1.81	1.82	0.82	17.93	8.0
HC2 Digene	156	0.18	0.14	1.04	0.15	0.16	1.30	3.42	2.21	0.18	18.90	10.0
HC2 Digene	115	0.20	0.50	1.41	0.16	0.29	1.58	6.30	2.36	0.22	16.78	10.0
HC2 Digene	585	0.54	1.34**	1.88	2.00**	2.12**	2.92	4.71	2.21	5.67**	22.21	2.0
HC2 Digene	584	0.20	0.21	1.42	0.20	0.36	1.38	6.79	3.16	0.20	18.99	10.0
HC2 Digene	13	0.34	0.37	1.75	0.40	0.27	1.38	10.51	2.61	0.26	18.84	10.0
HC2 Digene	344	0.24	0.21	1.04	0.50	0.23	1.20	4.16	1.70	0.38	11.89	10.0
HC2 Digene	405	0.59	0.24	1.28	0.47	0.23	1.38	4.53	2.05	0.51	15.77	10.0
HC2 Digene	31	0.34	0.25	1.69	0.36	0.31	2.43	6.36	2.83	0.45	34.82	10.0
HC2 Digene	315	0.40	0.70	1.12	0.40	0.30	1.20	4.50	2.40	0.50	15.20	10.0
HC2 Digene	147	0.56	0.47	1.72	0.28	0.55	1.38	6.67	2.68	0.46	18.40	10.0
HC2 Digene	407	0.57	0.52	1.14	0.77	0.46	1.60	3.86	2.29	0.45	13.97	10.0
HC2 Digene	11	0.15	0.26	1.02	0.20	0.13	1.13	3.51	1.63	0.19	12.85	10.0
HC2 Digene	230	0.23	0.19	1.06	0.18	0.20	1.22	4.09	1.82	0.22	15.56	10.0
HC2 Digene	471	0.16	0.14	1.43	0.15	0.17	1.36	5.67	2.70	0.18	18.75	10.0
HC2 Digene	NRL PV	0.35	0.33	1.67	0.23	0.39	1.73	7.27	2.64	0.35	18.63	10.0

EQA – External Quality Assurance, HPV – Human Papillomavirus, * – error caused by the low sensitivity of the detection method, ** – probable contamination of samples, HCT – Hybrid Capture 1 HPV test (Digene), HC2 – Hybrid Capture 2 HPV test (Digene), LR – low risk, HR – high risk, RLU/CO – relative light units/cut-off, ND – not done, NRL PV – National reference laboratory for papillomaviruses. Available at: http://www.papillomavirus.cz/eng/activities_eqa.html.

the detection of CIN2+ lesions. Therefore, in the EQA in 2000 we set the cut-off line just above the detection level for HCT. We recommended that all laboratories plan to switch to HC2 (Digene) (primarily because at that time no other commercial sets were available on the Czech market). The reaction was quite positive and as you can see from Table 3, the spectrum of diagnostic sets used in routine laboratories changed. In 2000, 50% (11/21) of laboratories in EQA used HCT, but this number decreased to 3 out of 22 in 2002 and just 1 in 23 laboratories in 2004. 2004 was the first year when 3 laboratories used PCR-based HPV DNA tests (HPV INNO-LiPA Innogenetics, Amplicor HPV Test Roche, DNA PCR Test Gentech).

Discussion

From the onset of EQA, we regularly detected mistakes in using even such robust sets as HC2. However, over the years, the performance of laboratories in EQA improved and in the last two years we have had no mistakes reported from the laboratories using HC2 or HC2 HR sets. In 2006, we had 6 laboratories using commercially available PCR-based sets. The results of EQA from laboratories which use PCR-based sets show bigger heterogeneity and confirm the need for participation in the EQA (Table 2)[3].

In the absence of an internationally available programme for EQA for HPV, we exchanged the samples with the Laboratory of Clinical and Epidemiological Virology, Rega Institute for Medical Research, University of Leuven, Belgium in 2002. In 2004 Instand (WHO Collaborating Centre for Quality Assurance and Standardization in Laboratory Medicine)[1] for the first time offered EQA for HPV and since then, NRL has been successfully participating in this programme. In 2004, Instand prepared samples which had a limited amount of HPV DNA (below the cut-off for HC2 Digene). Since we used different

TABLE 2
RESULTS OF EQA FOR HPV TESTING IN 2006[3]

Test	Laboratory number	Sample No.1		Sample No.2		Sample No.3		Sample No.4		Sample No.5		Rating
		LR RLU/CO	HR RLU/CO	LR RLU/CO	HR RLU/CO	LR RLU/CO	HR RLU/CO	LR RLU/CO	HR RLU/CO	LR RLU/CO	HR RLU/CO	
HC2 Digene	11	24.43	0.15	0.11	3.79	20.76	0.13	0.11	0.11	0.12	6.51	10.0
HC2 Digene	13	24.60	0.25	0.32	4.41	24.18	0.23	0.19	0.15	0.15	6.43	10.0
HC2 Digene	31	29.23	0.15	0.10	4.61	30.53	0.18	0.11	0.13	0.12	7.83	10.0
HC2 Digene	79	30.46	0.12	0.08	4.76	30.74	0.13	0.11	0.09	0.08	7.63	10.0
HC2 Digene	115	27.31	0.16	0.16	4.09	23.01	0.16	0.14	0.15	0.12	7.15	10.0
HC2 Digene	118	28.83	0.15	0.11	4.54	27.00	0.15	0.10	0.09	0.10	6.47	10.0
HC2 Digene	156	29.49	0.16	0.11	4.43	22.56	0.36	0.13	0.13	0.18	5.53	10.0
HC2 Digene	165	29.30	0.26	0.13	4.98	29.80	0.21	0.15	0.20	0.14	6.94	10.0
HC2 Digene	192	28.71	0.10	0.09	4.51	25.35	0.08	0.10	0.08	0.10	6.10	10.0
HC2 Digene	230	18.02	0.79	0.46	2.47	18.91	0.75	0.55	0.46	0.43	4.74	10.0
HC2 Digene	315	26.70	0.10	0.10	3.60	27.90	0.20	0.10	0.10	0.10	5.60	10.0
HC2 Digene	325	30.36	0.19	0.12	4.54	31.74	0.18	0.14	0.14	0.13	8.27	10.0
HC2 Digene	344	27.37	0.35	0.11	3.56	29.95	0.18	0.21	0.15	0.47	6.33	10.0
HC2 Digene	354	20.68	0.10	0.08	3.51	19.98	0.10	0.12	0.06	0.10	2.85	10.0
HC2 Digene	369	18.22	0.54	0.63	2.95	16.47	0.49	0.38	0.54	0.38	4.45	10.0
HC2 Digene	371	28.88	0.16	0.07	4.22	26.02	0.25	0.10	0.11	0.12	6.73	10.0
HC2 Digene	373	3.16	0.16	0.15	4.40	26.60	0.17	0.16	0.17	0.16	6.69	10.0
HC2 Digene	407	25.00	ND	ND	ND	31.00	ND	ND	ND	ND	6.00	10.0
HC2 Digene	585	27.46	0.26	0.45	4.38	23.70	0.27	0.23	0.24	0.18	7.45	10.0
HC2 Digene	734	29.37	0.16	0.09	4.85	27.72	0.14	0.09	0.13	0.10	7.17	10.0
HC2 HR Digene	584	–	0.19	–	4.45	–	0.14	–	0.12	–	7.38	10.0
HC2 HR Digene	736	–	0.13	–	5.15	–	0.14	–	0.21	–	8.21	10.0
HC2 Digene	NRL PV1	28.50	0.13	0.10	4.65	29.50	0.12	0.10	0.13	0.15	7.59	10.0
HC2 Digene	NRL PV2	26.70	0.16	0.11	4.51	30.18	0.14	0.15	0.10	0.11	7.41	10.0

PCR test	Laboratory No.	Sample No.1		Sample No.2		Sample No.3		Sample No.4		Sample No.5		Rating
		LR HPV	HR HPV	LR HPV	HR HPV	LR HPV	HR HPV	LR HPV	HR HPV	LR HPV	HR HPV	
HPV INNO-LiPA Innogenetics	211	neg**	HPV 31**	neg	HPV 16	HPV 6, 11**	HPV 18, 51, 52, 58, 66**	neg	neg	neg	HPV 16, 35, 45**	6.0
HPV INNO-LiPA Innogenetics	44	HPV 6	neg*	neg	HPV 16	HPV 6	neg	neg	neg	neg	HPV 16, 39	10.0
HPV INNO-LiPA Innogenetics	734	HPV 6	neg*	neg	HPV 16	HPV 6	neg	neg	neg	neg	HPV 16, 39	10.0
DNA PCR Test Gentech	16	poz	poz	neg	neg**	poz	neg	neg	neg	neg	poz	9.0
Amplicor HPV Test Roche	365	–	poz	–	poz	–	neg	–	neg	–	poz	10.0
Amplicor HPV Test Roche	716	–	neg*	–	poz	–	neg	–	neg	–	poz	10.0
Amplicor HPV Test Roche	NRL PV	–	poz	–	poz	–	neg	–	neg	–	poz	10.0
"in house" PCR GP5+/6+bio	NRL PV	HPV 6	HPV 33	0	HPV 16	HPV 6	0	0	0	0	HPV 16, 39	10.0

EQA – External Quality Assurance, HPV – Human Papillomavirus, * – borderline concentration of HPV DNA, ** – error, LR – low risk, HR – high risk, RLU/CO – relative light units/cut-off, ND – not done, NRL PV – National reference laboratory for papillomaviruses. Available at: http://www.papillomavirus.cz/eng/activities_eqa.html

TABLE 3
NUMBER OF LABORATORIES PARTICIPATING IN EQA FOR HPV BY TESTING YEAR AND THE DIAGNOSTIC SET

Year	Number of laboratories registered for the programme	Number of laboratories participating in the programme	Detection method					
			HCT Digene	HC2 Digene	HC2 HR Digene	HPV INNO-LiPA Innogenetics	Amplicor HPV Test Roche	DNA PCR Test Gentech
2000	22	21	11	10	–	–	–	–
2001	25	24	8	16	–	–	–	–
2002	28	22	3	19	–	–	–	–
2003	27	21	2	18	–	1	–	–
2004	25	23	1	19	–	2	–	1
2005	25	23	–	19	–	2	1	1
2006	29	28	–	20	2	3	2	1

EQA – External Quality Assurance, HPV – Human Papillomavirus, HCT – Hybrid Capture 1 HPV test (Digene), HC2 – Hybrid Capture 2 HPV test (Digene), HR – high risk, HPV INNO-LiPA – Line blot assay (Innogenetics), Amplicor HPV Test – Polymerase chain reaction HPV based test (Roche), DNA PCR Test – Polymerase chain reaction HPV based test (Gentech)

methods for assessment of EQA in our laboratory, this discrepancy was obvious and we sent our comments to the producers of EQA, which resulted in the change of the HPV DNA amount in the ECQ in 2005.

Conclusion

EQA in medical microbiology in the Czech Republic is well organized. It is coordinated by AD-CEM of the National Institute of Public Health in Prague. First samples were sent out in 1993. EQA for HPV has been available since 2000 and in 2006, 29 laboratories participated in this programme. As has been shown for other programmes, the EQA for HPV has proved to be useful, helping to improve the accuracy of analyses and contributing to the standardization of methods of HPV DNA testing. EQA for HPV has been well received by routine laboratories, demonstrated by a high number of these institutions voluntarily participating in EQA.

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OSIGURANJE KVALITETE TESTOVA NA HUMANI PAPILOMAVIRUS (HPV)

SAŽETAK

Vanjska provjera kvalitete (EQA, od engl. *External Quality Assurance*) je dobro organizirana u medicinskoj mikrobiologiji u Republici Češkoj. Koordinirana je od Odjela za akreditaciju centra za epidemiologiju i mikrobiologiju (AD-CEM, od engl. *Accreditation Department of the Centre of Epidemiology and Microbiology*) Nacionalnog instituta javnog zdravstva u Pragu. Od 1993. godine, kada su prvi uzorci poslani vani, broj programa i uključenih laboratorija je naglo narastao. EQA je za humani papilomavirus (HPV) bila u upotrebi od 2000. godine. Kako je bilo prikazano za druge programe, EQA se pokazala korisnom za HPV, pomažući tako povećanju točnosti analiza i doprinoseći standardizaciji metoda HPV-DNK-testova. EQA je dobro prihvaćena za HPV od strane laboratorija koji vrše rutinske analize, na što ukazuje dobrovoljno sudjelovanje velikog broja ovih institucija u EQA.

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Review

HPV Testing for Cervical Cancer Screening in Croatia

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A B S T R A C T

Opportunistic screening based on the Pap smear has been undertaken in Croatia since 1953. However, cervical cancer remains an important health problem in Croatia when compared to European countries with organised screening programmes. In Croatia, in addition to screening based on well established cytology, Human papillomavirus (HPV) testing is widely used as secondary test as a triage to borderline cytology and as a follow-up after treatment of severe cervical lesions. Many different approaches for HPV testing arose in Croatia over the last decade depending on the needs of each medical institution involved. Presently, there is an urgent need for better networking between the laboratories, the implementation of quality assessment and the adaptation of a uniform system of referring to and reporting of HPV testing. In conclusion, the best possible organisation for HPV testing would be essential for implementation of HPV testing as primary screening test in Croatia, an thus ultimately and hopefully, the more successful cervical cancer control.

***Key words**: Cervical cancer, human papillomavirus testing, screening programme, quality assessment*

Background

Cervical cancer remains an important health problem in Croatia where the incidence rate of 14.4/100,000 women recorded in 2004[1] is slightly lower than the average world incidence (16.2/100,000 women-year) but much higher than rates recorded in European countries with organised screening programmes.

Cervical cancer is highly amenable to screening because it has a long pre-clinical phase with precursor lesions that can be identified by cervical cytology (Papanicolaou or Pap smears) and that can be easily treated using simple procedures if they are detected at an early stage. Opportunistic screening based on the Pap smear has been undertaken in Croatia since 1953 when the first laboratory for gynaecological cytology was established in Zagreb[2]. Since then, a network of more than 30 laboratories has developed and now provides good coverage of a large part of the target population. Opportunistic screening has produced a decrease in cervical cancer incidence from 26/100,000 to 15/100,000 women-years between 1970 and 1990, although it remained almost stable thereafter[1].

The European Code Against Cancer states that all women from 25 years of age should participate in organised cervical screening programmes, the Council of the

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European Union has recommended that all Member States should implement organised cervical cancer screening programmes and the new European Guidelines now state that cervical cancer screening should only be offered within the context of an organised programme[3,4]. In 2003, a panel of Croatian experts prepared a proposal for the implementation of an organised screening programme that was submitted to the Croatian Ministry of Health and Social Welfare[5]. This proposal recommended that the programme should start by offering conventional Pap smears to all women from 25 to 64 years of age (app. 1.2 million women) who should be screened annually for the first 3 years but with the screening interval subsequently extended to 3 years for all women who have had 3 consecutive normal Pap smears. The proposal also recommended the implementation of liquid-based cytology with the extension of the screening interval to 5 years and the addition of HPV testing for women over the age of 30[5].

The implementation of an organised cervical screening programme in Croatia would be facilitated because many of the necessary elements are already in place:

- high quality cytology; gynaecological cytology is a mandatory sub-specialisation and cytotechnicians are required to participate in continuing education since 1967[2]. Also, gynaecological cytology is regularly reviewed and updated with the last improvement being a refinement of cytological classification to »Zagreb 2002« that was adapted from »Zagreb 1990« and the »National Cancer Institute Bethesda System 2001«[6].
- a well developed network of quality controlled gynaecologic cytology laboratories with extensive expertise in gynaecologic cytology
- a nationwide network of gynaecologists that currently offer screening to women,
- well established procedures for the diagnosis and treatment of women with abnormal smears[7],
- a tradition of expert colposcopy that has been in place since 1992,
- a network of laboratories with extensive experience in HPV testing for diagnostic purposes that has been in place since 1995, and
- the computerisation of the health system that is now in the process of being implemented.

However, opportunistic screening in Croatia does not include the entire target population and this is reflected in the mortality rate that has shown only a small decrease between 1970 and 2002 from 6/100,000 to 5/100,000 women-years[1] and the goal must be to reach the very low rate of 2–3/100,000 women-year that has been achieved by the Finnish screening programme[8]. As such, there is now an urgent need for the implementation of a properly organised, population-based screening programme that would effectively combine all the new technologies in a rational approach that would maximise the cost-effectiveness and equitably serve all the women of Croatia. Very importantly, this programme would also have to carefully consider the appropriate management of women who have been vaccinated against HPV and who would have a different risk profile from women who had not been vaccinated[9].

HPV Infections and Cervical Cancer

Persistent infection with HPV has now been confirmed as a necessary, although not sufficient cause of cervical cancer[10,11]. There are more than 130 well characterised HPV types, with approximately 40 types that can infect the genital mucosa. These have been classified into high-risk (hr) and low-risk (lr) HPV genotypes according to their association with cervical cancer and its precursor lesions; HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 were classified as high-risk or carcinogenic types, HPV 26, 53 and 66 as probably carcinogenic, while HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 were classified as low-risk types[12].

The genital HPV types are primarily transmitted by sexual contact and all women who have had a sexual relationship are at risk for both HPV infection and cervical cancer. Host factors as well as behavioural and environmental factors, may facilitate cancer development in women with a persistent HPV infection and the risk of cervical cancer increases with immunodeficiency, higher parity, tobacco smoking, co-infection with other sexually transmitted agents (human immunodeficiency virus [HIV], herpes simplex virus 2 [HSV 2], *Chlamydia trachomatis* and *Neisseria gonorrhoeae*) and long-term (>5 years) use of oral contraceptives[10]. However, the association between hrHPVs and cervical cancer is the strongest ever found for a human cancer with a relative risks for the development of cervical cancer ranging from 50–500 depending on the HPV genotypes, single or multiple HPV infections and the amount of virus (virus load)[10,11]. Also, cervical cancer will not occur in the absence of a persistent infection with one of the oncogenic HPV types[13].

Worldwide, HPV 16 is the most common hrHPV type as it is found in 60% of cervical cancer cases, while HPV type 18 is found in about 10%, types 45 and 31 in 4% each, and types 33, 52 and 58 each in another 2%[10]. HPV 16 and 18 are also associated with about 25% of the low grade cervical lesions (LSIL) and 50 to 60% of the high grade cervical lesions (HSIL). HPV 6 and 11 are responsible for about 10% of LSIL and about 90% of genital warts that do not represent additional risk for cancer developement[10].

HPV infection is very common in young women, but most infections are transient and resolve spontaneously in 6 to 24 months. Only a very small percentage of HPV infections will lead to precursor cervical lesions and only those that persist long-term pose a risk for the development of cancer. Cervical precursor lesions persist longer and progress more quickly in women with hrHPV infections than in women with lrHPV infections or those without HPV. Approximately 60% or more of cases of mild dysplasia resolve spontaneously and only about 10% progress to moderate or severe dysplasia within 2 to 4 years. In some cases, moderate or severe dysplasia may

occur without an earlier detectable mild dysplasia stage. Less than 50% of cases of severe dysplasia progress to invasive carcinoma, with much lower rates seen in younger women. Usually it takes 10 to 20 year for precursor lesions caused by hrHPV to progress to carcinoma and this is what makes cervical cancer a relatively easily preventable disease and provides the rationale for screening[10].

Pap Smear and HPV Testing

Cervical cancer is rare before the age of 30 years. Screening younger women detects many lesions that will regress spontaneously and leads to considerable overtreatment. Also, screening by Pap smear every three years is nearly as effective as yearly screening. According to the WHO recommendation if the resources are limited, screening every 5–10 years – or even just once between the ages of 35 and 45 years – will significantly reduce deaths from cervical cancer[11]. On the other hand, negative HPV test virtually excludes any risk of having significant prevalent cervical disease and provides the same degree of protection over 5 years that the accepted standard of a negative Pap smear provides over 2 years. Therefore, it is likely that HPV testing could also provide substantial cost savings for most European countries by reducing the screening frequency with no increase in risk for the women being screened[14]. However, HPV testing-based screening should not begin before 30 years of age.

Testing for HPV could be a useful cervical cancer screening tool and its use has been proposed for primary screening, triage of equivocal Pap smears and for the follow-up of patients after treatment for severe cervical lesions. Women who test positive are at high risk of developing cervical precursor lesions and cancer and they should be referred to more extensive diagnostic procedures. About 15–30% of women with normal cytology who are hrHPV positive will develop high-grade precancerous lesions, cervical intraepithelial neoplasia (CIN) grade 2 or 3 within 4 years of detecting the HPV infection[15]. In contrast, women who test negative or are lrHPV positive have almost no risk of developing cervical precancer or cancer and it is thus justifiable to offer such women less frequent screening.

HPV testing has been extensively evaluated and major international reviews have concluded that it 1) is more efficient than repeated cytology in the triage of ambiguous cytological lesions, 2) is at least as efficient as cytology as a primary screening test, and 3) is more efficient than cytology as a test of follow-up for recurrence after treatment of severe cervical lesions[10]. HPV testing as primary screening, at this time, is recommended for use only in pilot projects or other closely monitored settings. Several large-scale randomised controlled trials for evaluation of HPV testing for primary screening are at the moment conducted in Europe[9].

A comparative analysis of different studies on HPV testing for primary screening showed that HPV testing was substantially more sensitive in detecting CIN2+ than cytology (96.1% vs. 53.0%) but slightly less specific (90.7% vs. 96.3%). The sensitivity of HPV testing was uniformly high at all ages, whereas the sensitivity of cytology was substantially better in women over the age of 50 than in younger women (79.3% vs. 59.6%), while the specificity of both tests increased with age. These results support the use of HPV testing as the sole primary screening test, with cytology reserved for women who test HPV positive[16].

A good screening test should be accurate, reproducible, inexpensive, easy to perform and easy to follow-up, acceptable and safe. HPV testing meets all of these criteria, except for its high price, and this would probably decrease if the test is used on large-scale. At present, there is only one HPV test approved by the United States Food and Drug Administration (US FDA) and European Agency for the Evaluation of Medical Products (EMEA), the Hybrid Capture 2 test (HC2; Digene Co.), which uses a cocktail of 13 hrHPV types that are included within the 15 hrHPV types noted above.

Many other tests, commercial or in-house are polymerase chain reaction (PCR) based HPV tests, which uses general (consensus) primers that recognise most hr- and lrHPV types. The analysis of the PCR amplicon generated by consensus PCR by different methods (hybridization with specific probes, restriction fragment length polymorphism analysis, and sequencing) enables determination of HPV types as well as direct type-specific primer directed PCR. These methods of HPV genotyping, while sensitive and specific are too costly and cumbersome to incorporate into large-scale screening programmes. In the future, clinicians might benefit from knowing the number and the identification of the specific types present in order to follow for persistent infections and/or to test for cure after therapy, and also to monitor vaccinated women.

HPV Testing in Croatia

Laboratory network

In Croatia, several laboratories for molecular diagnostics offer detection and genotyping of HPVs[17–20]. These laboratories are located in Zagreb, Split, Rijeka and Osijek, and belong to public or to private health care system; only one laboratory is based in research setting. All laboratories are equipped with special clean room to avoid PCR amplicon contamination, specified equipment and reagents required for a specific test. The work is performed by highly trained technicians and supervised by medical doctors, molecular biologists or medical biochemists.

HPV testing methods

At present, there are several HPV testing systems that are used in the established laboratory services in Croatia. The first step in HPV testing is to collect an adequate sample for HPV-DNA determination. HPV testing can be performed using the same specimen collection me-

dium used for cytological examination (Thin-Prep), or dedicated collection medium specified by the manufacturer (Digene, Roche, and other). It should be noted that sampling error and processing of the collected sample could play an important role when using highly sensitive molecular assays.

PCR was traditionally the first implemented method for clinical use and is still used widely in most Croatian laboratories[17]. HC2 method was the first commercial method used in clinical laboratories but now, several other commercial assays (PCR-based methods) are also used: AMPLICOR HPV Test (Roche Co.) and TaKaRa PCR Human Papillomavirus Typing Set (TAKARA Mirus Bio Inc.). In addition, several laboratories offer in-house consensus and type- specific PCR, HPV detection and HPV genotyping by line blot assays (LiPA, Innogenetics; LA HPV genotyping; Roche). Sequencing of PCR amplicon is used in only research setting.

Standardization and quality assessment

Standardization, quality assessment and quality control are important issues in any routine diagnostic testing. They all serve to establish, maintain and guarantee a high level of quality in the performance of a laboratory to provide correct diagnoses that has a major influence on the management of disease. In the past, substantial efforts have been made by public and private organizations to established and assure a high quality of diagnostic procedures. Standardization in protocols and methods, together with regular participation in internal and external quality testing is essential for molecular diagnostic laboratories[21,22]. The challenge for a laboratory that wants to introduce this kind of testing is that it is very demanding because of two major reasons: first, the molecular diagnostic tests are technically demanding and require more expertise from the user compared to conventional tests and secondly, because of the extreme sensitivity of PCR and similar tests that can produce false positive results created by contamination[23]. Scientific, commercial or public institutions should provide panels with negative and positive control specimens which will be analysed on regular basis in diagnostic laboratories participating in a quality control scheme. Data processing and statistical analyses should be done by independent institutions that are responsible for supervising and licensing procedures.

The HPV test should be performed in clinical laboratories with a supervision of specialist of microbiology, cytology, pathology or a molecular biologist. Only tests that have been validated and standardized tests for use in clinical practice should be used and the laboratories should have a validated license to perform the specific test. Other, not clinically validated, HPV tests should be done only for research purposes[24]. Interpretation of the HPV test result should be done according to the standard »cut-off« point recommended by the test manufacturer.

The referral order for HPV testing should include, besides the general identification data, the date the sample was taken, the date of birth, the stage of the menstrual cycle, previous Pap smear findings and, if present, previous colposcopy, treatment and histological results.

The report of HPV test result should be composed of: 1) general patient data (name, date of birth), 2) referral diagnosis (from the referral order: Pap test result, other relevant colposcopic or histological results, 3) date of sample taking, 4) referral physician and medical institution, 5) method of HPV testing, 6) interpretation of HPV test result, and 7) optionally, recommendation of future procedures or follow-up. However, the recommendation should be given only by experts in the field of cervical lesions (gynecologic cytologists, pathologists and gynecologists) considering all clinical, cytological and pathological data available about a particular patient, respecting the accepted algorithms of follow-up or treatment of abnormal cytology and histology[7].

Conclusion

The higher sensitivity of HPV testing over cytology offers a number of advantages, including, most importantly, the potential of reducing cervical cancer rates while reducing the number of screens in a lifetime necessary to achieve this goal. In Croatia, in addition to screening based on well established cytology[2], HPV testing is widely used as secondary test as a triage to borderline cytology and as a follow-up after treatment of severe cervical lesions[7]. At present, screening is done only with the conventional Pap test, and HPV testing therefore requires an additional visit to gynaecologist which wastes both time and money. Moreover, many different approaches for HPV testing arose in Croatia over the last decade depending on the needs of each medical institution involved. Consequently, the implementation of HPV testing in Croatia is still very heterogeneous and uncontrolled. So, there is an urgent need for better networking between the laboratories, the implementation of quality assessment and the adaptation of a uniform system of referring to and reporting of HPV testing. In conclusion, the best possible organisation for HPV testing would be essential for implementation of HPV testing as primary screening test in Croatia, an thus ultimately and hopefully, the more successful cervical cancer control.

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HPV TESTIRANJE U PROBIRU RAKA VRATA MATERNICE U HRVATSKOJ – DANAS I SUTRA

S A Ž E T A K

Oportunistički probir temeljen na citološkom Papa-testu provodi se u Hrvatskoj od 1953. godine. Unatoč tome, u usporedbi s Europskim državama s organiziranim programima probira, rak vrata maternice u Hrvatskoj još uvijek predstavlja značajni zdravstveni problem. U Hrvatskoj, uz probir temeljen na priznatoj i dobro uhodanoj citologiji, HPV testiranje sve se više koristi kao sekundarni test razvrstavanja nakon graničnog citološkog nalaza te kao kontrolni test nakon liječenja težih promjena vrata maternice. Tijekom posljednjih deset godina u Hrvatskoj se pojavljuju brojni različiti pristupi HPV testiranju, ovisno o potrebama i stavovima pojedinih zdravstvenih ustanova koje ga primjenjuju. Stoga se danas javlja neophodna potreba za boljom povezanošću laboratorija, uspostavljanjem sustava kontrole kvalitete i zauzimanjem ujednačenog stava o indikacijama za HPV testiranje i načinu izdavanja nalaza HPV testa. U zaključku smatramo da će najbolje moguće uređen sustav HPV testiranja biti preduvjet za uvođenje HPV testa u primarni probir u Hrvatskoj, kao i uspješniju borbu za suzbijanje raka vrata maternice.

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Short communication

Prevalence of Human Papillomavirus among Croatian Women Attending Regular Gynecological Visit

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A B S T R A C T

Human papillomavirus (HPV) infection has been identified as major risk factor for cervical intraepithelial neoplasia (CIN) and invasive cervical cancer. About 40 HPV viral types are commonly found in the genital tract. Most HPV infections resolve spontaneously, while persistent infection with oncogenic types, namely HPV 16 and 18 is necessary for CIN to occur and progress to cancer. Cervical screening is presently based on the Pap smear that is designed to diagnose precancerous lesions and cervical cancer. The aim of this study was to investigate the prevalence of HPV DNA and to determine HPV types distribution among 361 women attending regular gynecological visit. There were 205 women (29±8 years old) without determined abnormal cervical lesions and 156 women (34±15 years old) with abnormal Pap smear; low grade squamous intraepitehelial lesions (LSIL, n=69), high grade squamous intraepithelial lesions (HSIL, n=72) and atypical squamous cells of undetermined significance (ASCUS, n=15). HPV DNA detection and genotyping was performed by Hybrid Capture 2 assay and additionally by consensus and type-specific primers directed PCR. The overall prevalence of high-risk HPV (hrHPV) in women with abnormal Pap smears was 67.9% (106/156), of which in ASCUS 33.4% (5/15), LSIL 62.3% (43/69) and HSIL 80.6% (58/72). In HPV positive specimens, HPV 16 was found as predominant type in 60.4% cases, followed by HPV 31 (8.5%), HPV 33 (6.6%) and HPV 18 (3.7%). In the group of women without obvious cervical changes the overall hrHPV prevalence was 35.6% with HPV 16 found in 43.8% cases, followed by HPV 31 (17.8%), HPV 33 (9.5%) and HPV 18 (6.8%). In both study groups, women with and without cervical lesions, the prevalence of HPV of indeterminate type was 14.2% and 13.7%, respectively. Our results indicate that cervical intraepithelial lesions are largely associated with HPV type 16, followed by HPV types 31, 33, 18 and HPV of indeterminate type. Although there is a significant difference in hrHPV DNA prevalence among two groups, no significant differences between particular hrHPV types distribution were observed.

Key words: *Cervical intraepithelial lesions, Human papillomavirus(HPV) detection, hybrid capture, HPV prevalence, polymerase chain reaction*

Introduction

Human papillomavirus (HPV) infection has been identified as major risk factor for cervical intraepithelial neoplasia (CIN) and invasive cervical cancer[1,2,]. Epidemiological studies indicate a strong association of high-risk human papillomavirus (hrHPV) genotypes with cervical carcinoma and malignant transformation of cervical epithelial cells[1,2,3].

There are about 40 HPV viral types that are commonly found in the genital tract[4]. The most prevalent hrHPV genotypes worldwide, which infect uterine cervix, are HPV 16 (~53%), followed by HPV 18 (~15%), HPV 45 (~9%), HPV 31 (~6%) and HPV 33 (~3%)[1]. Most HPV infections resolve spontaneously over 6 to 18 months, while viral persistence is necessary for CIN lesions to progress[5]. According to the Croatian National

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Cancer Registry[6], the incidence of cervical cancer in 2004 was 14.4 new cases *per* 100,000 women. There are only a few reports describing HPV genotype distribution in abnormal cervical Pap smears in Croatia[7,8]. Initial screening is presently based on the Pap smear and cytological examination. Cytology-based cervical cancer screening using Pap smears and new technologies such as liquid-based cytology have made significant impact on reducing cervical cancer rates worldwide[10]. Early detection and appropriate treatment of cervical lesions provide the best approach to prevent cervical cancer[11–13]. Treatment of pre-invasive lesions identified in screening programmes is very effective, but care should be taken to avoid unnecessary over-treatment. HPV testing and prophylactic vaccination will change substantially the use of cervical cytology, probably in favour to HPV testing as the primary test in secondary prevention[10].

Current studies evaluate the implementation of HPV testing in screening algorithms for the women with an increased risk for development of cervical cancer and those which could undergo unnecessary re-testing[14,15].

The aim of this study was to investigate the prevalence of HPV DNA and HPV types distribution among 361 women attending regular gynecological visit. According to cytological findings they were separated in the two study groups: women with normal and women with abnormal Pap smears.

Materials and Methods

This study was performed among 361 women attending regular gynecological examination in Zagreb and Rijeka County between 2004 and 2006. Informed consent was sign by all women who participated in the study. Cervical lesions were classified according to »Zagreb 2002« Uniform Classification of Uterine Cervix Cytological Findings in Croatia[9] adapted from the Bethesda system into normal epithelium, low-grade intraepithelial lesions (LSIL or CIN1) indicating a low risk of malignant transformation which may resolve spontaneously, high-grade intraepithelial lesions (HSIL, CIN2 or CIN3-carcinoma *in situ*) having a potential to progress to invasive cervical cancer, and finally borderline Pap smears, classified as atypical squamous cells of undetermined significance (ASCUS). Cervical smears were obtained from 156 women (34±15 years old) with LSIL (n=69) and HSIL (n=72) and ASCUS (n=15). All women from this group were diagnosed by cytological examination followed by subsequent HPV detection and genotyping. A second group of women consisted of 205 women (29±8 years old) undergoing regular preventive gynecological examinations. Women in this group had normal cytological smears and were also tested for the presence of HPV DNA, followed by subsequent genotyping of HPV DNA positive cases. All cervical smears were taken with cervical brush and collected to specimen transport medium (Digene Diagnostic, USA) and stored at +4 °C until testing. Samples were divided into aliquots for hybridization in the liquid phase and polymerase chain reaction (PCR) tests.

Hybrid capture HPV assay

The Hybrid Capture assay (HC2, Digene Diagnostic, USA) is based on signal amplification by chemiluminescent detection[16]. Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. The RNA:DNA hybrids are captured onto the surface of a tube coated with antibodies specific for hybrids and detected with a chemiluminescent substrate. The intensity of the light emitted denotes the amount of target DNA in the specimen. RNA probe mix for the detection of hrHPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) was used according to the manufacturer's instructions.

DNA extraction and PCR analysis

DNA was isolated by NucleoSpin®Tissue kit (Macharey-Nagel, Duren, Germany) according to the manufacturer's instructions. Successful DNA extraction was confirmed by the presence of β-globin gene sequence[17]. Detection of HPV DNA was performed by PCR using E6 and E7 consensus primers pU-1M/pU-2R, which are specific for HPV 16, 18, 31, 33, 35, 52 and 58 types[18] (Human Papillomavirus Typing Set, Takara Biomedicals, Japan). HPV typing of HPV 16, 18 and 33 was performed by PCR with primers chosen within the E6 region (Human Papillomavirus Detection Set, Takara Biomedicals, Japan)[19], while HPV 31 was amplified with primers chosen within the E7 region of the HPV genome[3].

PCR products of 228 to 268 bp for generic amplification, and 100 and 140 bp for type-specific amplification were resolved by electrophoresis on 1.5 to 2% agarose gel.

Statistical analysis

HPV prevalence was expressed as percentage of HPV positives against all cases tested for HPV. The prevalence of individual hrHPV genotypes was determined as single infection. Multiple hrHPV infections were defined as two hrHPV genotypes. The distribution of non-continuous cytological variables *versus* HPV status was analysed with the Chi-square test (χ^2). P values of <0.05 were used as the cut-off for statistical significance.

Results and Discussion

High-risk HPV DNA was detected in 179 and 154 out of 361 cervical smears by HC2 and PCR, respectively (Table 1). It was demonstrated that correlation between HC2 and PCR was between 85.8% (91/106) to 86.3% (63/73) This data were additionally confirmed by type-specific PCR amplification which failed to detect any HPV type in 13.7% and 14.2% of cases in the group with CIN and the group with normal cervical findings, respectively, and was referred as HPV of indeterminate types (Table 1).

In 25 women, the positive HPV-DNA results obtained by HC2 test were not confirmed by type-specific PCR amplification for HPV types 16, 18, 31 and 33, suggesting a presence of another HPV type which could be detected by

TABLE 1
HIGH-RISK HUMAN PAPILLOMAVIRUS PREVALENCE AND TYPES DISTRIBUTION IN WOMEN WITH (N=156) AND WITHOUT (N=205) CERVICAL ABNORMALITIES

	n	hrHPV negative		hrHPV positive		HPV 16		HPV 18		HPV 31		HPV 33		HPV indeterminate*		Multiple infection**	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
ASCUS	15	10	66.7	5	33.3	3	60.0	0	-	1	20.0	0	-	0	-	1	20.0
LSIL	69	26	37.7	43	62.3	24	55.8	1	2.3	3	6.9	3	6.9	9	20.9	3	6.9
HSIL	72	14	19.4	58	80.6	37	63.8	3	5.2	5	8.6	4	6.9	6	10.3	3	5.2
Women with abnormal Pap smears (34±15 years)	156	50	32.1	106	67.9	64	60.4	4	3.7	9	8.5	7	6.6	15	14.2	7	6.6
Women with normal Pap smears (29±8 years)	205	132	64.4	73	35.6	32	43.8	5	6.8	13	17.8	7	9.5	10	13.7	6	8.2

hrHPV – high-risk human papillomavirus, *HPV indeterminate: HPV types other than 16, 18, 31, 33, **Multiple HPV infections: positive for HPV 16/18, 16/31 and 18/31

the probes of broader HPV type spectrum in the HC2 assay[16].

Comparison of the HC2 and PCR with hrHPV primers demonstrated concordant results in as many as 86.0% (154/179) of HPV-DNA positive samples. Therefore, PCR method with E7 consensus primers pU-1M/pU-2R which achieved sensitivity comparable to HC2 is also a good method for screening assessments. Development of methods for simple, rapid and accurate detection of HPV DNA has a central role in many strategies designed to reduce the risk of cervical cancer[20,21].

High-risk HPV infection was present in 73 out of 205 (35.6%) women with normal Pap smear (control group) and in 106 out of 156 (67.9%) women with abnormal Pap smear. The hrHPV prevalence is significantly different between these two groups (χ^2=37.060, p≤0.001) (Table 1). Statistically significant difference in hrHPV prevalence was detected in women with LSIL – 62.3% (43/69) and those with HSIL – 80.6% (58/72) compared to women with ASCUS – 33.3% (5/15) (χ^2=14.511, p≤0.001 for trend).

Out of all HPV positive specimens HPV 16 was the predominant type in all cytological entities detectable in 60.0% of ASCUS, 55.8% of LSIL and 63.8% of HSIL as well as in 43.8% women with normal Pap smears (Table 1). In most studies HPV 16 was found to be the most prevalent HPV genotype in cervical cancer, precursors lesions and cytologically normal Pap smears[5,22]. The association of HPV types and histological type of the cancer is well established; HPV16 being the most frequently found genotype in squamous cell carcinoma (SCC), while HPV18 in adenocarcinoma[23]. Our previous study demonstrated the high overall prevalence of HPV-DNA in cervical neoplasia (>90%)[24]. In CIN3 and SCC, HPV 16 was the most common hrHPV type, identified in 65% and 52% of cases, respectively[24]. Grce et al. have demonstrated that among Croatian non-pregnant women increase in hrHPV prevalence is associated with CIN grade changes from 1 to 3 (35.1%, 64.6% and 81.0%, respectively) with HPV 16 being predominant type in all cervical lesions[7].

HPV 18 was detected in 3.7% (4/106) of women with intraepithelial lesions and in 6.8% (5/73) specimens of women with normal smears. HPV 31 and 33 are detected in 8.5% (9/106) and 6.6% (7/106) cases of CIN, respectively, and in 17.8% (13/73) and 9.5% (7/73) of normal smears. The high prevalence of HPV 31 (17.8%) was found in women with normal Pap smears, although the most relevant studies report average prevalence between 5 and 8%[1,3,7,8]. Hadžisejdić et al.[24] have demonstrated the unexpected high prevalence of HPV 31 of 10% and 26% in CIN3 and SCC, but in almost half of these cases as part of multiple infection.

HPV of indeterminate type was found in 14.2% (15/106) and 13.7% (10/73) of cases from abnormal and normal Pap smear groups, respectively. In LSIL cytological group, the prevalence of indeterminate HPV type 20.9% was the highest one, although with no statistical significance. The possible explanation for this finding could be the fact that HPV of indeterminate type could be low- as well as high-risk HPV types; there is always a possibility of cross-hybridization of HC2 RNA probes and in case of PCR with generic primers amplification of a large number of different HPV types.

Low prevalence of multiple infections in LSIL and HSIL as well as in normal Pap smears was found in 6.9%, 5.2% and 8.2% cases, respectively. These data suggest that single hrHPV infection in LSIL and HSIL (>90%) are significant for establishing and maintaining proliferative growth of epithelial cells[25].

Conclusion

Our results indicate that cervical intraepithelial lesions are largely associated with HPV types 16, 31 and 33 followed by HPV 18 and HPV of indeterminate type. A significant difference between hrHPV prevalence among the group of women attending regular examination with normal cytology and the group of women with abnormal Pap smear was observed. However, no significant differences between particular hrHPV types distribution were

observed between the two groups of women. The significance of higher prevalence of HPV 31 type in women with normal Pap smears as well as indeterminate HPV type in low-grade intraepithelial lesions should be considered in future epidemiological studies of hrHPV prevalence in borderline cervical intraepithelial lesions.

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PREVALENCIJA HPV U CERVIKALNIM BRISEVIMA UZETIM PRIGODOM REDOVITOG GINEKOLOŠKOG PREGLEDA

SAŽETAK

Infekcija humanim papilomavirusom (HPV) je jedan od glavnih rizičnih čimbenika za nastanak cervikalne intraepitelijske neoplazije (CIN) i invazivnog karcinoma vrata maternice. Oko 40 HPV tipova je utvrđeno u urogenitalnoj regiji žena. Iako u većini slučajeva infekcija prolazi spontano, trajna infekcija s HPV tipovima visokog rizika, posebice HPV-16 i HPV-18 je neophodna za nastanak CIN-a i invazivnog karcinoma. Probiranje na prekancerozne lezije i karcinom vrata maternice danas se temelji na citološkom pregledu Papa-razmaza. Cilj i svrha ove studije je bila da se ispita prevalencija HPV-DNK i odredi raspodjela HPV tipova visokog rizika u skupini žena (n=361), koje su došle na redovni ginekološki pregled i pristale da sudjeluju u studiji. U skupini je bilo 205 žena životne dobi 29±8 godina s urednim Papa-nalazom i 156 žena u dobi 34±8 godina s promjenama u Papa-nalazu; s niskim stupnjem intraepitelijske lezije (LSIL, engl. *Low grade Squamous Intraepithelial Lesions*) 68 žena, s visokim stupnjem intraepitelijske lezije (HSIL, engl. *High grade Squamous Intraepithelial Lesions*) 72 žene i s atipičnim nalazom pločastih stanica neodređenog značaja (ASCUS, engl. *Atypical Squamous Cells of Undetermined Significance*) 15 žena. Određivanje HPV-DNK i tipizacija provedeni su metodama Hybrid Capture 2 (HC2) i lančanom reakcijom polimeraze (PCR, engl. Polymerose Chain Reaction) temeljenim na koncenzus i tip-specifičnim početnim oligonukleotidima. Ukupna prevalencija HPV-DNA visokog rizika u žena s abnormalnim Papa-razmazom bila je 67,9% (106/156) i to s ASCUS-om 33,4% (5/15), LSIL-om 62,3%

(43/69) i HSIL-om 80,6% (58/72). Od svih HPV-pozitivnih uzoraka u toj skupini, HPV-16 ja nađen u 60,4% žena, HPV--31 u 8,5%, HPV-33 u 6,6% i HPV-18 u 3,7% žena. U skupini žena bez promjena na vratu maternice, ukupna prevalencija HPV-DNK visokog rizika je utvrđena u 35,6% žena, s udjelom HPV-16 od 43,8%, HPV-31 s 17,8%, HPV-33 s 9,5% i HPV-18 s 6,8%. U obje skupine, prevalencija neodređenog HPV-tipa bila je 14,2% i 13,7%. Naši rezultati su pokazali da su intraepitelijska oštećenja vrata maternice udružene s HPV-tipovima visokog rizika i to 16, 31, 33 i 18 u više od 85% slučajeva, dok preostali dio predstavljaju netipizirani HPV-tipovi.

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Human Papillomavirus DNA Typing in the Cervical Specimens among Women of Split and Dalmatian County

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A B S T R A C T

Human papillomavirus (HPV) infection is the main cause of cervical cancer, the second most common cancer in women worldwide. More than 200 types of HPV have been described, and within this range more than 40 types attack epithelium of genital tract. The types that were most commonly related to the development of cervical cancer are called high-risk types (HR HPV). There are very few studies about HPV prevalence in Croatia and there is an absolute lack of data for Split and Dalmatian County. Therefore, during a 6 month period, we routinely screened 570 women for HPV DNA at the Educational Public Health Institute of Split and Dalmatian County. HR HPV was detected in cervical exfoliated cells, by using Hybrid Capture 2 HPV DNA test. Out of the total number of tested women, 200 (35%) of them were positive to HR HPV. Polymerase chain reaction (PCR) based assays were employed for HR HPV genotyping in positive specimens. The following frequency was observed: HPV 16 in 10%, HPV 18 in 6.1%, HPV 31 in 2.6%, HPV 33 in 1.9%, HPV 52 in 1.4%, HPV 59 in 0.7%, HPV 45 in 0.4% specimens, while 11.9% of tested specimens currently remained untyped. It is necessary to expand this study to a larger number of women, in order to better evaluate genital HPV types distribution among women in this region.

Key words: *Human papillomavirus (HPV), high-risk genotypes, Split and Dalmatian County, Croatia*

Introduction

Cervical cancer is the second most common cancer in women worldwide. Infection with Human papillomavirus (HPV) is the main cause of cervical cancer[1]. HPV is the most common sexually transmitted viral disease and one of the most frequent causal agents of sexually transmitted diseases. Genital HPV infection is rarely reported (there is no legal obligation). While its prevalence is assumed to be higher than 20 million, the incidence of HPV infection only in the United States ranges from one million to 5.5 million per year[2]. The prevalence of HPV DNA in cervical cancer cases is 99.7%[3], while in cervical specimens of women without cervical cancer ranges from 5% to 20%[4–6]. More than 200 types of HPV are known, and within this range more than 40 attack epithelium of genital tract. The types that are most commonly related to the development of cervical cancer, so called oncogenic or high-risk types (HR HPV), are: 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 70[2,7]. The most common HPV types in cervical cancer are: HPV type 16 (46% to 63%) and HPV type 18 (10% to 14%)[7]. There are very few studies in Croatia about HPV prevalence related only to cervical specimens of women with abnormal Papanicolaou (Pap) smear, with the most common types: HPV type 16 (11.4% to 20.2%) and HPV type 31 (5% to 17.8%)[8–10]. Concerning the absolute lack of data for Split and Dalmatian County, we initiated a testing for HPV in order to evaluate the prevalence in our region.

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Materials and Methods

In the Educational Public Health Institute of Split and Dalmatian County, during the 6 months period in 2006, HPV screening tests were done in 570 women. The age range of the study was 18 to 62 years (the average age 40 years). The study population included all women referred by their gynecologists for HPV DNA testing to our laboratory, as a survey of general population (with unknown cytological diagnosis). HR HPV was detected in cervical exfoliated cells, by using Hybrid Capture 2 (HC2) HPV DNA test (Digene Corporation, Gaithersburg, MD USA). HC2 test was used in screening specimens for high-risk types of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), which had been approved by Food and Drug Administration (FDA, USA) for detection of HPV DNA in cervical specimens for diagnostic purpose. This test cannot differentiate individual types of HPV within the group[2].

Only HR HPV positive specimens analyzed by HC2 test were sent for further genotyping. DNA was extracted from cervical cells, by using reagents Nucleo Spin Tissue (Machery-Nagel, Düren, Germany). Genotypes were distinguished by polymerase chain reaction (PCR) based assays using primer sequences for HR HPV types: 16, 18, 31, 33, 45, 52, and 59 (Table 1). Positive and negative controls were included in each reaction. PCR reactions were performed according to the manufacturer instruction on PCR system (Applied Biosystems, Foster City, USA). PCR products were detected using agarose gel electrophoresis (3% agarose gel). The specific primers for amplification of the sequence containing E6 region of HPV types: 16, 18 and 33 were used (TaKaRa, Bio. Inc., Japan)[11–12]. Amplification products yield DNA fragment of 140 bp for HPV types: 16, 18 and 141 bp for HPV type 33. The presence of HPV types: 31, 45, 52 and 59 was detected by using primers for amplification of the sequence containing E7 region, described by Walboomers et al.[3], yielding a PCR product of 100 bp.

TABLE 1
PRIMER SEQUENCES USED FOR HIGH-RISK HPV GENOTYPING

HPV Type	Primer sequence (5'–3')
HPV 16	GTTTGCAGCTCTGTGCATA
HPV 16	CATTTTATGCACCAAAAGAGAACTGCAATG
HPV 18	GTGTTCAGTTCCGTGCACA
HPV 18	TGAGAAACACACCACAATACTATGGCGCGC
HPV 33	GTCTCCAATGCTTGGCACA
HPV 33	CATTTTGCAGTAAGGTACTGCACGACTATG
HPV 31	GGGCTCATTTGGAATCGTGTG
HPV 31	AACCATTGCATCCCGTCCCC
HPV 45	CCCACGAGCCGAACCACAG
HPV 45	TCTAAGGTCCTCTGCCGAGC
HPV 52	GCAGAACAAGCCACAAGCAA
HPV 52	TAGAGTACGAAGGTCCGTCG
HPV 59	CTCCGAGAATGAAAAAGATGAA
HPV 59	GCTGAAGTTGATTATTACA

HPV – Human Papillomavirus

Results

During routine HPV testing in Split and Dalmatian County, total of 570 women were tested, and 200 (35%) of them were positive to HR HPV, when samples were assessed by HC2 test. Only HR HPV positive specimens by HC2 were further genotyped by PCR based assays. Our analysis revealed the frequency of HPV 16 in 10%, HPV 18 in 6.1%, HPV 31 in 2.6%, HPV 33 in 1.9%, HPV 52 in 1.4%, HPV 59 in 0.7%, HPV 45 in 0.4% specimens (Table 2). Among HPV positive sample, HPV 16 was the more abundantly found types in 28.5% cases, followed by HPV 18 (17.5%), 31 (7.5%), 33 (5.5%), 52 (4%), 59 (2%) and 45 (1%), while 68 (34%) specimens remained with unresolved genotype (Table 2). Multiple HPV infections were found in two cases out of 200 (1%): HPV 16 and 33, and HPV 18 and 31, respectively.

TABLE 2
HIGH-RISK HPV FOUND IN THE CERVICAL SPECIMENS AMONG WOMEN IN SPLIT AND DALMATIAN COUNTY

HPV Type	Number	% of tested samples (N=570)	% of positive specimens (N=200)
16	57	10.0	28.5
18	35	6.1	17.5
31	15	2.6	7.5
33	11	1.9	5.5
52	8	1.4	4.0
59	4	0.7	2.0
45	2	0.4	1.0
others	68	11.9	34.0

HPV – Human Papillomavirus

Discussion

Having in mind the absolute lack of data for Split and Dalmatian County, we screened HPV prevalence in our region. Our preliminary study included 570 women who underwent routine screening as a survey of general population with either normal or abnormal Pap smears. Our results showed that 35% of them were positive for HR HPV. These positive samples detected by HC2 were further genotyped by type-specific primer-directed PCR for 7 most common types of HR HPV (Table 1). In our study, among HPV positive sample the most commonly found HPV types were: HPV 16 in 28.5%, HPV 18 in 17.5%, and HPV 31 in 7.5% specimens, while 68 (34%) specimens currently remained undetermined (Table 2).

The prevalence of HR HPV in cervical specimens of women without cervical cancer ranges from 5% to 20% worldwide, while in healthy women with normal Pap smears it is approximately 10%[4–6]. Among European women with low grade lesions found in Pap smears, HPV prevalence in exfoliated cells is 67.8%, with the most common HPV types: HPV type 16 in 19.4% specimens

and HPV type 31 in 10.4% specimens. Prevalence of HPV type 18 in Europe is 5.1%, but ranges from 0% to 31.4%, compared by region[13]. Very few studies about HPV prevalence in Croatia were conducted. The studies presented by Grce et al. analyzed only women with abnormal Pap smears. According to these studies in Croatia, HPV prevalence in cervical specimens of women with abnormal Pap smears ranges from 43% to 64%, with the most common types: HPV type 16 (11.4% to 20.2%) and HPV type 31 (5% to 17.8%)[8–10]. In our study, we had unexpectedly high number of specimen without genotype (34%), in comparison to studies by Grce et al. that reported from 23.7% to 49% of untyped HPV DNA positive samples[9,10]. This result might be due to presence of other rare types of HPV, insufficient quantity of DNA in the specimen or due to differences between detection methods (HC2 and PCR). The integrity of DNA from 68 specimens with unresolved HPV genotype was further tested for β-actin by PCR, and 13 (19.1%) did not yield amplification product.

Our results of HR HPV genotyping are slightly different from those found in other studies conducted in Croatia due to several reasons. In previous studies regarding HPV prevalence in Croatia, mainly women with abnormal Pap smears were tested for the presence of HPV and typed for a limited number of HPV types (HPV 6/11, 16, 18, 31 and 33)[8–10]. In our study, population included women who were referred by their gynecologists for HPV DNA testing to our laboratory (with unknown cytological diagnosis). Furthermore, in this study HPV DNA positive samples by HC2 were further genotyped for more HR HPV types (HPV 16, 18, 31, 33, 52, 59 and 45) than those reported in previous studies. The frequency of HPV types in the study of Grce et al. 2001[9] conducted on 1,874 cervical specimens collected in the region of Zagreb and this study are similar for HPV types 16 (12 versus 10%) and 33 (2.7 versus 1.9%), but discordant for HPV 18 (2.4 versus 6.1%) and 31 (5.1 versus 2.6%). The reason for these discrepancies could be (1) small number of analyzed sample in this study, (2) the choice of type-specific primers for DNA amplification, and (3) different distribution of HR HPV types in both Counties. Split and Dalmatian County is an important tourist region of Croatia with a high number of tourists passing through. It is also a maritime centre with the port and significant number of seamen that are carriers of HPV types gained from all over the world and spread within this County. Due to these reasons, it is important to continue this study on larger number of specimens and to enlarge the spectrum of genotyping of HR HPV in order to evaluate HPV genital types distribution among women in this region, especially in the context of HPV vaccine application.

Conclusion

The data obtained from our study indicate that HPV 16 and 18 are the most common HPV types found in the cervical specimens among women of Split and Dalmatian County as in most studies worldwide. However, low abundant HR HPV types differ between regions in Croatia. In our opinion, it is necessary to expand this study to a larger number of women in order to obtain a better distribution of HR HPV types in Split and Dalmatian County.

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RASPODJELA POJEDINIH TIPOVA HUMANOGA PAPILLOMA VIRUSA U UZORCIMA OBRISKA VRATA MATERNICE ŽENA SPLITSKO-DALMATINSKE ŽUPANIJE

S A Ž E T A K

Infekcija uzrokovana human papillomavirusom (HPV) je glavni čimbenik razvoja karcinoma vrata maternice, drugoga po učestalosti među karcinomima u žena u svijetu. Poznato je više od 200 tipova HPV-a, od kojih više od 40 tipova napada epitel spolnoga sustava. Tipovi koji se najčešće povezuju s razvojem karcinoma vrata maternice nazivaju se visokorizični tipovi (HR, engl. *High Risk* HPV). U Hrvatskoj je, do sada, napravljeno svega nekoliko studija o prevalenciji HPV-a, a ne postoje nikakvi podatci za Splitsko-dalmatinsku županiju. Zbog toga smo, u Nastavnom zavodu za javno zdravstvo Splitsko-dalmatinske županije, napravili probir 570 žena na HPV DNK, u razdoblju od 6 mjeseci. HR HPV dokazan je u obriscima vrata maternice, metodom HC2 (Hybrid Capture 2, Digene). Od ukupnoga broja testiranih žena njih 200 (35%) je bilo pozitivno na visokorizične tipove HPV-a. Daljnjom genotipizacijom HR HPV pozitivnih uzoraka, metodom lančane reakcije polimerazom (PCR, engl. *Polymerase Chain Reaction*), utvrđena je učestalost tipa 16 u 10% uzoraka, tipa 18 u 6,1%, tipa 31 u 2,6%, tipa 33 u 1,9%, tipa 52 u 1,4%, tipa 59 u 0,7%, tipa 45 u 0,4% uzoraka, dok su 11,9% uzorka, za sada, ostala netipizirana. Smatramo da je neophodno proširiti studiju na veći broj ispitanica, te nastaviti praćenje proširenosti pojedinih tipova HPV-a u žena ove regije.

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Short communication

Genital Human Papillomavirus Infection in Women from the Zagreb Region

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A B S T R A C T

Human papillomavirus (HPV) infection is the most common sexually transmitted infection, especially among young, sexually active individuals. As persistent infection with oncogenic types may lead to cervical cancer, HPV testing is a useful tool to screen for women at risk for subsequent development of cervical cancer. The aim of the study was to determine the prevalence of high-risk HPV (hrHPV) infection in different age groups of cytologically selected women from the Zagreb region, and to evaluate the frequency and results of repeat hrHPV testing. During a one-year study period (November 2005 to November 2006), a total of 3,440 cervical samples from women attending gynecological services of public and private health care systems were received. They were tested for 13 hrHPV genotypes by the polymerase chain reaction based AMPLICOR HPV test (Roche Molecular Systems). The overall prevalence of hrHPV was 34.6%. Most samples were obtained from women aged 21–30 years (44.2%), followed by the 31–40 (27.6%), 41–50 (15.7%), 51–60 (5.3%) and ≥61 (2.4%) age groups. Out of 3,227 cervical samples obtained from women of known age, 4.9% were obtained from the group of girls younger than 21, in which the highest prevalence of hrHPV (49.4%) was found. A similar prevalence was observed in women aged 21–30 (45.1%). The prevalence gradually decreased with age. During the study period, repeat hrHPV testing was performed in samples from 66 women at different intervals. Out of 28 women that were hrHPV negative on initial testing, only five women turned positive on repeat testing. Out of 38 women that were positive on initial testing, in one-third hrHPV could not be detected on repeat testing. As expected, hrHPV infection was highly prevalent in female adolescents and young women. Further investigation on repeat hrHPV testing is needed to assess virus clearance and rate of newly acquired infection.

Key words: *High-risk human papillomavirus (hrHPV), prevalence, AMPLICOR HPV Test, repeat hrHPV testing*

Introduction

Human papillomavirus (HPV) infection is the most common sexually transmitted infection[1]. Among more than 100 known HPV genotypes, there are 40 that affect anogenital mucosa[2]. According to their potential to increase the risk of cervical cancer, they are divided into low-risk group, which is usually associated with benign lesions such as condylomata acuminata, and high-risk HPV (hrHPV) genotypes, which have a role in cervical carcinogenesis[3]. High-risk HPV types are demonstrated in almost 100% of cervical carcinomas[4]. The infection is especially prevalent among sexually active adolescents and young adults, but usually of short duration in these age groups[5]. Only persistent infection, which is more common in older women, may lead to cervical cancer and its precursor lesions, cervical intraepithelial neoplasia 2/3 (CIN 2/3). The precise role of HPV in the etiology of cervical cancer is unknown and the host immune system is considered to be of crucial importance in HPV clearance or development of persistence after primary infection[6,7].

HPV testing has a recognized role in improvement of cervical-cancer screening programmes, evaluation of women with unclear or low-grade cytological abnormalities, and follow-up of patients treated for CIN[3,8,9]. The only test currently approved by the U.S. Food and Drug Ad-

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ministration for HPV detection is Hybrid Capture 2 HPV DNA test (HC2) (Digene Corporation, Gaithersburg, MD, USA). This signal amplification assay detects 13 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and five low-risk (6, 11, 42, 43 and 44) HPV types. Although evaluation of its laboratory performance has confirmed its reliability and reproducibility, several recent studies showed a significant analytical inaccuracy, mainly due to the cross-reactivity of its high-risk probe cocktail[10–12]. In 2003, a newly developed polymerase chain reaction based AMPLICOR HPV test (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was marketed. This target amplification test detects the same 13 hrHPV types as HC2 assay, with simultaneous detection of human β-globin gene, which allows assessment of cellular adequacy, extraction and amplification of each processed specimen. Some recent studies demonstrated the HC2 assay and AMPLICOR HPV test to give comparable results, both being suitable for routine use[13,14]. In a recent study, the AMPLICOR HPV test demonstrated even higher analytical sensitivity and specificity[13]. While the higher analytical specificity of AMPLICOR HPV in comparison to that of HC2 can be considered clinically beneficial, the clinical importance of the higher analytical sensitivity of AMPLICOR HPV is still a matter for extensive professional discussion[13,15].

The aim of the study was to determine the prevalence of hrHPV infection in different age groups of women from the Zagreb region using AMPLICOR HPV test, and to evaluate the frequency and results of repeat hrHPV testing in the same population.

Materials and Methods

Patients and clinical specimens

During the one-year study period (November 2005 to November 2006), a total of 3,440 cervical cell specimens for hrHPV testing were received at the Laboratory of Molecular Microbiology, Zagreb Institute of Public Health. The specimens were obtained from cytologically selected women attending gynecological services of public and private health care systems in the Zagreb region. The median age of the women was 31 (range, 15–73) years. For 213 specimens no data on the patients' age were available. Cervical samples were collected by 46 gynecologists using Cervex-Brush (Rovers Medical Devices). Upon sampling, the brush was washed in a ThinPrep vial containing PreservCyt solution (Cytyc Corporation, Boxborough, MA, USA). During the study period, repeat hrHPV testing was performed in samples from 66 women at different time intervals. The indication for repeat testing was clinical evaluation of the patients.

Specimen preparation

HPV DNA was isolated from the PreservCyt solution using AmpliLute Liquid Media Extraction kit (AMPLICOR HPV test, Roche Molecular Systems) according to the manufacturer's instructions. Briefly, HPV DNA was released by lysing cervical cells under denaturing conditions at elevated temperatures in the presence of proteinase K, chaotropic agent and detergent, isolated and purified over columns with silica-based membrane using vacuum pressure, and eluted with elution reagent. During the procedure, the human β-globin gene was concurrently isolated, allowing assessment of cellular adequacy, extraction and amplification of each processed specimen.

Amplification

Polymerase chain reaction (PCR) based AMPLICOR HPV test (Roche Molecular Systems) is designated to amplify HPV DNA from 13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The test amplifies 165 bp long nucleotide sequence within the polymorphic L1 region of the HPV genome with a master mix containing biotin labeled primers. An additional primer pair is added to allow simultaneous amplification of the human β-globin gene (268 bp amplicon). PCR was performed with a final reaction volume of 100 μL, containing 50 μL of AMPLICOR HPV master mix and 50 μL of isolated DNA, on GeneAmp PCR System 9700 with gold block (Applied Biosystems, Foster City, CA, USA) using AMPLICOR defined parameters: 2 min at 50 °C and 9 min at 95 °C, followed by 40 cycles of: 30 s at 95 °C, 45 s at 54 °C and 30 s at 72 °C, with a final extension at 72 °C for no longer than 1 h.

Hybridization and detection

After amplification, the amplicons were chemically denatured to form single-stranded DNA. Two separate 96-microwell plates, one coated with HPV high-risk probes and the other with β-globin specific oligonucleotide probes, were used for HPV and β-globin detection. After adding of AMPLICOR hybridization buffer and denatured amplicons to appropriate wells of both detection plates, hybridization reaction occurred. Following hybridization and washing procedures, bound hybrids were detected with a biotin avidin-horseradish peroxidase assay. The absorbance at 450 nm was measured immediately using an automated microwell plate reader (Anthos 2010, ASYS Hitech, BIOCHROM Group, Great Britain). According to AMPLICOR instructions, HPV absorbance reading of ≥0.20, accompanied with any value of β-globin result, was considered positive for the presence of hrHPV. HPV result of <0.20 and β-globin result of ≥0.20 was considered negative for the presence of hrHPV. Test result could not be interpreted if both (HPV and β-globin) absorbance values were <0.20. HPV DNA, if present, could not be detected because of inadequate cell content of specimen or inadequate extraction or amplification procedure. In that case, another aliquot of original specimen was retested and, if the same result appeared, new specimen collection was recommended.

Statistical methods

Proportions were compared by the χ^2-test. A p value <0.01 was considered statistically significant. The 95% confidence interval for a proportion was calculated ac-

cording the Wilson procedure with a correction for continuity[16]. The decrease of hrHPV prevalence with age was estimated by the χ^2-goodness of fit to rectangular distribution using STATISTICA 7.1 (StatSoft Inc., Tulsa, OK, USA).

Results

Results of this study conducted during the one-year period in 3,440 cervical samples tested for the presence of 13 hrHPV types by AMPLICOR HPV test showed the overall hrHPV prevalence to be 34.6%. Out of 3,227 cervical specimens from women of known age, hrHPV was detected in 1,120 (34.7%) samples. In four (0.1%) samples, HPV test results could not be interpreted. Out of 213 cervical samples from women with no age data available, hrHPV was detected in 70 (32.9%) samples (χ^2= 0.24, p>0.01).

Out of 3,227 cervical samples obtained from women of known age, 158 (4.9%) were obtained from the group of girls younger than 21, in which the highest prevalence of hrHPV (49.4%) was recorded. Most cervical specimens were obtained from women aged 21–30 (1,425 samples, 44.2%); in this group hrHPV was detected in 642 (45.1%) patients. The prevalence of hrHPV gradually decreased with age (χ^2=1,531.3, df=5, p<0.01). In the 31–40 age group (27.6% of study patients), the recorded prevalence was 28.9%, followed by 19.3% in the 41–50 age group and 13.5% in the 51–60 age group. In women older than 60, the prevalence of hrHPV was 28.9% (Table 1). There was a statistically significant difference in the distribution of hrHPV genotypes according to age groups (χ^2=183.96, df=5, p<0.01), which was due to the higher prevalence of hrHPV in younger women (≤30 years).

Out of 3,440 cervical samples tested for hrHPV, 1,168 (34%) of samples were diagnosed as atypical squamous cells of undetermined significance (ASCUS), 468 (13.6%) of samples were diagnosed as dysplasia levis or CIN 1, 152 (4.4%) of samples were diagnosed as CIN 2 (n=107), CIN 3 (n=43) or carcinoma in situ (n=2), and for 1,652 (48%) of samples the informations regarding cervical abnormalities were not available. In the ASCUS group hrHPV was detected in 432 (37%) patients. The prevalence of hrHPV increased with the severity of cervical lesions. In the CIN 1 group hrHPV was detected in 201 (43%) and in the CIN 2/3 group in 111 (73%) patients (χ^2=158.1, p<0.01).

During the study period, repeat hrHPV testing was performed in samples from 66 women at different intervals. Out of 28 women found to be hrHPV negative on initial testing, only five women turned positive on repeat testing. Six women underwent retesting within three months of initial testing, all of them being negative for hrHPV again. Of twelve women that underwent re-testing within 3–6 months, hrHPV was detected in three cases. In seven women, repeat testing was performed within 6–9 months, one of them being positive for hrHPV. Three women underwent retesting within 9–12 months, with hrHPV detected in one case.

On repeat testing, hrHPV could not be detected in one third of 38 women found hrHPV positive on initial testing. Repeat testing within three months was performed in 11 women, two of them negative; within 3–6 months in 15 women, six of them negative; within 6–9 months in nine women, four of them negative; and within 9–12 months in three women, all of them positive.

Discussion and Conclusion

The present study assessed the prevalence of hrHPV types among cytologically selected women from a large, well-defined region in Croatia, tested during the one-year period at Zagreb Institute of Public Health. In 3,440 tested women with cytologically abnormal smears, the prevalence of hrHPV was 34.6%.

The prevalence of hrHPV is known to decrease with age, from 20% among women aged 20–30 to 5% in women aged >30, as estimated in previous studies in pregnant and non-pregnant women with cytomorphologically normal cervical smears[17–19]. Although the prevalence of hrHPV is considerably higher among women with cytologically abnormal smears, a higher rate of hrHPV is still recorded in women aged <30 than in those from older age groups irrespective of the grade of cytological abnor-

TABLE 1
RESULTS OF CERVICAL SAMPLES TESTED FOR 13 HIGH-RISK HPV GENOTYPES BY AMPLICOR HPV TEST ACCORDING TO PATIENTS AGE

Age (years)	Total n=3,227	NT[a]		Positive[b]		
		n=4	%	n=1,120	%	95%CI[c]
≤20	158	0	0	78	49.4	41.4–57.4
21–30	1,425	2	0.1	642	45.1	42.5–47.7
31–40	889	2	0.2	257	28.9	26.0–32.0
41–50	508	0	0	98	19.3	16.0–23.1
51–60	171	0	0	23	13.5	8.9–19.7
≥61	76	0	0	22	28.9	19.4–40.7

HPV – human papillomavirus, [a]NT – not possible to interpret, [b]χ^2=183.96, df=5, p<0.01, [c]CI – confidence interval

mality. A recent study of the distribution of HPV types in ThinPrep Papanicolaou (Pap) tests classified according to the »Bethesda 2001 terminology« in correlation with patient age detected one or more of 13 hrHPV types in 53% of samples diagnosed as atypical squamous cells of undetermined significance (59% of patients aged <30 and 45.5% of patients aged ≥30), 55.5% of samples diagnosed as low-grade squamous intraepithelial lesion (60% of patients aged <30 and 44% of patients aged ≥30), 80% of samples in which the high-grade squamous intraepithelial lesion could not be ruled out; and 87.5% of samples diagnosed as high-grade squamous intraepithelial lesion[20]. In the present retrospective study, most cervical specimens were obtained from women aged 21–30 (44.2%), in which group hrHPV was detected in 45.1% of patients. Limitations of the study included the unavailability of cytological, clinical and epidemiological data for all patients, and involvement of a great number of gynecologists and cytologists, which resulted in variable classification of cervical abnormality. Additional prospective investigation of samples cytologically diagnosed according to the »Bethesda 2001 guidelines« is needed.

As expected, hrHPV infection was found to be highly prevalent in female adolescents and young women from the Zagreb region. Similar findings were recorded in a study conducted in 1998 and 1999 in Croatia, when 466 women with minor and moderate cervical abnormalities were tested. The prevalence of hrHPV was found to be 66% in women aged <20, 57% in women aged 20–29, and 65% in women aged 30–39[21]. In this study, the decline of hrHPV prevalence with age was less pronounced because of the small number of subjects, especially those over age 50. In another study published in 2001, assessing the prevalence of HPV in cervical specimens obtained from 1,874 women with abnormal Pap smears collected during the 1996–1998 period, the HPV positivity rate declined with age[22]. In a more recent study, the same authors demonstrated the presence of HPV in cervical samples to significantly increase with the severity of cervical lesions[23]. The same was observed in the present study which is valuable because of the great number of women included, yielding relevant epidemiological data for this geographical region. However, properly designed, target studies are necessary to overcome the shortcomings of the present, retrospective study.

During the one-year study period, only 66 women were tested on multiple occasions, which cannot be considered a relevant sample, therefore further investigation with repeat hrHPV testing is needed to assess virus clearance and rate of newly acquired infection in correlation with patient age and cytological diagnosis.

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INFEKCIJA GENITALNIM HUMANIM PAPILOMAVIRUSIMA U ŽENA SA ZAGREBAČKOG PODRUČJA

S A Ž E T A K

Infekcija humanim papilomavirusima (HPV) najčešća je spolno prenosiva bolest, poglavito u mladih, seksualno aktivnih osoba. Kako ustrajna infekcija može dovesti do raka vrata maternice, testiranje na HPV je korisno sredstvo u probiranju žena koje su pod povećanim rizikom od razvoja karcinoma vrata maternice. Cilj ovoga rada bio je utvrditi prevalenciju infekcije HPV visokog rizika (hrHPV, engl. *high risk* HPV) u različitim dobnim skupinama citološki odabranih žena sa zagrebačkog područja, te analizirati učestalost i rezultate ponovljenih testiranja na hrHPV. U promatranom jednogodišnjem razdoblju (studeni 2005. do studeni 2006.) ukupno je zaprimljeno 3.440 uzoraka obrisaka vrata maternice žena iz ginekoloških ambulanata domova zdravlja i privatnih ginekoloških ordinacija. Uzorci su testirani na prisutnost 13 genotipova hrHPV testom AMPLICOR HPV (Roche Molecular Systems), koji se temelji na lančanoj reakciji polimerazom. Ukupna prevalencija hrHPV iznosila je 34,6%. Većina uzoraka zaprimljena je od žena u dobnoj skupini od 21–30 godina (44,2%), a slijedile su dobne skupine žena od 31–40 (27,6%), 41–50 (15,7%), 51–60 (5,3%) i ≥61 godine (2,4%). Od ukupno 3.227 obrisaka vrata maternice zaprimljenih od žena poznate dobi 4,9% je otpadalo na populaciju djevojaka mlađih od 21 godine. U toj dobnoj skupini utvrđena je najveća prevalencija infekcije hrHPV (49,4%). Slična prevalencija uočena je u žena dobne skupine od 21–30 godina (45,1%). U starijim dobnim skupinama prevalencija se postupno smanjivala. U promatranom razdoblju testiranje na hrHPV ponovljeno je u različitim vremenskim razmacima na uzorcima dobivenim od 66 žena. Od 28 žena koje su na prvom testiranju bile negativne samo ih je pet bilo pozitivno na ponovnom testiranju. Od 38 žena koje su na prvom testiranju bile pozitivne u jedne trećine se hrHPV se nije mogao utvrditi kod ponovnog testiranja. Kako se je očekivalo, infekcija hrHPV najučestalija je u adolescentica i mladih žena. Potrebna su daljnja istraživanja o ponavljanim hrHPV testiranjima kako bi se mogla procijeniti stopa iščezavanja virusa i stopa novo stečenih infekcija.

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Retrospective Study of the Prevalence of High-Risk Human Papillomaviruses among Croatian Women

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A B S T R A C T

The infection with Human papillomavirus (HPV) is the necessary cause for cervical cancer. There are at least 15 High-Risk (HR) HPV types that are significantly associated with progression of cervical intraepithelial neoplasia to cervical cancer. Since previous studies showed that the prevalence of HPV in cervical cancers varies among different geographic regions, we wanted to investigate the prevalence of HPV types in Croatia, especially low abundant HR HPV types. By means of consensus primers directed polymerase chain reaction (PCR), we analysed cervical DNA samples of 2,136 Croatian women, mostly with abnormal cervical smears, in order to detect the presence of HPV. Type-specific primers were then used to determine Low-Risk (LR) HPV types 6/11 and HR HPV types 16, 18, 31, 33, 45, 52 and 58. Out of 2,136 specimens, 1,255 (58.8%) were positive for HPV. More than half of positive samples were typed (64.5%) and 35.5% still remained untyped. Multiple HPV infections were found in 10.3% of the cases. The most prevalent type, including both single and multiple infections, was HPV 16 with the prevalence of 15.9%, followed by HPV types 31, 6/11, 33, 18, 52, 45 and 58 with 8.7%, 7.1%, 4.5%, 3.8%, 2.3%, 1.2% and 1.1%, respectively. The significant increase of frequency from Low-grade Squamous Intraepithelial Lesions (LSIL) to High-grade Squamous Intraepithelial Lesions (HSIL) was observed for HR HPV types 16, 18, 31 and 33 but not 45, 52 and 58. The frequency of unknown HPV types was almost the same in cervical specimens of women with LSIL and those with HSIL, 19.8% and 21.1%, respectively. The prevalence of HPV infection rate decreased significantly with patient age from 68.5% (age group 12 to 24 years) to 38.8% (age group 45 to 54 years). But, in women aged 55 or older the overall prevalence increased to 56.6%. Our results indicate that prevalence of HR HPV types in Croatia is similar to other countries. We suggest that HPV positive women in Croatia should be closely monitored by typing for HR HPV types: 16, 18, 31, 33, 45, 52 and 58.

Key words: *human papillomavirus (HPV), high-risk (HR) types, Croatia*

Introduction

Cervical cancer is the second most common malignancy after breast cancer among women worldwide. More than 80% of cases occur in developing countries. In 2002, about 493,000 cervical cancer cases (incidence rate 16/100,000) and about 273,000 deaths from cervical cancer (mortality rate 8.9/100,000), respectively, were recorded worldwide[1]. The incidence and mortality rates in Croatia in 2002 represents the intermediate value of the highest and lowest rates noted in Europe, with 431 cases (incidence rate 18/100,000) and 209 deaths (mortality rate 8.7/100,000) from cervical cancer, respectively[2,3]. This indicates that cervical cancer represents a major health concern in Croatia that needs to be improved.

Epidemiological studies have clearly established human papillomavirus (HPV) infection as the prerequisite for cervical cancer[4]. Studies in 22 countries coordinated by the International Agency for Research on Cancer (IARC), identified HPV DNA in almost all (99.7%) of about 1,000 cases of cervical cancer[5]. To date, more than 200 different HPV types have been recognized on the basis of DNA sequence data[6]. More than 100 HPV genotypes are well characterized[7]. Over 40 types infect the human anogenital area, of which some can cause genital warts and others can lead to precursor cervical lesions, CIN (cervical intraepithelial neoplasia) and cervical cancer[7]. At least 15 HPV types are significantly associ-

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ated with progression of CIN to cervical cancer and are considered carcinogenic and named High-Risk (HR) HPV types[8]. Thus, HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 were classified as HR or carcinogenic types, HPV 26, 53 and 66 as probable carcinogenic types, while HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 as Low-Risk (LR)[8]. In contrast to HR HPV types, LR types are associated with benign genital warts and only occasionally found in cervical carcinomas. According to the epidemiological and the phylogenetic studies, most HR HPV types associated with lesions of similar pathologies are phylogenetically related to either HPV 16 (31, 33, 35, 52 and 58) or HPV 18 (39, 45, 59 and 68)[7].

The prevalence of HPV types in cervical cancers varies among different geographic areas[4,9–10]. In a worldwide survey, HPV 16 was found to be the most prevalent type in cervical cancer (51.0%), followed by HPV 18 (16.2%) and other types such as HPV 45, 31, 33, 58 and 52 (collectively accounting for 18.3% cases)[4]. In Europe, beside HPV 16, types 18, 33, 31 and 45 were the most frequently found HR HPV types in cervical cancer[4]. Infection with HPV 45 was found to be third most frequent in Africa, after HPV 16 and 18. HPV 52 and 58 are less frequent in Europe, but not uncommon worldwide, especially in Asia, being the most frequently found HR HPV types after 16 and 18[4].

There is limited data regarding the prevalence of low abundant HR HPV types in Croatia. Therefore, the aim of this study was to evaluate the prevalence of HR HPV types 45, 52 and 58 among Croatian women on the archival DNA samples where HPV types 6/11, 16, 18, 31 and 33 were previously determined[11,12].

Material and Methods

Study population

Two thousand one hundred and thirty six Croatian women, mostly with abnormal cervical smears, were enrolled in this study. The cervical specimens for HPV detection and typing were collected in different gynaecological hospital clinics in Zagreb, Croatia[11,12]. The cytological diagnosis[13] of the study population consisted of 0.7% (15/2,136) normal Pap smears, 24.8% (529/2,136) ASCUS (atypical squamous cells of undetermined significance), 28.8% (615/2,136) LSIL (Low-grade Squamous Intraepithelial Lesions) and 40.9% (874/2,136) HSIL (High-grade Squamous Intraepithelial Lesions). Furthermore, there were 4.8% (103/2,136) women with unknown cytological diagnosis.

DNA preparation

DNA from cervical cell samples was isolated as described previously[11,12]. Briefly, cervical cell suspensions were collected and treated with proteinase K (100 μg/ml in lysis buffer, 10 mM Tris-HCl; pH 7.5, 1 mM EDTA, pH 7.9; 0.5% SDS) overnight at 37°C or 2h at 56°C. Standard phenol-chloroform extraction and ethanol precipitation were used for DNA purification. Pelleted DNA was resuspended in 50–100 μL of deionised sterile water and stored at –20°C until further analysis[14]. In order to determine the quality and the quantity of isolated DNA, each DNA was analysed by electrophoresis on 1% agarose gels stained with ethidium bromide and spectrophotometrically[14].

HPV detection and typing

Cervical DNA samples were tested for the presence of HPV DNA by PCR based method as previously described[11,12–15]. Briefly, two sets of consensus primers: MY09/MY11, degenerated primers, and L1C1/L1C2-1/L1C2-2 primers were used for HPV DNA detection. To internally control the quality of the target DNA and the absence of PCR inhibitors, β-globin specific primers were used in the multiplex PCR with MY09/MY11 primers. Type-specific primers for HPV 6/11, 16, 18, 31 and 33 were also used in two separate multiplex PCRs, i.e. HPV 6/11 with 31 and HPV 16, 18 with 33. All amplification reactions were performed on 100 ng of each DNA in a total volume of 20 μl under the optimized conditions as previously described[11,12–15].

In this study, archival DNA samples were additionally examined for the presence of HPV 45, 52 and 58 with type-specific primers directed PCR, also [5,16]. The reaction mixtures contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.15 μM of each type-specific primer, 0,12 U AmpliTaq Gold DNA Polymerase (Roche) and 100 nm of each DNA in a total volume of 20 μl. The amplification conditions for HPV 52 and 58 consisted of 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec, while for HPV 45 it consisted of 35 cycles of denaturation at 95°C for 40 sec, annealing at 55°C for 40 sec and extension at 72°C for 45 sec, with in both cases an initial denaturation at 95°C for 10 min and a final extension of 7 min.

Aliquots (10 μl) of each PCR product were resolved by electrophoresis in a 2% agarose gel stained with ethidium bromide[14]. The amplified products were identified by UV irradiation of the gels, and photographed by Image Master VDS (Pharmacia Biotech).

Statistical analysis

The standard Chi-square (χ^2) test was used. Two-tailed p values were calculated in 2 x 2 tables using the GraphPad Prism (version 4.00) (GraphPad Software, San Diego, California). All tests were two sided and the significance level was set at $p < 0.05$[17].

Results

The average age of the study population was 30.6 (ranging from 12 to 75 years of age). The distribution of patient cytological diagnosis according to patient age is shown in Figure 1.

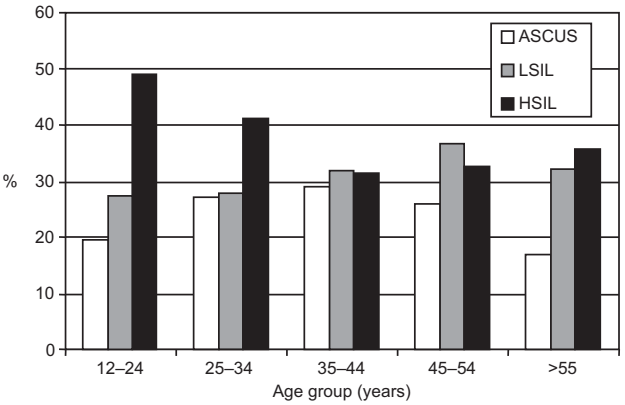

Fig. 1. Distribution of patient diagnosis according to patient age. ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous cell intraepithelial lesion, HSIL – high-grade squamous cell intraepithelial lesion.

Distribution of different HPV types

All DNA specimens prepared from cervical cell samples were β-globin positive and as such suitable for further HPV DNA detection and typing by PCR. Out of 2,136 DNA samples, 1,255 (58.8%) were positive for any HPV type (Tables 1 and 2).

More than half (64.5%) of HPV positive samples were typed. In 445 (35.5%) samples HPV type was not determined (HPV X). Multiple HPV infections with two or more HPV types were found in 10.3% (129/1,255) of the cases, mainly among younger women (mean age 26) (Table 2).

LR HPVs (types 6 or 11) appeared as single infections only in 4.9% (104/2,136) cases. Among HR HPVs, type 16 was the most frequently found as single infection in 11.9% (254/2,136) of the cases, followed by HPV types 31, 33, 18, 52, 45 and 58 found in 5.7% (121/2,136), 2.8% (60/2,136), 2.6% (55/2,136), 2.2% (46/2,136), 1.0% (22/2, 136), and 0.9% (19/2,136) of the cases, respectively (Tables 1 and 2).

Out of 129 multiple HPV infections (Table 3), there were 82 (63.6%) co-infections of two or three HR HPVs and 47 (36.4%) co-infections of LR HPV (type 6 or 11) and one or two HR HPV types. The co-infection of HPV types 16 and 31 or 16 and 6/11 were the most frequent, present in 25.6% (33/129) and 19.4% (25/129) cases, respectively.

Prevalence of different HPV types

HPV 16 was present in 85 multiple infections and in 254 single infections, which increases the total prevalence of HPV 16 in the study population to 15.9% (339/2, 136). HPV 31 was the second most frequent HPV type, being represented in 65 multiple infections and 121 single infections, with a final prevalence of 8.7% (186/2,136) (Figure 2). The prevalence, including both single and multiple infections, of HPV types 6/11, 18, 33, 45, 52 and 58 was 7.1% (151/2,136; not shown), 3.8% (82/2,136), 4.5% (95/2,136), 1.2% (25/2,136), 2.3% (49/2,136) and 1.1% (23/2,136), respectively (Figure 2).

TABLE 1
HPV TYPE SPECIFIC PREVALENCE IN THE GROUP OF PATIENTS WITH DIFFERENT CYTOLOGICAL FINDINGS

HPV type	Cytological diagnosis										Total	
	Unknown		Normal		ASCUS		LSIL		HSIL			
	N	%	N	%	N	%	N	%	N	%	N	%
HPV 6/11	5	4.9	0	0.0	29	5.5	29	4.7	41	4.7	104	4.9
HPV 16	10	9.7	0	0.0	49	9.3	46	7.5	149	17.1	254	11.9
HPV 18	3	2.9	0	0.0	8	1.5	13	2.1	31	3.6	55	2.6
HPV 31	5	4.9	0	0.0	26	4.9	21	3.4	69	7.9	121	5.7
HPV 33	3	2.9	0	0.0	7	1.3	15	2.4	35	4.0	60	2.8
HPV 45	0	0.0	0	0.0	8	1.5	6	1.0	8	0.9	22	1.0
HPV 52	1	1.0	0	0.0	15	2.8	11	1.8	19	2.2	46	2.2
HPV 58	0	0.0	0	0.0	3	0.6	7	1.1	9	1.0	19	0.9
Any HR	22	21.4	0	0.0	116	21.9	119	19.4	320	36.6	577	27.0
HPV X	17	16.5	3	20.0	119	22.5	122	19.8	184	21.1	445	20.8
Multiple	7	6.8	1	6.7	22	4.2	28	4.6	71	8.1	129	6.0
Positive	51	49.5	4	26.7	286	54.1	298	48.5	616	70.5	1,255	58.8
Negative	52	50.5	11	73.3	243	45.9	317	51.5	258	29.5	881	41.3
Total	103	4.8	15	0.7	529	24.8	615	28.8	874	40.9	2,136	100

ASCUS – atypical squamous cells of undetermined significance, LSIL – low grade squamous cell intraepithelial lesion, HSIL – high grade squamous cell intraepithelial lesion, HPV – human papillomavirus, HR – high-risk, HPV X –unknown HPV type

TABLE 2
HPV TYPE SPECIFIC PREVALENCE BY AGE

HPV type	Age group (years)												Total	
	Unknown		12–24		25–34		35–44		45–54		≥55			
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
HPV 6/11	5	8.6	44	6.4	33	4.2	9	2.4	10	5.6	3	5.7	104	4.8
HPV 16	11	19.0	97	14.0	96	12.3	35	9.4	9	5.1	6	11.3	254	11.9
HPV 18	2	3.5	16	2.3	27	3.5	5	1.3	3	1.7	2	3.8	55	2.6
HPV 31	3	5.2	55	8.0	42	5.4	14	3.7	4	2.3	3	5.7	121	5.7
HPV 33	1	1.7	25	3.6	20	2.6	9	2.4	3	1.7	2	3.8	60	2.8
HPV 45	0	0.0	3	0.4	13	1.7	5	1.3	1	0.6	0	0.0	22	1.0
HPV 52	1	1.7	11	1.6	23	2.9	6	1.6	5	2.8	0	0.0	46	2.2
HPV 58	0	0.0	7	1.0	9	1.2	2	0.5	0	0.0	1	1.9	19	0.9
Any HR	18	31.0	214	30.9	230	29.5	76	20.3	25	14.0	14	26.4	577	27.0
HPV X	13	22.4	153	22.1	164	21.0	72	19.3	31	17.4	12	22.6	445	20.8
Multiple	3	5.2	63	9.1	53	6.8	6	1.6	3	1.7	1	1.9	129	6.0
Positive	39	67.2	474	68.5	480	61.5	163	43.6	69	38.8	30	56.6	1,255	58.8
Negative	19	32.8	218	31.5	301	38.5	211	56.4	109	61.2	23	43.4	881	41.3
Total	58	2.7	692	32.4	781	36.6	374	17.5	178	8.3	53	2.5	2,136	100

HPV – human papillomavirus, HR – high-risk, HPV X – presence of unknown HPV type

TABLE 3
MULTIPLE HPV INFECTIONS

HPV types	Cases (N)	%
16, 31	33	25.6
6/11, 16	25	19.4
16, 33	14	10.9
18, 31	11	8.5
6/11, 31	7	5.4
31, 33	6	4.7
6/11, 18	5	3.9
6/11, 33	5	3.9
16,18	4	3.1
16, 31, 33	3	2.3
18, 33	2	1.6
45, 52	2	1.6
6/11, 31, 33	2	1.6
16, 18, 31	2	1.6
16, 58	1	0.8
33, 58	1	0.8
45, 58	1	0.8
52, 58	1	0.8
6/11, 16, 18	1	0.8
6/11,16, 31	1	0.8
6/11, 18, 33	1	0.8
16, 18, 33	1	0.8
Total	129	100

HPV – human papillomavirus

Prevalence of HR HPV types in HSIL

When both single and multiple infections are included HPV 16 was present in 23% (201/874) of HSIL samples HPV types 31, 33, 18, 52, 58 and 45 were found in 12.0% (105/874), 6.1% (53/874), 5.0% (44/874), 2.3% (20/874), 1.1% (10/874) and 0.9% (8/874) of the cases, respectively (Figure 3).

HPV distribution according to patient diagnosis

The prevalence of HPV increased significantly (χ^2 = 74, d.f. = 1, $p < 0.0001$) from 48.5% to 70.5% with the severity of cytological findings (Table 1 and Figure 4). While LR HPV types 6/11 were equally distributed among LSIL (4.7%) and HSIL (4.7%), HR HPVs were almost two times more frequent in HSIL (36.6%) than in LSIL (19.4%) (for presence of any HR HPV, χ^2 = 51.75, d.f. = 1, $p < 0.0001$). HPV type 16, as a single infection, was the most frequent, found in 7.5% of cases in LSIL compared to 17.1% in HSIL (χ^2 = 29.0, d.f. = 1, $p < 0.001$). Multiple HPV infections were also two times more frequent in HSIL than in LSIL, 8.1% and 4.6%, respectively. The frequency of unknown HPV types (HPV X) was almost the same in cervical specimens of women with LSIL and those with HSIL, 19.8% and 21.1%, respectively.

HPV distribution according to patient age

The frequency of HPV infections decreased significantly (χ^2 = 94.2, d.f. = 4, $p < 0.0001$) with patient age from 68.5% (age group 12 to 24 years) to 38.8% (age group 45 to 54 years) (Table 2 and Figure 5). But, the prevalence of HPV in women aged 55 or older increased

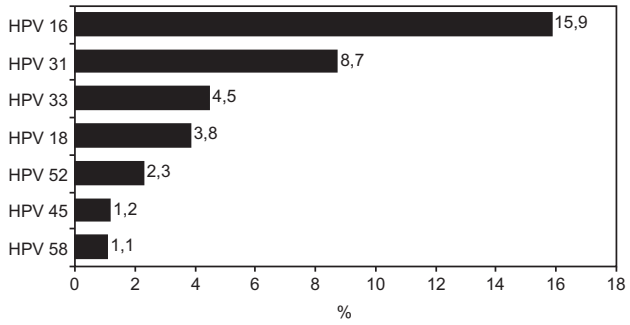

Fig. 2. Prevalence of specific high-risk human hapillomavirus (HPV) types including single and multiple infections in the whole study population.

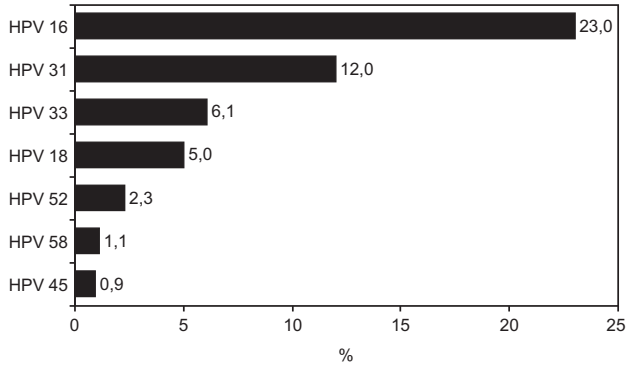

Fig. 3. Prevalence of specific high-risk human papillomavirus (HPV) types including single and multiple infections in high--grade squamous cell intraepithelial lesion (HSIL) samples.

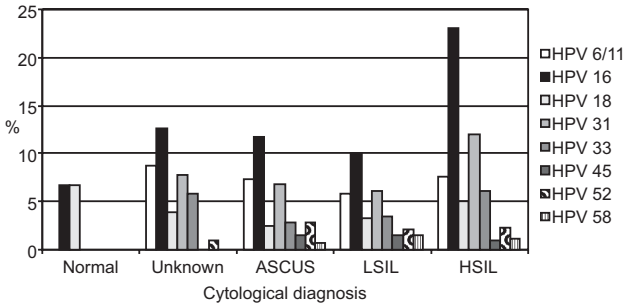

Fig. 4. Prevalence of human hapillomavirus (HPV) types including single and multiple infections according to cytological diagnosis. Unknown – diagnosis not known, ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous cell intraepithelial lesion, HSIL – high-grade squamous cell

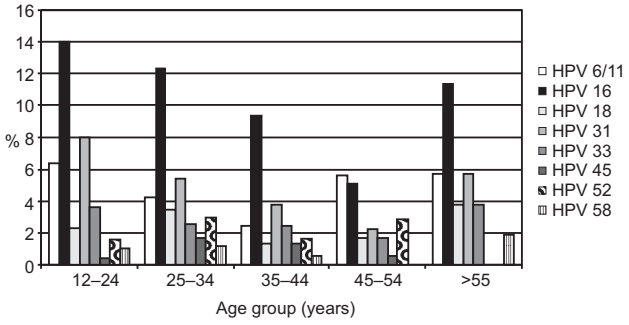

Fig. 5. Age dependent prevalence of human hapillomavirus (HPV) types including single and multiple infections.

to 56.6%. The observed trend is statistically significant for HPV 16 ($\chi^2 = 10$, d.f. = 1, p = 0.0013), HPV 31 ($\chi^2 = 10$, d.f. = 1, p = 0.0013), presence of any HR HPV ($\chi^2 = 22$, d.f. = 1, $p < 0.0001$) and presence of multiple infection ($\chi^2 = 28$, d.f. = 1, $p < 0.0001$).

Discussion

This study was designed to investigate the prevalence of low abundant HR HPV types 45, 52 and 58 among Croatian women. We analysed archival DNA samples from 2,136 cervical specimens collected from Croatian women that were previously tested for the presence of LR HPV types 6 or 11 and HR HPV types 16, 18, 31 and 33[11,12,15].

The overall detection of HPV was 58.8%. Out of 1,255 positive samples, 64.5% were typed and in 35.5% of samples the type was not determined. Those samples gave positive results with general primers, but they were negative with type-specific primers used in this study.

The geographic variation of the distribution of different HPV types is well established in some regions[4,18–20]. Worldwide, in squamous cell carcinoma HPV 16 was the most prevalent type varying from 46% in Asia to 63% in Europe, while HPV 18 was the second most common HPV type. HPV 33, 31 and 45 in Europe and HPV 45 in Africa were the third HPV types most frequently associated to cervical squamous cell carcinoma. HR HPV 58 and 52 were found to be less frequent in Europe, but not uncommon worldwide, especially in Asia, where these two types took the third and fourth place after HPV 16 and 18.

Herein, we found that HPV 16 was the most prevalent type among Croatian women, as expected, and it was present in 15.9% cases of both single and multiple HPV infections. HPV 31 was the second most prevalent HPV type appearing in 8.7% both single and multiple HPV infections, followed by HR HPVs 33, 18, 52, 45 and 58 (Figure 2). The distribution of HR HPV types in the precancerous lesions is slightly different than those in cervical cancer cases. According to a meta-analysis of Clifford et al.[21] overall HPV prevalence was higher in squamous cell carcinoma of the cervix (SCC) than in HSIL, 87.6% *versus* 84.2%, respectively (SCC : HSIL ratio 1.04). HPV 16 was found to be the most common type in both SCC (54.3%) and HSIL (45.0%), with a ratio of 1.21. HPV 18 was also more prevalent in SCC (12.6%) than in HSIL (7.0%), with a ratio of 1.79. Interestingly, HPV 45 was the third type with a higher ratio (1.85) than those observed with HPV 16 and 18. It was also more frequent in SCC (4.2%) than in HSIL (2.3%). All other analysed HR HPVs (including HPV 31, 33, 52 and 58) had ratios less than 1, i.e. between 0.1 and 0.6[21]. These results suggested

that HSIL, when associated with HPV 16, 18 or 45 is more likely to progress to SCC than HSIL associated with other HR HPV types[10,21]. This evidence pointed out that women infected with HPV 16, 18 or 45 may require closer surveillance than women infected with other HR HPV types.

When we compared our prevalence analysis of HR HPV types in HSIL (including both single and multiple infections) with a worldwide meta-analysis of Clifford et al.[21], we found some discrepancies (Figure 3). HPV 16 was, as expected, the most frequently found type in HSIL samples in both studies but with almost twice lower prevalence in our study than those of Clifford et al., 23% and 45%, respectively. Similarly in both studies, HPV 31 was on the second place with the prevalence of 12.0% in our study *versus* 8.8% in Clifford et al. study. HPV 33, followed these two HPV types in both studies with, in this case, similar prevalence of 6.1% in this study and 7.2% in Clifford et al. study. Further, we found slightly lower incidence of HPV 18 in HSIL samples, in 5.0% cases *versus* 7.1%. The major discrepancy was the prevalence of HPV 58. While it was rather frequently found type (6.9%) in Clifford et al. study, we found much lower incidence among Croatian women (1.1%). We also noticed unexpected low prevalence for HPV 52 (2.3%) and HPV 45 (0.9%) compared to Clifford et al. study (5.2% and 2.3%, respectively). These discrepancies could be explained by (1) low number of analysed samples in this study, (2) different determination of cytological diagnosis HSIL[13], (3) the choice of primers sets for type-specific HPV amplification[5,12,16] or (4) our data represent a real picture of HPV prevalence among Croatian women. However, these observations clearly point out that further analysis with larger number of samples and alternative method of typing need to be carried out in the future to clarify this dilemma.

Herein, we found a strong correlation between the distribution of HR HPV infections and the severity of cervical lesions, i.e. the frequency of HR HPVs (type 16, 18, 31 and 33) increased with increasing severity of squamous intraepithelial lesions-SIL, from 19.4% in LSIL to 36.6% in HSIL ($\chi^2 = 74$, d.f. = 1, $p < 0.0001$). HPV 16 is the most predictive virus type of SIL and it is present in 7.5% cases of LSIL and 17.1% cases of HSIL ($\chi^2 = 29$, d.f. = 1, $p < 0.0001$). This result is not surprising, and matches data found in previous reports[22,23–24]. The progression from LSIL to HSIL is strongly associated with the presence of HR HPVs, while no progression is associated with the presence of LR HPV types or the absence of HPV[25], which further stresses the importance of HPV DNA typing.

The frequency of unknown HPV types (HPV X) was approximately the same in cervical swabs of women with LSIL and those with HSIL, 19.8% versus 21.1%, respectively. Although type-specific primers that we used in this study were detecting mainly group of HR HPV types expected in Europe, we would like to continue our study and determine the unknown HPV types[11,12].

In this study, all 129 multiple HPV infections contained at least one HR HPV type. There were 63.6% co-infections of two or three HR HPV types, while 36.4% co-infections of LR and HR HPV types (Table 3). The co-infection of HPV types 16 and 31 was most frequently found followed by the co-infection of HPV 16 and 6/11. The frequency of co-infections follows the frequency of individual HPV types found in the study population (Figures 2 and 3). HPV types 45, 52 and 58 were rarely found in multiple infections.

Herein, multiple infections were two times more frequent in HSIL than in LSIL, 8.1% *versus* 4.6%, what is not surprising because multiple HPV infections were mainly represented by co-infections of two or three HR HPVs or HR- and LR HPVs. In the similar study population in Taiwan, Chang et al.[26] found adverse results, i.e. decreasing frequency of multiple HPV infections with increasing severity of cervical lesions. However, a similar decrease of multiple infection frequency can be observed from CIN 2 to CIN 3, if the HSIL category is classified accordingly to the Croatian classification of cervical smears[13] (data not shown). In both cases, the HPV infections associated with LSIL still represents transient infections, while the HPV infections associated with HSIL, especially CIN 3 most probably persistent infections[27].

In this study, the frequency of HPV infections decreased significantly (χ^2 for trend = 72.42, d.f. = 1, $p < 0.0001$) with patient age. Women under the age of 24 years exhibited the highest rate of HPV infections (68.5%), while women between 45 and 54 years of age exhibited the lowest rate of HPV infections (38.8%). Multiple HPV infections were also found mainly among younger women (mean age 26). This trend of high prevalence in young age after the beginning of sexual activity and then a steady decline in subsequent age group is observed in other studies[27]. In this study, an increase of HPV prevalence in women aged 55 or older to 56.6% was also observed. This trend of gradual fall and then increase in older age has been observed for any HR HPV, particularly HPV 16, HPV 18, 31 and 33 but not 45, 52 and 58, and multiple HPV infections (Table 2 and Figure 5). Interestingly, HPV 45 and 52 were not found among women ≥ 55, while HPV 58 was found mostly in the age group ≥ 55 but absent in the age group 45–54 years of age; the reason of that observation could be a small number of analysed specimens in these age groups. Further analysis with larger number of samples is needed in these age groups.

In conclusion, the distribution of HPV infections among Croatian women is similar to most countries with HPV 16 being the most prevalent type followed by HPV 31 in precancerous lesions. HPV 33, 18 and 52 are all at the third place of prevalence in this study group, while HPV 45 and 58 remain low abundant HR HPV types. As HPV detection of HR types is recommended for women with equivocal cytology and for follow-up after treatment of high-grade cervical lesions according to the Croatian diagnostic and therapeutic guidelines for management of women with premalignant cervical lesions[28], we suggest to perform HPV typing for HR HPV types: 16, 18, 31, 33, 45, 52 and 58 in HPV positive women in order to improve the monitoring process and prevent the cancer development.

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RETROSPEKTIVNA ANALIZA UČESTALOSTI VISOKORIZIČNIH TIPOVA HUMANOG PAPILOMAVIRUSA (HPV) KOD ŽENA U HRVATSKOJ

SAŽETAK

Infekcija humanim papilomavirusom (HPV) glavni je uzrok raka vrata maternice. Postoji najmanje 15 visokorizičnih tipova HPV-a koji su značajno povezani s napredovanjem cervikalne intraepitelne neoplazije (CIN) prema raku vrata maternice. Budući da su prijašnje studije pokazale da je učestalost HPV-a različita među različitim zemljopisnim područjima, naš cilj je bio ispitati učestalost tipova HPV-a u Hrvatskoj, posebno rijetkih visokorizičnih tipova HPV-a. Metodom lančane reakcije polimerazom (PCR), pomoću konsenzus početnica, a kako bismo ustanovili prisutnost infekcije HPV-om, analizirali smo uzorke DNK 2.136 žena u Hrvatskoj, uglavnom s abnormalnim nalazom brisa vrata maternice. Tip-specifične početnice korištene su za detekciju sljedećih tipova HPV-a: niskorizičnih 6/11 te visokorizičnih 16, 18, 31, 33, 45, 52 i 58. Od ukupno 2.136 uzoraka, 1.255 (58,8%) je bilo pozitivno, a 881 (41,3%) uzorak je bio negativan na prisutnost nekog tipa HPV-a. Više od polovine pozitivnih uzoraka bilo je tipizirano (64,5%), dok kod 35,5% nije utvrđen tip HPV-a. Višestruke infekcije HPV-om su pronađene u 10,3% slučajeva. Utvrđeno je da je najučestaliji tip HPV 16 s učestalošću od 15,9%, uključujući jednostruke i višestruke infekcije. Slijedili su ga tipovi HPV-a: 31, 6/11, 33, 18, 52, 45 te 58 s 8,7%, 7,1%, 4,5%, 3,8%, 2,3%, 1,8% te 1,1% učestalosti. Uočeno je značajno povećanje učestalosti od blagih (LSIL, engl. Low-grade Squamous Intraepithelial Lesions) do težih (HSIL, High-grade Squamous Intraepithelial

Lesions) promjena vrata maternice za visokorizične tipove HPV-a 16, 18, 31 i 33, ali ne i za HPV 45, 52 i 58. Učestalost nepoznatih tipova HPV-a (HPV X) bila je gotovo jednaka u uzorcima vrata maternice žena sa citološkom dijagnozom LSIL i HSIL, 19,8% i 21,1%. Uočeno je značajno smanjenje broja HPV-a s porastom dobi ispitanica, od 68,5% (dobna skupina od 12 do 24 godine) prema 38,8% (dobna skupina od 45 to 54 godine). Međutim, kod žena od 55 godina i starijih ukupna učestalost infekcija HPV-om je porasla na 56,6%. Ovi rezultati učestalosti visokorizičnih tipova HPV-a u Hrvatskoj se podudaraju s onima zabilježenim u drugim zemljama. Smatramo važnim HPV-pozitivne žene u Hrvatskoj tipizirati barem na prisustvo visokorizičnih tipova HPV-a: 16, 18, 31, 33, 45, 52 i 58 kako bi im se pružila maksimalna pozornost.

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Distribution of Human Papillomavirus Types in Different Histological Subtypes of Cervical Adenocarcinoma

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A B S T R A C T

Little information is available regarding distribution of HPV types in different histological subtypes of adenocarcinoma (AC). Thus, in this study we examined the frequency of high-risk (hr) HPV types in AC, adenocarcinoma in situ (AIS) and adenosquamous carcinoma (ADSQ). A total of 102 cases of primary cervical adenocarcinoma (26 AIS and 76 invasive AC) obtained from pathology files from 1995–2006 were histologically subtyped. Our results demonstrated that endocervical type occupied the major subtype of AC (22/66) followed by ADSQ (17/66) where as in the group of AIS endocervical type (12/23) was followed by intestinal type of AIS (7/23). Successful DNA extraction was obtained in 89 samples; 81 out of 89 (91.0%) tested positive for HPV DNA. The prevalence of HPV DNA in AIS, AC and ADSQ was 91.3% (21/23), 90.9% (60/66) and 94.1% (16/17), respectively. We found HPV 18 type to be the most predominant type in AIS (11/21) and AC (17/60) followed by HPV of undeternmined type in AIS (3/21) and HPV 16 in AC (9/60) as the sole viral type. HPV 18 was most frequently detected type in all histological subtypes of AIS and AC. We have detected HPV DNA in all 5 samples of clear cell carcinoma (CCC), although other studies have reported a highly variable prevalence of HPV DNA in CCC. The most prevalent HPV type in ADSQ was HPV-16 followed by HPV 33 as single type. The observed overall predominance of HPV 18 in AIS ($\chi^2 = 6.109$, $p \leq 0.025$) and AC ($\chi^2 = 8.927$, $p \leq 0.01$) as well as of HPV 16 in ADSQ ($\chi^2 = 10.164$, $p \leq 0.01$) was statistically significant. Our data revealed statistically significant predominance of single hrHPV infections in AIS (16/21; $\chi^2 = 11.523$, $p \leq 0.001$) and AC (37/60; $\chi^2 = 6.533$, $p \leq 0.025$) whereas multiple hrHPV infections were more abundant in AC comparing to AIS (23/81and 5/81, respectively; $\chi^2 = 13.989$, $p \leq 0.001$).

Key words: *cervical adenocarcinoma, adenocarcinoma in situ, histological subtypes, HPV prevalence frequency and typing*

Introduction

There are two major histological types of invasive cervical cancer: squamous cell carcinoma (SCC) and adenocarcinoma (AC). Incidence rate of SCC has been declining in recent years[1] but unfortunately incidence of cervical AC is increasing in developed countries, despite their widespread screening programmes. Recent reports indicated that AC accounts for approximately 20–25% of uterine cervical cancer compared with only 5–15% in the past[2]. Many countries report steady increase in incidence rates especially among younger women, since early 1970s[2–6]. Although effective screening programmes contribute to decreasing incidence rates of SCC but neither improved surveillance or classification of ACs appears to account for all of the increase in ACs. There are more than 40 HPV types known to infect female genital tract, and a subset of at least 15 of these are known to have a strong oncogenic potential[11]. Therefore, HPV types have been classified according to their association with cervical cancer and precursor lesions into oncogenic or high-risk (hr) and low-risk (lr) HPV types[12]. Cervical squamous neoplasia has shown to be strongly associated with hrHPV infection with studies showing more than 90% of cancers containing HPV DNA[13,14]. Although, association of hrHPV with cervical AC has been studied less extensively, reports in the past decade indicate similar association, suggesting causal relationship between hrHPV and cervical

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AC[15,16]. Major limitation is that number of patients with cervical ACs involved in these studies was small. Another reason why causal relationship between HPV infection and cervical AC is further complicated is the fact that AC encompasses several heterogeneous histological subtypes. The majority of tumours are mucionus AC including endocervical and intestinal type, while minor component of cervical AC includes variety of histological subtypes such as endometrioid, clear cell serous, villoglandular, minimal deviation, mesonephric adenocarcinoma and mixture of those subtypes. Specific histological subtype is clear cell carcinoma (CCC) which accounts for 2–7% of cervical AC and if presenting in young patients usually involves association with diethylstilbestrol (DES) exposure *in utero*[17,18] and HPV DNA negativity. Other patients with CCC have no known risk factors and occur in an older age group[18].

While hrHPV infection appears to be required for the development of both SCC and AC, the distribution of hrHPV types seen in these two forms of disease varies from study to study. Whereas HPV 16 is the most frequently involved in the development of SCC, HPV 18 alone or in combination with HPV 16 is reported to be a predominant type in AC[19] and adenocarcinoma *in situ* (AIS). Little information is available regarding geographic variation of HPV types in different histological subtypes of AC. Herein we conducted a retrospective study in order to evaluate the frequency of HPV types in adenocarcinoma *in situ* (AIS), AC and adenosquamous carcinoma (ADSQ).

Materials and Methods

Case selection and histological subtyping

A total of 102 specimens of primary cervical adenocarcinoma were (26 AIS and 76 invasive AC) obtained from the Department of Pathology, University of Rijeka, Croatia, which were collected from 1995 to 2006. Paraffin embedded sections of cone or hysterectomy specimens were reviewed by two pathologists. Each case was histologically subtyped according to the standard histological criteria[20] and associated lesions of the squamous epithelium were recorded as well. Endocervical type was the most common invasive adenocarcinoma, found in 22 cases, followed by adenosquamous carcinoma (17 cases), intestinal (12 cases), and endometrioid type (8 cases). Five cases were diagnosed as clear cell carcinoma, 3 cases were signet-ring carcinoma, 3 cases as serous and one case as villoglandular and glassy cell carcinoma. Mixed mucinous adenocarcinoma was found in 4 cases. Associated CIN I–III lesions were found in 11 cases. According to morphological criteria 15 AIS were suptyped as endocervical, 7 cases as intestinal, 2 cases as endometrioid and one as adenoaquamous and mixed type. Depending on tissue availability, successful DNA extraction, defined by presence of β-globin gene sequence, was found in 89 samples, 3 cases of AIS and 10 cases of AC were excluded from further analysis.

DNA extraction and PCR analysis

Total DNA was isolated from formalin fixed, paraffin embedded samples. Great care was taken on sample sectioning to avoid any contamination between the samples. Depending on the amount of biopsy material embedded in paraffin, 4–10 sections (5 μm thick) were placed in a microcentrifuge tube. The sections were deparaffinized by adding 1 mL of xylene and heating at 55°C for 30 minutes, followed by centrifugation and subsequent removal of the supernatant. Upon dewaxing with three washes of xylene, 1 mL of 100% ethanol was added to remove residual xylene. The tissues were dried at 37°C for 30 minutes and DNA was isolated using NucleoSpin®Tissue kit (Macharey-Nagel, Duren, Germany) according to the manufacturer's instructions.

Detection of HPV DNA was performed using E6 and E7 consensus primers (Human Papillomavirus Typing Set, Takara Biomedicals, Japan). The HPV types in positive samples were further characterized by using type specific primers amplifying sequences of HPV 16, 18 and 33 within E6 and E7 open reading frame (ORF) (Human Papillomavirus Detection Set, Takara Biomedicals, Japan) and HPV 31, 45, 52, 59 and 68 in the E7 ORF[14].

Statistical analysis

HPV prevalence was expressed as percentage of all cases tested for HPV in different histological groups of AC (accounted only once). The overall prevalence of individual hrHPV types was determined as they appeared as either single or within multiple infections. Multiple hrHPV infection was defined as two or more hrHPV types. The differences of the means of the continuous variables were analyzed with the Student's *t*-test and the distribution of non-continuous histological variables *versus* HPV status was analyzed with the Chi-square test (χ^2). *P* values of <0.05 were used as the cut-off for statistical significance.

Results

As shown in Table 1. endocervical type represented the major subtype of AC (22/66) followed by ADSQ (17/66) where as in the group of AIS endocervical type (12/23) was followed by intestinal type of AIS (7/23). The average age of patients with AIS, AC and ADSQ was 41.82, 48.9 and 44, respectively. Statistical analysis revealed that average age of the patients with AIS and AC (41.82 years *versus* 48.9 years) was significantly different (Student's t-test; $t=2.55$, degrees of freedom 87, $p\leq0.05$). Looking at different histological subtypes the average age of patients with intestinal AC and AIS was the youngest (35.8 and 39.28 years respectively). Cervical intraepithelial neoplasia (CIN), ranging from CIN I to CIN III accompanied AIS and AC in 27 cases (16/23 and 11/66, respectively).

We were able to successfully extract DNA from 89 samples and 81 (91.0%) tested positive for HPV DNA. HPV DNA prevalence according to different histological subtypes of AC, AIS and ADSQ is presented in Table 1.

TABLE 1
VARIOUS HISTOLOGICAL SUBTYPES OF CERVICAL ADENOCARCINOMA AND HIGH RISK HPV PREVALENCE

Diagnosis	n	Age range years	Average age years	CIN component		HPV negative		HPV positive		Single HPV infection		Multiple HPV infections*	
				n	%	n	%	n	%	n	%	n	%
Endocervical	22	26–74	48.59	6	27.3	2	9.1	20	90.9	15	75	5	25
Intestinal	5	30–40	35.8	1	20	1	20	4	80	2	50	2	50
Endometrioid	8	29–82	54.37	–	–	–	–	8	100	4	50	4	50
Clear cell	5	42–73	63.8	–	–	–	–	5	100	4	80	1	20
Serous	3	38–78	55.67	1	33.3	2	66.7	1	33.3	1	100	–	–
Villoglandular	1	40	40	–	–	–	–	1	100	1	100	–	–
Mixed	4	37–69	48.75	1	25	–	–	4	100	2	50	2	50
Glassy cell	1	65	65	–	–	–	–	1	100	–	–	1	100
Adenosqumaous	17	21–73	44	2	11.8	1	5.9	16	94.1	8	50	8	50
All invasive adenocarcinoma	66	21–82	48.9	1	116.7	6	9.1	60	90.9	37	61.7	23	38.3
Endocervical	12	26–56	45.5	10	83.3	1	8.3	11	91.7	7	58.3	4	33.3
Intestinal	7	30–46	39.28	5	71.4	–	–	7	100	6	85.7	1	14.3
Mixed	1	40	40	1	100	–	–	1	100	1	100	–	–
Adenosqumaous	1	47	47	–	–	–	–	1	100	1	100	–	–
Endometrioid	2	43	43	–	–	1	16.7	1	50	1	100	–	–
All adenocarcinoma *in situ*	23	26–56	41.82	16	69.6	2	8.7	21	91.3	16	76.2	5	23.8

CIN – cervical intraepithelial neoplasia, HPV – human papillomavirus, *Multiple HPV infections: positive for > 1 high risk HPV

The prevalence of HPV DNA in AIS, AC and ADSQ was 91.3% (21/23), 90.9% (60/66) and 94.1% (16/17), respectively. We have detected HPV DNA in all 5 samples of CCC although other studies have reported a highly variable prevalence of HPV DNA in CCC[17,18,21]. Also, patients with CCC belong to oldest age group (63.8 years) compared to all other histological subtypes of AC (Table 1).

While HPV types 16, 18, 31 and 33 were detected in single or multiple infections we did not detect HPV types 45, 52, 59 and 68 in any tested samples. All samples that were HPV DNA positive but were not typed as HPV 16, 18, 31, 33, 45, 52, 59 or 68 were marked as HPV X. We found HPV 18 type to be the most predominant type in AIS (11/21) and AC (17/60) followed by HPV X in AIS (3/21) and HPV 16 in AC (9/60) as the sole viral type (Table 2). Also, HPV 18 was most frequently detected type in all histological subtypes of AIS and AC. On the other hand the most prevalent HPV type in ADSQ was HPV 16 followed by HPV 33 as single type. The observed overall predominance of HPV 18 in AIS (χ^2= 6.109, p≤ 0.025) and AC (χ^2=8.927, p≤0.01) and of HPV 16 in ADSQ (χ^2=10.164, p≤0.01) was statistically significant (Table 3).

When we analysed the distribution of HPV 16 and 18 in endocervical AIS and AC together we found statistically significant prevalence of HPV 18 (21/31; χ^2=6.458, p≤0.025). Also, prevalence of HPV 18 in intestinal AIS and AC was statistically significant (9/11; χ^2=4.701, p≤ 0.05) (Table 3). However, there is no statistically significant prevalence of HPV 18 in remaining histological subtypes of AIS and AC.

Combining together single and multiple HPV infections, HPV 18 was detected in 68.2% of AC (30/44) and 66.7% of all AIS (14/21) cases whereas HPV 16 in 36.4% (16/44) and 28.6% (6/21), respectively. Single HPV type infections were statistically significant in AIS (16/21; χ^2=11.523, p≤0.001) and AC (37/60; χ^2=6.533, p≤0.025) while equal number of single and multiple HPV infections was detected in ADSQ (8/16). However we found statistically significant higher number of multiple infections in AC compared to AIS (23/81and 5/81, respectively; χ^2=13.989, p≤0.001).

Discussion

When we examined the number of AC cases diagnosed at our Department of pathology (which covers large region of Rijeka county), we noticed increasing trend of glandular cervical carcinomas during 1995–2006 period of time with predominant number of cases in women 29–49 years of age (data not shown). These data are in accordance with increasing trend of this neoplasia seen in developed countries[2,6].

It is well established that hrHPV is causal factor in development of SCC and its precursor lesions[22] with studies reporting almost 100% prevalence of HPV in SCC[11,13,23]. However, causal linkage of HPV infection to the cervical AC has not been considered as strong as it is for SCC due to more variable and generally lower HPV prevalence rates (60%–96%)[18,19,22,24–26]. Recent more sensitive extraction and detection techniques have made it possible to identify the higher rate of HPV infection in

TABLE 2
HIGH-RISK HPV TYPES DISTRIBUTION ACCORDING TO HISTOLOGICAL SUBTYPES

Diagnosis	HPV positive	HPV 16		HPV 18		HPV 31		HPV 33		HPV X*		HPV 16 & 18		HPV 18 & 31		HPV 18 & 33		HPV 16 & 31		HPV 16 & 33		> 2 hr-HPV**	
	n	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Endocervical	20	3	15	10	50	1	5	–	–	1	5	2	10	1	5	–	–	–	–	–	–	2	10
Intestinal	4	–		2	50	–	–	–	–	–	–	2	50	–	–	–	–	–	–	–	–	–	–
Endometrioid	8	–		2	25	–	–	–	–	2	25	1	12.5	1	12.5	–	–	–	–	–	–	2	25
Clear cell	5	–		2	40	–	–	1	20	1	20	–	–	–	–	–	–	–	–	–	–	1	20
Serous	1	–		–	–	1	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Villoglandular	1	–		1	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mixed	4	1	25	–	–	–	–	–	–	1	25	1	25	–	–	–	–	–	–	–	–	1	25
Glassy cell	1	–		–	–	–	–	–	–	–	–	–	–	–	–	1	100	–	–	–	–	–	–
Adenosqumaous	16	5	31.3	–	–	–	–	3	18.7	–	–	1	6.3	–	–	–	–	1	6.3	3	18.7	3	18.7
All invasiveadeno-carcinoma	60	9	15	17	28.3	2	3.3	4	6.7	5	8.3	7	11.7	2	3.3	1	1.7	1	1.7	3	5	9	15
Endocervical	11	1	9.1	4	36.3	–	–	–	–	2	18.2	1	9.1	1	9.1	–	–	1	9.1	–	–	1	9.1
Intestinal	7	1	14.3	4	57.1	–	–	–	–	1	14.3	–	–	–	–	–	–	–	–	–	–	1	14.3
Mixed	1	–		1	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Adenosqumaous	1	–		1	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Endometrioid	1	–		1	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
All adenocarcinoma *in situ*	21	2	9.5	11	52.3	–	–	–	–	3	14.3	1	4.8	1	4.8	–	–	1	4.8	–	–	2	9.5

HPV – human papillomavirus, *Includes HPV types other than 16, 18, 31, 33, 45, 52, 59, 68; **Includes multiple high risk HPV infections (HPV 18, 16 &31, HPV 18, 16 & 33, HPV 16, 31 &33, HPV 16, 18, 31 &33)

AC with frequencies reaching 85% or more[18,27]. Although DNA extractions from older (>10 years) paraffin embedded tissue blocks can be sometimes cumbersome we were able to detect HPV DNA in large majority of cases (91%) consistent with previous findings[18,28]. We screened our samples for only 8 hrHPV types because combined together, as we showed previously[29], they cover almost 90% of overall HPV type found in cervical carcinoma.

TABLE 3
OVERALL PREVALENCE OF HIGH-RISK HPV TYPES IN VARIOUS HISTOLOGICAL SUBTYPES OF CERVICAL ADENOCARCINOMA

Diagnosis	HPV positive	HPV 16		HPV 18		HPV 31		HPV 33		HPV X*	
	n	n	%	n	%	n	%	n	%	n	%
Endocervical	20	7	35	15	75	3	15	2	10	1	5
Intestinal	4	2	50	4	100	–	–	–	–	–	–
Endometrioid	8	3	37.5	5	62.5	3	37.5	2	25	2	25
Clear cell	5	1	20	3	60	–	–	2	40	1	20
Serous	1	–	–	–	–	1	100	–	–	–	–
Villoglandular	1	–	–	1	100	–	–	–	–	–	–
Mixed	4	3	75	1	25	1	25	1	25	1	25
Glassy cell	1	–	–	1	100	–	–	1	100	–	–
All invasive adenocarcinoma	44	16	36.4	30	68.2	8	18.2	8	18.2	5	11.4
Adenosquamous	16	13	81.3	4	25	3	18.7	8	50	–	–
Endocervical	11	4	36.4	6	54.5	2	18.2	1	9.1	2	18.2
Intestinal	7	2	28.6	5	71.4	1	14.3	1	14.3	1	14.3
Mixed	1	–	–	1	100	–	–	–	–	–	–
Adenosquamous	1	–	–	1	100	–	–	–	–	–	–
Endometrioid	1	–	–	1	100	–	–	–	–	–	–
All adenocarcinoma *in situ*	21	6	28.6	14	66.7	3	14.3	2	9.5	3	14.3

HPV – human papillomavirus, *Includes HPV types other than types 16, 18, 31, 33, 45, 52, 59 & 68

HPV infection is required for the development of both SCC and AC, however, distribution of HPV types seen in these two forms of the diseases differ. HPV 16 type is the most frequently involved in the development of SCC[30] of the cervix where as studies describing HPV type prevalence in AC are in disagreement. Some report HPV 16 and HPV 18 type to have equal prevalence[30,18], while others show predominance of HPV 18 in AC[26]. In this study we found that, in overall, HPV 18 statistically significantly prevails in AIS and AC, while in ADSQ HPV 16 is the most frequent type. We found HPV 18 predominantly in endocervical and intestinal AIS and AC, however there is no statistically significant prevalence of HPV 18 in remaining histological subtypes of AIS and AC. Large meta-analysis study[16] showed that the highest risks of developing AC were associated with HPV 18 (OR=410) followed by HPV 16 (OD=164). In context of these findings our results support the hypothesis of HPV 18 being the most important risk factor in developing of cervical AC. Existing data on the HPV DNA detection in cervical CCCs are opposing, with some studies indicating HPV DNA presence[17,21,28], while others were unable to identify HPV DNA[18,31]. In our study we found all 5 CCC cases to be HPV DNA positive. Therefore our results are in line with those that reported positive findings but exact cause for the discrepancy in the HPV DNA detection from CCCs is still unclear.

Our data revealed statistically significant predominance of single hrHPV infections in AIS and AC, however, other studies showed similar results only for AC[18,27]. Presented results support hypothesis of Zielinski et al.[27] that invasive growth of glandular epithelial cells is triggered by the action of a single HPV type rather than a potentially synergistic action of multiple HPVs. However, in our study we found rather high prevalence of multiple HPV infections in AIS and invasive AC, 23.8% and 38.2% cases respectively (Table 1), that can not be overlooked. The fact that multiple infections are even more abundant in invasive AC than in the precursor lesion, might indicate that multiple infection could contribute to cancer development in a more aggressive way than a single infection with hrHPV.

Conclusion

Our study revealed that the most prevalent hrHPV type in AIS and AC is HPV 18 while HPV 16 is predominant type in ADSQ. Analysis of various histological subtypes of AIS and AC demonstrate that in endocervical and intestinal carcinoma HPV 18 is predominant type, whereas in remaining histological subtypes such predominance was not detected. We have detected HPV DNA in all 5 samples of CCC although other studies have reported a highly variable prevalence of HPV DNA in CCC. Our data revealed statistically significant predominance of single hrHPV infections in AIS and AC whereas multiple hrHPV infections were more abundant in AC comparing to AIS.

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RASPODJELA TIPOVA HUMANOG PAPILOMAVIRUSA U RAZLIČITIM HISTOLOŠKIM SUPTIPOVIM ADENOKARCINOMA VRATA MATERNICE

S A Ž E T A K

Budući da su podaci u svijetu o prevalenciji HPV tipova u različitim histološkim suptipovma adenokarcinoma (AC) vrlo oskudni, proveli smo ovu studiju s ciljem da ispitamo prevalencije HPV tipova u AC, adenokarcinomu *in situ* (AIS) i adenoskvamoznom karcinomu (ADSQ) u Hrvatskoj. Ukupno 102 arhivska uzoraka primarnog adenokarcinoma vrata maternice (26 AIS i 76 AC), prikupljeno je na patologiji tijekom 1995–2006, i histološki suptipizirano. Dobiveni podaci su pokazali da je najzastupljeniji suptip AC endocervikalni (22/66), zatim ADSQ (17/66), dok je u AIS-u endocervikalni suptip utvrđen u 12/23, a intestinalni u 7/23 AIS-a. DNK je uspješno izolirana iz 89 uzoraka. U 81 uzorku (91%) dokazana je HPV DNK. Utvrđena je HPV DNK prevalencija u AIS-u 91,3%, u AC 90,9 % i ADSQ 94,1%. U slučajevima infekcije samo jednim tipom utvrdili smo da je HPV 18 najučestaliji u AIS-u (11/21) i AC (17/60), a slijede ih grupa neodređenog tipa HPV-a u AIS-u (3/21) te HPV 16 u AC (9/60). HPV 18 je i najučestaliji genotip u svim histološkim suptipovima AIS-a i AC. Također smo dokazali HPV DNK u svih 5 uzoraka karcinoma svjetlih stanica (CCC) iako su druge studije pokazale različite podatke o prevalenciji HPV DNK u CCC. Najučestaliji pojedinačni HPV tipovi u ADSQ su HPV 16 i HPV 33. Dokazano je da je ukupna prevalencija HPV 18 u AIS-u i AC, te HPV 16 u ADSQ statistički značajna; HPV 18 u AIS-u ($\chi^2 = 6.109$, $p \leq 0.025$) i AC ($\chi^2 = 8.927$, $p \leq 0.01$) i HPV 16 u ADSQ ($\chi^2 = 10.164$, $p \leq 0.01$). Infekcija s jednim HPV tipom je statistički značajno učestalija u AIS-u (16/21; $\chi^2 = 11.523$, $p \leq 0.001$) i AC (37/60; $\chi^2 = 6.533$, $p \leq 0.025$), dok je višestruka HPV infekcija učestalija u AC-u u odnosu na AIS (23/81 i 5/81, $\chi^2 = 13.989$, $p \leq 0.001$).

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Short communication

Combined Analysis of HPV DNA and $p16^{INK4a}$ Expression to Predict Prognosis in ASCUS and LSIL Pap Smears

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A B S T R A C T

Human papillomavirus (HPV) is known to play an important etiological role in the genesis of cervical cancer, but only a very small proportion of infected women develop invasive cervical cancer. The purpose of cervical cancer prevention is early diagnosis of its precursors. The molecular detection of HPV DNA as a diagnostic test to cervical carcinogenesis gave a low positive predictive value as compared to the use of biomarkers. $p16^{INK4a}$ has been proposed as putative surrogate biomarkers that would allow identification of dysplastic cervical epithelia. Serial consecutive cervical smears were test for high-risk HPV, stained with immunocytochemistry for $p16^{INK4a}$ and followed-up for 36 months. The aim of the study was to evaluate the immunohistochemical expression of $p16^{INK4a}$ as a marker of progression risk in low-grade dysplastic lesions of the cervix uteri. In the present series, significant p16 overexpression was observed in the group that progressed from low to high-grade squamous intraepithelial lesion when compared with the group that did not progress. In conclusion, overexpression of $p16^{INK4a}$ acts as potential biomarkers for cervical cancer progression from premalignant lesions.

Keywords: *cervical intraepithelial neoplasia, cervical cancer, human papillomavirus, $p16^{INK4a}$*

Introduction

Epidemiological and molecular studies over the past two decades have demonstrated that high-risk human papillomavirus (HR-HPV) types are etiologically related to the progression to cervical cancer. Although more that 85 types of HPV have been detected in the genital mucosa, in the majority of HPV-infected individuals, the virus is eliminated. A substantial proportion of HPV lesion regresses spontaneously over 6–18 months period. Several studies have shown that viral persistence is necessary for cervical intraepithelial neoplasia (CIN) lesions to progress or in fact be maintained.

Although HPV testing has been successfully used and proposed for triaging to colposcopy those patients with minor cytologic abnormalities, its positive predictive value (PPV) is suboptimal and a substantial proportion of patients are still referred unnecessarily to colposcopy. In the ALTS study[1] the PPV for CIN3 of a positive HR-HPV test in a patient with and atypical squamous cells of undetermined significance (ASCUS) Pap was only 10%.

Identifying other molecular events associated with progression from low (L)- to high (H)-grade squamous intraepilelial lesions (SIL) is a crucial area of research, as it may further improve selection of HPV-positive patients really worthy of assessment and treatment.

The use of modulators involved in the cell cycle as biomarkers of HR-HPV infected cells may be an important tool in the future to identifying those smears containing HSIL of patients that might progress and develop to cervical carcinoma.

The $p16^{INK4}$ is a tumour suppressor protein that inhibits the function of cdk4 and cdk6, which in turn regulate the G1 checkpoint. CDK/cyclin-D phosphorylate the retinoblastoma protein (pRb), resulting in a conformational change, with the release of E2F from Rb. Thus, inactivation of either p16 or Rb function allows the cell to enter the S phase after only a brief pause as the G1 checkpoint. In addition, the E6 HPV oncoprotein has the ability to bind p53, resulting in its degradation, and the E7 gene product inactivates the pRb pathway.

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Pathogenic activity of HR-HPV indicated by p16 expressions on smears could be a strategy to identify patients at major risk to develop cervical lesions.

The p16^{INK4a} immunostaining has been suggested as a tool for triaging women with low-grade or borderline cytology; p16 could be particularly interesting among women with LSIL cytology, where triage by HPV is inefficient. Several studies[2,3] reported a differential expression of p16 in HSIL, LSIL and normal cervical epithelial cells.

The p16^{INK4a} has been shown to be associated with HPV-infected high grade lesions but its PPV and sensitivity in prospective follow-up for relevant outcomes (> CIN2) has yet to be determined.

In our previous report[4] we assessed the accuracy of p16 and HR-HPV testing in identifying high-grade cervical lesions in 283 cervical samples (ThinPrep) on a consecutive series of women referred to colposcopy for abnormal cytology (≥ASCUS). In this follow-up study we analyzed the role of immunocytochemical expression of p16 in HPV infected women as prognostic markers of the progression of SIL.

Subjects, Material and Methods

Initially we assessed p16^{INK4a} immunostaining and HR-HPV in 283 patients consecutively referred to colposcopy for cytologic evidence of LSIL or ASCUS within the Florence (Italy) District screening programme for cervical cancer.

HPV and p16 testing were performed in the whole series prior to colposcopy assessment: cervical material was collected using ThinPrep® (Cytic Corp., Boxborough, MA), allowing for multiple slide preparation and residual fluid. Laboratory operators performing the testing were blinded to the colposcopy assessment outcome. The patients without CIN2 or more were invited regularly for follow-up cytology; if cytology was ASCUS or more colposcopy was performed.

The 238 out of 252 patients were followed for 36 month period and then stratified according the cytology results and final outcome. Final outcome was defined according to colposcopy-directed biopsy result (<CIN2 or >CIN2) and was assumed to be negative in the presence of negative colposcopy, indicating no biopsy. Observed differences were tested by the Chi-square test, with statistical significance set at $p < 0.05$.

HPV and p16 testing were performed in the whole series prior to colposcopy assessment: cervical material was collected using ThinPrep® (Cytic Corp., Boxborough, MA), allowing for multiple slide preparation and residual fluid. Laboratory operators performing the testing were blinded to the colposcopy assessment outcome.

HPV Testing

From each specimen, 2 mL of residual ThinPrep fluid was used, and DNA extraction was carried out using a QIAmp DNA Mini Kit (Qiagen Corporation, Venlo, the Netherlands) according to the manufacturer's protocol. Polymerase chain reaction (PCR) analysis was performed according to a previously described protocol[5] using primers for the *E6/E7* region of HR HPV types (HPV 16, 18, 31, 33, 35, 45, 52 and 58). For a quality control of DNA extraction, the primer set PC04 and Gh20 was employed to amplify a 268-base pair (bp) fragment of the human beta globin gene in all specimens. In each PCR reaction, negative and positive controls were introduced.

p16^{INK4a} Testing

From each specimen, 2 mL of residual ThinPrep fluid was used for a cytospin preparation; after cytocentrifugation (5 min at 1000 rpm), slides were air dried for 10 minutes, then treated with spray fixation reagent, containing polyethylene glycol, and immunostained within 24 hours. Before they were immunostained, all spray-fixed specimens were incubated in 50% volume/volume alcohol, followed by one washing step in deionized water. For immunostaining, CINtec^{tm} p16 Cytology kit (Dako Cytomation, now Dako A S, Glostrup, Denmark) was used, according to the manufacter's protocol. In brief, smears were treated with 3% hydrogen peroxide and then submitted for epitope retrieval at 95–99 °C for 40(±1) minutes; after cooling, the p16^{INK4a} antibodies were applied for 30 (±1) minutes and then a reagent for observation and substrate-chromogen solutions were added. Hematoxylin was used as counter stain. The methodology differs from other studies on p16[6,7], but we used the same monoclonal antibody and believe that the results are comparable. The choice of cytospin preparation was essentially aimed at a more efficient use of the residual ThinPrep fluid. Before the study, we made a comparison of p16^{INK4a} testing on cytospin and ThinPrep preparations on limited numbers of negative and positive samples (data not shown), and we observed no differences.

Slides were read by two investigators blinded to final outcome, and a minimum of 500 cells in different fields were analyzed. A negative result was defined if no cells immunoreactive to the p16^{INK4a} antibody were in evidence. Slides showing positive staining for p16 were categorized on the basis of the percentage of positive cells as: <5%, 5–10% or >10%. The cellular staining site was also evaluated and categorized as 1) nuclear, 2) cytoplasmic, or 3) nuclear plus cytoplasmic.

Results

At recruitment[4] we assessed the accuracy of p16 and HR-HPV testing in identifying high-grade cervical lesions in 283 cervical samples (ThinPrep) on a consecutive series of women referred to colposcopy for abnormal cytology (≥ASCUS). The results were compared with colposcopy and biopsy findings. HPV positivity rate was 44.2% among <CIN1, and 89.2% among ≥CIN2 patients (Chi-square for trend $<10^{-6}$). The sensitivity, specificity, and PPV of HPV testing for ≥CIN2 were 89.2% (25 of 28), 47.8 (122of 255) and 15.8% (28 of 158), respectively. P16 positivity rate was 25.3% among <CIN1, 57.4% among CIN1 and 88.0% among ≥CIN2 patients (square for trend $<10^{-6}$). Sensitiv-

TABLE 1
HPV TEST AT RECRUITMENT ACCORDING TO THE CYTOLOGICAL DIAGNOSIS DURING THE FOLLOW-UP

	N (%) of HR-HPV negative	N (%) of HR-HPV positive	Total – N (%)
Normal	100 (92.6)	89 (83.2)	189 (87.9%)
ASCUS	3 (2.8)	2 (1.9)	5 (2.3%)
LSIL	3 (2.8)	10 (9.3)	13 (6.04%)
HSIL	2 (1.8)	6 (5.6)	8 (3.7%)
Total	108 (50.2%)	107 (49.8%)	215

HR – high-risk, HPV – human papillomavirus, ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous intraepilelial lesions, HSIL – high-grade squamous intraepilelial lesions

TABLE 2
P16 AT RECRUITMENT ACCORDING TO THE CYTOLOGICAL DIAGNOSIS DURING THE FOLLOW-UP

	N (%) of p16^{INK4a} negative	N (%) of p16^{INK4a} positive	Total – N (%)
Normal	58 (87.9)	31 (77.5)	89 (84.0)
ASCUS	1 (1.5)	1 (2.5)	2 (1.9)
LSIL	6 (9.1)	4 (10)	10 (9.4)
HSIL	1 (1.5)	4 (10)	5 (4.7)
Total	66 (62.3)	40 (37.7)	106

HR – high-risk, HPV – human papillomavirus, ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous intraepilelial lesions, HSIL – high-grade squamous intraepilelial lesions

ity for ≥CIN2 was 88% (22 of 25), specificity was 61.2% (79 of 129) and PPV was 30.5% (22 of 72).

The patients with cytological abnormalities but without CIN2/3 lesions were followed-up, for 36 months by cytology and colposcopy was performed if cytology was ASCUS or worse.

Between 252 patients, 215 women have had a follow-up procedure: 108/122 (88%) with a negative result for HR-HPV and 107/130 with a positive result for HR-HPV.

After 36 months (Table1), 8 (7.4%) women with a negative result for HR-HPV at recruitment, still displayed abnormal smears (3 ASCUS, 3 LSIL and 2 HSIL). Final outcome of these cases was: 2 CIN2 (1.8%), 3 CIN1 (2.8%) and 3 Negative (2.8%).

Between women with a positive result for HR-HPV at enrolment (Table1), after 36 months of follow-up, 18 (16.8%) women still displayed abnormal smears (2 ASCUS, 10 LSIL and 6 HSIL). Final outcome of these cases was: 9 CIN2+ (8.4%), 3 CIN1 (2.8%) and 6 Negative (5.6%), ($p < 0.05$).

Considering p16 immunostaing, 66/79 (84%) p16 negative and 40/50 (80%) p16 positive have had follow-up (Table 2). During the follow-up of p16 negative women 3 (3/66=4.5%) CIN2 were founded, while 6 CIN2 (6/40= 15%) were founded in p16 positive women ($p<0.05$).

At the enrolment the number of p16 positive cells did not correlate with the probability of ≥CIN2 (<5% = 36.3%, 5–10% = 27.2%, >10% = 36.3) and was no further considered a relevant variable. No evident differences in pattern or intensity of p16 expression were observed between the specimens with high grade lesions and without high lesions at follow-up.

Discussion

HPV DNA testing appears useful in the triage of equivocal Pap-smears; however studies could not demonstrate a high level of specificity of HPV DNA testing for clinically significant cervical disease. The p16 immunostaining has been suggested as a tool for triaging women with low-grade or borderline cytology; p16 could be particularly interesting among women with LSIL cytology, where triage by HPV is inefficient. Moreover, an obvious problem in using HPV testing as a screening tool is that a sizable proportion of normal women are HPV positive; however a report[8] suggested that about 15% of women in annual screening programme who concurrently have a negative Pap test and a positive oncogenic HPV test will have a subsequent abnormal Pap test within 5 years.

Nevertheless, HPV testing also identified many transient HPV infections that are not associated with high-grade CIN. Several studies based on molecular markers associated with HPV infection could facilate and optimise diagnosis in a screening setting. It may be possible to detect clinically important disease with risk of progression towards dysplasia and carcinoma, and consequently, improve patient care by combining test results from molecular markers with either cytology or HPV or both.

Several studies[2–4] reported a differential expression of p16 in HSIL, LSIL and normal cervical epithelial cells.

In this study, we evaluated the potential of p16 immunocytochemical expression to predict the course of cytological cervical abnormalities associated with HR-HPV types.

In the present series, significant p16 overexpression was observed in the group that progressed from LSIL to

HSIL when compared with the group that did not progress. To our knowledge, there are a few studies with a prospective follow-up design carry out to evaluate SIL progression and the association of p16 overexpression in cervical specimens in a screening setting for cervical cancer.

Although p16 protein and HPV infection may be detected in low-grade lesions or reactive changes that undergo spontaneous regressions, Wang[9] et al. found that the risk for CIN progression or HPV persistent is higher for women with diffuse staining for p16 protein compared with those without diffuse staining in tissue samples. Negri[10] also found that CIN1 cases with diffused p16 staining had significant higher tendency to progress to a high-grade lesion that p16 protein negative cases.

Conclusion

This study suggest that the combined use of p16 protein and HPV testing may be useful in identifying cervical cells with minor abnormalities and a high risk of progressing to cervical neoplasia, and also defining cases requiring early management, or at least close surveillance. Our study demonstrated that p16 evaluation on ThinPrep samples could be useful as a biomarker for progressive malignancy in HPV-related cytological abnormalities. Patients with ASCUS or LSIL and HR-HPV and p16 positive test were a major risk to develop high grade lesion compared with patients HR-HPV negative or HR-HPV positive but with p16 negative.

These findings suggest that among HPV positive patients, there is a subgroup that may be at increased risk of progression to invasive cancer and should be followed-up more closely. Theoretically, all HR HPV-associated lesions should express p16^{INK4a}. It is unclear why some CIN2 samples that are HPV DNA positive were also p16^{INK4a} negative. We might assume that there are other mechanisms of p16^{INK4a} regulation besides HPV infection, such as promoter methylation, that could occur in cervical cancer. However, according to previous report in the literature, pRb inactivation via the p16/cdk-cyclin/ RB pathway and increase in p16 expression in HPV- transformed cells is an important mechanism for cervical carcinogenesis.

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KOMBINIRANA ANALIZA DNA HPV-A I EKSPRESIJE P16 U PREDVIĐANJU PROGNOZE PAPA-NALAZA ASCUS I LSIL

SAŽETAK

Poznato je da humani papilomavirus (HPV) igra važnu etiološku ulogu u nastanku raka vrata maternice, no samo mali udio inficiranih žena zaista i razvije invazivni rak vrata maternice. Cilj prevencije raka vrata maternice je rana dijagnoza njegovih prekursora. Molekularna detekcija DNK HPV-a kao dijagnostički test za karcinogenezu raka vrata maternice je dala slabo pozitivne prediktivne vrijednosti u usporedbi s upotrebom bioloških biljega. p16^{INK4a} je predložen kao osnovni biljeg koji bi omogućio identifikaciju promjena epitela vrata maternice. Grupa uzastopnih obrisaka vrata maternice je testirana na visokorizične HPV, obojana imunocitokemijski za detekciju p16^{INK4a} i praćena 36 mjeseci. Cilj studije je bio procijeniti imunohistokemijsku ekspresiju p16^{INK4a} kao biljega rizika progresije u oštećenjima niskog stupnja stanica vrata maternice. U toj grupi je zabilježena znatno povećana ekspresija p16 za skupinu koja je prešla iz oštećenja niskog stupnja u oštećenja stanica vrata maternice visokog stupnja u usporedbi sa skupinom kod koje nije došlo do progresije. Zaključili smo da povećana ekspresija p16^{INK4A} djeluje kao mogući biološki biljeg koji ukazuje na progresiju oštećenja vrata maternice u premaligni i maligni stadij.

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Short communication

Evaluation of p16^{INK4a} in Cervical Lesion of Premenopausal and Postmenopausal Women

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A B S T R A C T

Pap smears of postmenopausal women are often misdiagnosed because of the difficulty in distinguishing atrophic epithelial cells groups only by morphological criteria. In this study we investigated the diagnostic application of immunocytochemical staining of p16^{INK4a} on conventional Pap smear. A total of 137 cervical specimens were enrolled in this study, of which 77 and 60 cervical smears were taken from premenopausal and postmenopausal women, respectively. Two cervical smears were taken simultaneously in 68 women, one for conventional cytology and the other for immunostaining. Additional 69 cervical smears were taken from the archive, decolorized and then used for immunostaining. In premenopausal women 1 out of 14 (7.1%) with negative cytology, 7 out of 24 (29.2%) with low grade squamous intraepithelial lesion (LSIL), all 35 (100%) with high grade squamous intraepithelial lesion (HSIL) and all 4 (100%) with squamous cell carcinoma (confirmed by histopathology) had positive staining to p16^{INK4a}. In postmenopausal women p16^{INK4a} positivity was observed in 4 out of 7 (57.1%) cases of LSIL, 12 out of 14 (85.7%) cases of HSIL and all 4 out of 5 (80%) different cases of carcinoma (1 cervical adenosquamous carcinoma and 3 cervical squamous cell carcinoma in situ confirmed by histopathology), but none of 34 smears with normal cytology. Twenty smears with normal cytology chosen for the negative control in this study were from the group of postmenopausal women and were as expected negative for p16^{INK4a} immunostaining. In the group of postmenopausal women, 16 out of 60 (26.7%) cases the cytological diagnosis was established on the basis of p16^{INK4a} immunostaining as being HSIL. From our preliminary study on a limited number of samples, we can however conclude that p16^{INK4a} immunostaining is a very useful tool for cytological diagnosis enabling to distinguish HSIL from normal, reactive or inflammatory changes.

Key words: *postmenopausal, cervical, dysplasia, immunostaining, p16^{INK4a}*

Introduction

It is well established that persistant infection with oncogenic Human papillomavirus (HPV) causes preneoplastic and neoplastic changes of the cervical epithelium, leading towards carcinoma, especially in middle aged women[1]. In postmenopausal age, women have received little attention concerning this problem. Improvements in life standards and better health care have led to increasing population of elderly women who come to their regular gynaecological exam and their Papanicolaou (Pap) smears[2–3]. In spite of the fact that there is almost 20% of women infected with HPV[4], only a few of them develop preneoplastic lesion, and even fewer develop cancer. Smith and al.[5] found that 16% of persistent HPV infections exist in the elderly group of women, suggesting that one can predict development of a preneoplastic lesion that can progress toward cancer. However, problems in diagnosis such as aging-related changes, reparative changes and interpretation difficulties may lead towards an incorrect diagnosis. Pap smears of postmenopausal women are often misdiagnosed because of the difficulty in distinguishing atrophic epithelial cell groups only by the morphological criteria. Those criteria are more viable when premenopausal women are concerned, referred to better oestrogen effect, hence better maturation. In this study we investigated whether immunocytochemical staining of p16^{INK4a}, performed on conventional Pap smear

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could be of any diagnostic help in solving this dilemma. Thus, we performed p16^{INK4a} immunostaining on specimens taken from premenopausal and postmenopausal women in order to make an accurate diagnosis in those cases, which were impossible for us to distinguish on the basis of cytomorphological criteria, i.e. to distinguish a high grade cervical lesion from atrophy or reactive changes.

Examinees, Materials and the Methods

We analysed 137 cervical smears, which consisted in 69 archival Pap slides and 68 cervical specimens collected from women who attended our clinic in 2006 for their regular check-up. They were divided into two age groups. Seventy-seven premenopausal women were in group I (median age 34.5) and 60 postmenopausal women (median age 63) in group II, of which 20 women with normal cervical smears were used as a control group.

Two simultaneous cervical smears were taken by a cytobrush and a wooden spatula, one for conventional cytology and the other for immunostaining. Archival Pap slides were decolorized in 0.5% HCl, rinsed with distilled water and then immunocytochemically stained with CINtec™ p16^{INK4a} Kit (DAKOCytomation, Denmark) according to the manufacturer instructions. There were no differences in staining reaction or intensity of reaction between decolorized and freshly taken smears. According to DAKO instructions at least 1% of cells should be stained for the reaction to be considered positive. Cells are regarded as stained if brownish-like granules were found in nuclei and/or in a cytoplasm.

Pap smears were classified by the »Bethesda system«[6] into a normal epithelium, low (LSIL) and high grade intraepithelial cervical lesion (HSIL) and squamous cell carcinoma (SCC). Among postmenopausal women, there was one case of recurrent adenosquamous carcinoma and metastatic transitional cell carcinoma. In 16 postmenopausal women we could not diagnose based on the morphological criteria; in those women diagnosis was established after the p16^{INK4a} immunostaining.

In total, fifty-five women underwent colposcopy and biopsy, and a consequent cold knife conisation or hysterectomy was performed; in each case histological verification was made. Unfortunately, the patient with metastatic transitional cell carcinoma died.

The distribution of positivity rate versus diagnosis was analyzed with the standard Chi–square (χ^2) test. The statistical significant differences was set at $p<0.025$.

Results

The distribution of cytological diagnoses and p16^{INK4a} immunostaining in premenopausal women is as follows: 18.2% (14/77), 31.2% (24/77), 45.5% (35/77) and 5.2% (4/77) women had a negative cytology, LSIL, HSIL, SCC, respectively (Table 1). P16^{INK4a} immunostaining was observed in 7.1% (1/14), 29.2% (7/24) and 100% (35/35 and 4/4) of premenopausal women with negative cytology, LSIL, HSIL and SCC, respectively. The observed frequencies were significantly different ($\chi^2=52.24$, $p<0.0001$). The positive p16^{INK4a} immunostaining of one of the 4 SCC later histologically confirmed, and of normal metaplastic cells is shown in Figure 1 and 2. In this age group we succeed to diagnose all smears according to viable morphological signs and well established criteria for every grade of intraepithelial lesion.

TABLE 1
P16^{INK4a} FINDINGS ACCORDING TO DIAGNOSIS OF PREMENOPAUSAL WOMEN

p16^{INK4a}	N (%) of cytological diagnosis				
	Normal	LSIL	HSIL	Carci-noma	Total
Negative	13 (92.9)	17 (70.8)	0	0	30 (39)
Positive	1 (7.1)	7 (29.2)	35 (100)	4 (100)	44 (61)
Total	14 (18.2)	24 (31.2)	35 (45.5)	4 (5.2)	77 (100)

LSIL – low grade squamous intraepithelial lesion, HSIL – high grade squamous intraepithelial lesion, *cervical squamous cell carcinoma *in situ* confirmed by histopathology, $\chi^2 = 52.24$, $p < 0.0001$

The distribution of cytological diagnoses and p16^{INK4a} immunostaining in postmenopausal women is as follows: 52.3% (34/60), 10.8% (7/60), 21.5% (14/60) and 7.7% (5/60) women had a negative cytology, LSIL, HSIL, SCC, respectively (Table 2). P16^{INK4a} immunostaining was observed in 57.1% (4/7), 85.7% (12/14) and 80% (4/5) of postmenopausal women with LSIL, HSIL and SCC, respectively, while none with those with normal cytology. The observed frequencies were significantlly different ($\chi^2=40.97$, $p<0.0001$).

In 16 postmenopausal women we had difficulties for classifying a certain cell groups as neoplastic because of their resemblance to mucosal atrophy or because of the presence of strong degenerative, inflammatory or reactive changes. In 8 women, after p16^{INK4a} immunostaining we were able to distinguish HSIL from reactive changes (Figure 3). Among these women one case of previously diagnosed adenosquamous carcinoma, was p16^{INK4a} positive, indicating the possibility of recurrent carcinoma.

Discussion

In spite of good screening methods based on Pap smear and well organized screening programmes in many countries, women all around the world continue to develop cervical disease. The incidence rate of cervical cancer in developing countries is increasing due to the aging population, while in Western Europe first peak is between 30 and 40 years of age and the second peak occurs around 60 years of age[7]. Genital infection with oncogenic or high-risk (hr) HPV is established as one of the main events in pathogenesis of cervical and other genital tumours[1]. After the first contact with the virus,

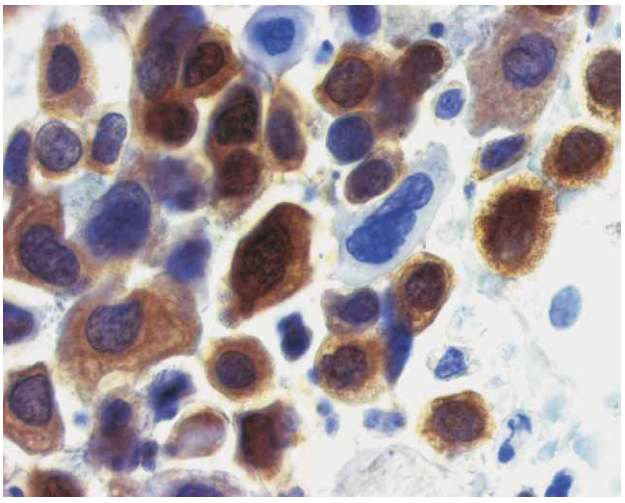

Fig 1. p16^{INK4a} staining of malignant squamous cells found in a smear of a premenopausal woman

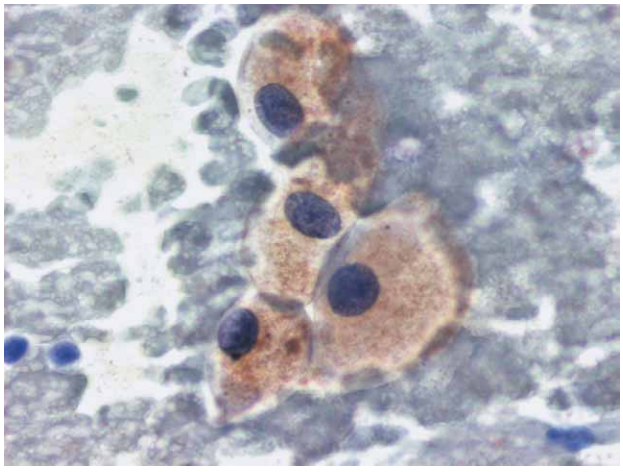

Fig 2. p16^{INK4a} staining of normal metaplastic cells found in a smear of a premenopausal woman

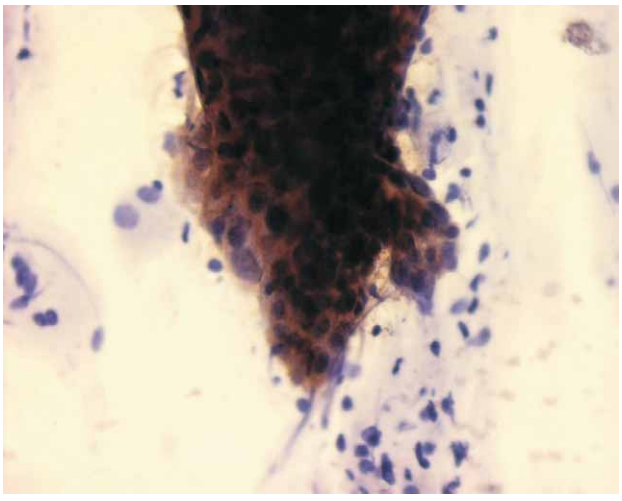

Fig 3. p16^{INK4a} staining of high grade squamous intraepithelial lesion (HSIL) found in a smear of a postmenopausal woman

TABLE 2
P16^{INK4a} FINDINGS ACCORDING TO DIAGNOSIS OF POSTMENOPAUSAL WOMEN

p16^{INK4a}	N (%) of cytological diagnosis				
	Normal	LSIL	HSIL	Carcinoma	Total
Negative	34 (100)*	3 (42.9)	2 (14.3)	1** (20.0)	40 (66.7)
Positive	0	4 (57.1)	12 (85.7)	4*** (80.0)	20 (33.3)
Total	34 (52.3)	7 (10.8)	14 (21.5)	5 (7.7)	60 (100)

LSIL – low grade squamous intraepithelial lesion, HSIL – high grade squamous intraepithelial lesion, *include 20 women as control group, **transitional cell carcinoma confirmed by histopathology, ***1 case of cervical adenosquamous carcinoma and 3 cases of cervical squamous cell carcinoma *in situ* confirmed by histopathology, χ^2=40.97, p<0.0001

viral replication is limited to superficial layers of the cervical epithelium that represents the acute, transitory phase. If viral E6 and E7 oncogene products are over-expressed in a basal or parabasal layers, they will interfere with the regulation of the host cell cycle, inducing genetic instability. The probability of progression of cervical precancerogenic lesions is considered greater with the longer persistence of papillomavirus infection. Almost 100% of all cervical cancers can be attributed to certain hrHPV types[8]. The highest proportion of cervical squamous cancers is associated with HPV type 16, followed by type 18 which is more common in adenocarcinomas, than HPV types 31, 33, 45, 52, 58 and others[1,7].

There are lots of regulating proteins involved in the cell cycle control. One of them, p16^{INK4a}, has been shown to be over-expressed after the cell has been infected by hrHPV types. Expression of the viral E7 oncogene leads to functional inactivation of tumour suppressor retinoblastoma protein (pRB), which in turn, results in strong over-expression of the cyclin-dependent kinase inhibitor p16^{INK4a}. This indicates that an active expression of the viral E7 oncogene is present in dysplastic cells[4].

There are a lot of data about HPV prevalence in younger women, but not much about elderly population. Smith et al.[5] showed that, opposite to a common belief, the prevalence rate of HPV in postmenopausal women was almost as high as in younger women and the association between hrHPV and consecutive abnormal Pap smear was 36%. Bosch and Harper[7] on the other hand summarised the results from cohort studies and found that hrHPV infection persisted in 50% cases. Bruner and Davey[9] had similar results in their group of women aged 60 years and over in contrast with 20% of hrHPV positive infection in younger groups (40 and 50 year old women). That leads towards conclusion of Massad et al.[10] that older women more frequently have a higher abnormality grade, while younger women had more lesions connected with transient infection.

In our study, following »Bethesda recommendations« we classified lesions as normal including inflammatory and reactive changes, as LSIL presented with or without koilocytotic changes, as HSIL and as carcinoma. Among premenopausal women with normal cervical smears p16--positive metaplastic cells were noted in one patient. It is known that some metaplastic and endocervical normal cells could be positively stained due to unfinished differentiation of the cells. Staining intensities of the normal, metaplastic cell are weaker than in dysplastic[10,12], and staining was observed only in the cytoplasm, while the nucleus showed no staining reaction. After p16^{INK4a} immunostaining, most (70.8%) of dysplastic cells in LSIL were stained negative in a group of premenopausal women. There were only 7 (29.2%) LSIL smears with a stained reaction in the nucleus and cytoplasm of the cells (Figure 1). All of these premenopausal women with HSIL were hrHPV positive (data not shown). Our results showed p16^{INK4a} overall positivity of 61% that is similar to the results of Bose et al.[13], although higher percentage up to 78% were reported previously[14,15]. We agree with the statement of Bose that p16^{INK4a} does not have a role in the diagnosis of LSIL but it might be useful for confirming the diagnosis of HSIL since our study showed positive staining in all HSIL. The role of p16^{INK4a} as a predictive and prognostic factor is to be confirmed by additional large scale studies.

Correct cytological diagnosis of cervical abnormalities in older women is a much greater problem because of the morphological similarities between atrophic epithelial cells and cells from high-grade squamous lesion. Therefore one should keep in mind several cytomorphologic findings such as: clean smear background, maturation index, nuclear/cytoplasmatic ratio, presence of hyperchromasia and then incorporate that in physiologic and pathologic changes of women's genital tract. Saad et al.[16] divided impact factor analysis to individual (degree of cell maturation, patient age, and smear background) and the other ones that dramatically influenced accurate diagnosis. The following factors were suggested: nuclear membrane features, hyperchromasia with nuclear/cytoplasmatic ratio favouring nucleus and abnormal single cell arrangement. These cytomorphologic findings lead us to an »uncertain« cytological diagnosis in postmenopausal smears with profound inflammatory or degenerative changes. By performing p16^{INK4a} immunostaining, in case that it is positive in the nucleus and cytoplasm, we will be able to classify some of these »uncertain« lesions as likely to be HSIL, which could be further confirmed by histology. In cytologically negative smears of postmenopausal women, p16^{INK4a} staining was negative as well.

Conclusion

From our preliminary study on a limited number of samples, it appears that although p16^{INK4a} is not helpful in the diagnosis of LSIL in younger women, in postmenopausal women it may be useful for confirming cervical lesion even if cytological diagnosis was mild dysplasia. To avoid »uncertain« diagnosis when the rate of possible false-negative diagnoses as well as false-positive cytology is high, we think that the use of p16^{INK4a} staining would be helpful for identification of adequate diagnosis.

For elderly women with squamous atypia an oestrogen treatment is often suggested, maturation effect can make an abnormal cell more viable and abnormal reactive changes disappear. That involves repeated patient's discomfort of physical exam as well as a certain dose of stress. To minimize that we suggest taking two simultaneous smears during Pap sampling; it will allow to perform normal Pap and p16^{INK4a} immunostaining. In this way, we will be able to distinguish more accurately normal, non-specific reactive or inflammatory changes from neoplastic changes in postmenopausal women Furthermore, p16^{INK4a} immunostaining would be useful in a routine laboratory practice for analysis and follow-up of abnormal cervical smears in general. Further population-based studies will be necessary to analyse predictive value of p16^{INK4a} protein in cervical smears in both groups of women.

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PROCJENA P16^{INK4a} U LEZIJAMA VRATA MATERNICE KOD ŽENA U GENERATIVNOJ DOBI I POSTMENOPAUZI

S A Ž E T A K

Česte su pogrešne citološke dijagnoze cervikalnih razmaza žena u postmenopauzi zbog sličnosti u morfološkom izgledu stanica dubljih slojeva epitela i neoplastično promijenjenih stanica. U našoj studiji istražili smo primjenu imunocitokemijskog bojanja sa p16^{INK4a} na cervikalnim razmazima u svrhu rješavanja tog problema. Imunocitokemijski smo obojali uzorke 77 žena u generativnoj dobi i 60 uzoraka žena u postmenopauzi. Kontrolnu skupinu su sačinjavali 20 urednih citoloških razmaza bez izraženih upalnih ili degenerativnih promjena iz skupine žena u postmenopauzi. Kod 68 pacijentica prilikom uzimanja uzoraka učinili smo 2 razmaza: jedan za citološku analizu a drugi za imunocitokemijsko bojanje. Šezdesetdevet predhodno uzetih uzoraka smo odbojali i pripremili za imunoreakciju. Kod žena generativne dobi 14 pacijentica je imalo uredan Papa-test i opažen je p16^{INK4a} pozitivitet kod jednog uzorka (7,1%); 24 uzoraka je ocijenjeno kao SIL (skvamozna intraepitelna lezija) niskog stupnja i među njima je bilo 7 (29,2%) p16^{INK4a} pozitivno obojenih uzoraka. Svih 39 uzoraka SIL visokog stupnja, od kojih 4 karcinoma pločastih stanica *in situ* naknadno histološki utvrđenih, pozitivno se obojilo. Pozitivitet p16^{INK4a} kod razmaza starijih žena je rastao u slijedećem nizu: 0%, 57,1%, 85,7% i 80% kod 34 urednih razmaza, 4 razmaza niskog i 12 visokog SIL te 4 karcinoma naknadno histološki utvrđenih (1 adenokarcinom, 3 karcinoma pločastih stanica *in situ*). U skupini od 16 pacijentica, točna citološka dijagnoza SIL-a visokog stupnja postavljena je tek nakon imunocitokemijske reakcije. Na temelju našeg preliminarnog istraživanja na malom broju uzoraka, možemo zaključiti da uključivanje p16^{INK4a} imunocitokemijskog bojanja u redovitu laboratorijsku praksu može pomoći u razlikovanju reaktivnih ili degenerativnih promjena od neoplastičnih lezija cervikalnog epitela.

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Review

Development of Prophylactic HPV Vaccines

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A B S T R A C T

Since several years it has been accepted that persistent infection with certain (so called-high risk: HR) types of Human papillomaviruses (HPV) represents a strong risk factor for cervical cancer. The most frequent HR HPV types 16 and 18 account for about 70% of this tumour, which is the second most frequent malignancy in women worldwide. Several studies in animal papillomavirus models revealed that protection against infection is conferred by neutralizing antibodies directed against conformational epitopes of the major structural protein L1. Such antibodies can most efficiently be induced by immunization with virus-like particles (VLP) that assemble spontaneously following expression of L1 in recombinant vectors. Large-scale production of HPV 16 and 18 VLPs proved to be successful facilitating, a few years ago, first clinical trials on safety and immunogenicity. In the meantime more than 25,000 women have been included into several efficacy trials which demonstrated protection against persistent infection with HPV 16 and 18 and against the development of precursor lesions to cervical cancer. Although the ultimate proof of success, i.e. reduction of cancer incidence still requires the immunization of large populations and many years of follow-up, the existing data are so persuasive that the responsible agencies in several countries permitted the licensing of the first HPV vaccine in 2006. Several questions such as the duration of protection, the need development of for post-exposure vaccination strategies and availability of such vaccine in low-budget countries are open and will be discussed.

Key words: *human papillomavirus (HPV) vaccines, cervical cancer*

Preventive vaccines are based on the induction of virus-neutralizing antibodies. For the papillomaviruses this has first been achieved by immunization with formalin-fixed wart extracts and subsequently by inactivated purified viruses[1]. In demand of an appropriate infection model, it was difficult to determine the neutralizing activity of serum antibodies. The first *in vitro* assay for virus neutralization was the inhibition of focus formation of mouse cells in culture by BPV virions[2]. A more sophisticated neutralization assay was introduced by Kreider and coworkers who grafted normal human tissue infected *in vitro* with HPV 11 or HPV 16 under the renal capsule of immuno-deficient mice where typical lesions develop[3]. Infection is then monitored by phenotypic analysis and by HPV-specific RT-PCR. Only very recently, a potentially high throughput neutralization assay on the basis of pseudovirions containing a reported gene became available which allows detection of neutralizing antibodies in an *in vitro* setting[4]. Neutralizing antibodies are directed against the major structural protein L1 and, to a much lesser degree, against the minor capsid protein L2. For induction of neutralizing antibodies, the L1 protein is required to be presented in a correctly folded conformation. This became only possible with the production of L1 virus-like particles (VLPs). In 1986 it had already been shown that purified VP1 of mouse polyomavirus spontaneously assembles into virus-like particles[5]. This inspired in the early 1990s the production of papillomavirus-like particles by different researchers. Zhou and colleagues used a vaccinia virus expression system to produce HPV 16 L1 VLPs, however the yield of this system was extremely low and did not allow further characterization of the antigen e.g. by immunological studies[6].

Shortly after, it was demonstrated that HPV 1, BPV 1 and HPV 11 VLPs can be produced much more efficiently compared to HPV 16 VLPs leading to the speculation whether this might be due to intrinsic properties of the L1 protein of different PV types[7,8,9]. After a report by Kirnbauer and colleagues it became clear, however, that the HPV 16 L1 gene of the original isolate by Dürst et al. harboured a point mutation rendering its encoded L1 protein virtually assembly defective[10]. The HPV 16 prototype genome, used by numerous researchers worldwide

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was derived from a tumour biopsy, in which the HPV 16 genome was integrated into the cellular genome and thus had been without any selection pressure for the maintenance of intact structural genes. When HPV 16 genomes obtained from virus-producing lesions were analyzed, the respective L1 proteins assembled with much greater efficiency into VLPs. Sequence analysis revealed that this difference was based on a single histidine to aspartic acid exchange at position 202 of the HPV 16 L1 protein. VLPs were subsequently produced successfully in insect cells, later also in yeast, in plants and even in cell free systems. Meanwhile, VLPs of several animal and human papillomaviruses have been produced, i.e. cottontail rabbit papillomavirus, rabbit oral papillomavirus, BPV 1 and 4, canine oral papillomavirus and HPV types 1, 2, 6, 8, 11, 13, 16, 18, 31, 33, 39, 45, 58 and 59[11,12,13–15], for review see[16]. The production of VLPs in larger quantities made structural and immunological studies possible.

The data obtained from several animal models fostered the concept of prophylactic vaccines against HPV infections. Virus-like particles proved to be prime candidates for prophylactic vaccination since they induce high--titer conformational antibodies of neutralizing capacity both in non-human primates and in man[2,17–25]. By passive transfer of IgG from immunized animals it was shown that they confer protection against experimental challenge[26].

The first clinical trial with VLPs (of HPV 11) involving human subjects was initiated in 1996 (for reference see Inglis et al.[27]). HPV 11 VLPs used in this study were produced in insect cells. The success of this trial initiated further HPV vaccine development. In several phase I clinical trials safety and immunogenicity of HPV 11, 16 and 18-specific VLPs generated by expression of the L1 protein in yeast or in insect cells were demonstrated[17,19,20–22]. Three VLP-based vaccines (HPV 16, HPV 16 + 18, HPV 6 + 11 + 16 + 18) were brought into clinical efficacy trials. The so far only licensed vaccine provided by Merck & Co (Gardasil™) contains in addition to the most relevant cancer-associated HPV types 16 and 18 also VLPs of the low-risk HPV types 6 and 11 aiming for protection against genital warts whereas the product developed by GlaxoSmithKline has only the two high-risk HPVs. The data obtained from immunization of about 25,000 young women receiving either vaccine or placebo demonstrated after more than 4 years of follow-up protection against incident and persistent infection with HPV 6/11, and/or HPV 16 an 18 and the lesions associated therewith (external genital lesions, high grade CIN, adenocarcinoma in situ)[28–32]. Longer follow-up and large scale population-based efficacy trials will be required to evaluate the potential of these vaccines to reduce the incidence of cervical cancer[33,34]. Based on the existing data, however, the regulatory agencies in different countries have approved. It will be more difficult to evaluate the efficacy towards genital warts since the information about the incidence of this disease is rather scarce (http://wrongdiagnosis.com/g/genital warts). The second product manufactured by GlaxoSmithKline (consisting of HPV 16 + HPV 18 VLPs) is expected to enter the market soon.

After introduction of the vaccines there are several open questions that can be solved only after wide application in different countries and longer time of follow-up. The critical issues include the acceptance of the HPV--specific vaccines within different cultures[35,36] including the question whether the vaccine will be marketed as preventive measures against a sexually transmitted disease or as anti-cancer prophylaxis[37]. Other issues are the duration of immune protection and the participation of vaccinated women in Pap-screening programs since HPV 16 and 18 accounts for about 70% of cervical cancer cases[38] hence the risk for this disease by other high-risk HPV types is not negligible. Therefore, vaccinated women need to be educated about the necessity to keep up with the screening program although the intervals of the visits can possibly be extended[39]. Obviously prophylactic vaccination will be highly desirable in areas where screening programs are not offered to the general population and where cervical cancer is a major public health problem. The high price clearly is prohibitive and joint efforts of scientists (developing more cost effective protocols), providers and politicians (discussing multi-tiered pricing) and public and philanthropic foundations (providing large amounts of money) are needed to make the vaccines available to the populations of highest need.

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RAZVOJ PROFILAKTIČKOG CJEPIVA PROTIV HPV-a

S A Ž E T A K

Od prije nekoliko godina je prihvaćena tvrdnja da trajna infekcija s određenim (tzv. visokorizičnim; HR od engl. high risk) tipovima humanog papilomavirusa (HPV) predstavlja jaki faktor rizika za razvoj raka vrata maternice. Najčešći HR HPV-i su tipovi 16 i 18, povezani s oko 70% ovog tumora, koji je na drugom mjestu najčešćih oboljenja kod žena u svijetu. Nekoliko studija na modelima animalnih papilomavirusa je pokazalo da je zaštita protiv ove infekcije neutralizirajučih antitijela protiv konformacijskih epitopa glavnog strukturnog proteina L1. Ta antitijela mogu biti vrlo učinkovito inducirana imunizacijom sa česticama sličnim virusima (VLP, engl. Virus-Like Particles) koje se spontano nakupljaju nakon ekspresije L1 u rekombinantnim vektorima. Velika skala proizvodnje VLP-ova od strane HPV 16 i 18 se pokazala uspješnom, olakšavajući tako, prije nekoliko godina, prve kliničke pokuse o sigurnosti i imunogenosti. U međuvremenu, više od 25.000 žena je bilo uključeno u nekoliko kliničkih pokusa o zaštiti protiv trajne infekcije tipovima HPV-a 16 i 18 te o sprječavanju razvoja oštećenja koja prethode raku vrata maternice. Iako je krajnji dokaz uspjeha smanjenje stope pojavnosti raka, potrebno je još cijepiti velike skupine te ih godinama pratiti. Ipak, sadašnji podaci su toliko uvjerljivi da su zadužene agencije u nekoliko zemalja dopustile uvođenje prvog cjepiva protiv HPV-a u 2006. g. Neka pitanja, kao što je trajanje zaštite, potreba za razvojem strategija nakon cijepljenja i dostupnost ovog cjepiva u siromašnijim zemljama, su još otvorena za diskusiju.

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Review

Vaccine Regulations in Croatia

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A B S T R A C T

In this paper legal prerequisites for vaccine licensure in Croatia are discussed. The Croatian legislation concerning vaccine licensing, marketing authorisation and utilization is reviewed. The procedures for including a vaccine into the Mandatory Childhood Vaccination Programme are also discussed with focus on Human papillomavirus (HPV) vaccines. Non-obligatory vaccination recommendations are given when according to professional opinion; vaccination is beneficial for the vaccinee. There is little doubt that HPV vaccines should be recommended for preadolescent girls in Croatia. However, reaching a decision on its possible introduction into the Childhood Vaccination Programme will require careful consideration of the larger picture and a comparison of the cost-effectiveness of a mandatory vaccination against other competing public health priorities.

Key words: *vaccination, regulations*

Introduction

The first prerequisite for a vaccine to be legally used in Croatia is licensure. The Agency for Medicinal Products and Medical Devices (AMPMD) is responsible for the licensing procedure and for marketing authorization of vaccines[1]. However, it is very important to understand that licensing and marketing authorization only guarantee the quality, safety and efficacy of vaccines but this does not determine the way a vaccine will be used.

Each lot of vaccine regardless of the manufacturer is subject to quality control performed by the AMPMD, Division for Immunological Medicinal Products. Further, the AMPMD undertakes a variety of other tasks including postmarketing surveillance and this is done in collaboration with the Croatian National Institute of Public Health which is responsible for surveillance of adverse events following immunization. Based on this surveillance, the AMPMD can request that the manufacturer review and change the summary of product characteristics, as well as the prescribing information.

The way a vaccine will be used depends on a number of epidemiological, regulatory and financial factors. Vaccines may be mandatory for the entire eligible population such as specific birth cohorts according to the Childhood Vaccination Programme[2], or for specific at-risk groups such as hepatitis B vaccination for health care workers. Then, there are vaccines that are recommended for specific groups such as typhoid fever vaccination for travellers to endemic areas and influenza vaccination for the elderly, health care workers and chronically-ill patients.

Mandatory Vaccinations

Each year, the Minister of Health announces the Childhood Vaccination Programme (»the Programme«) based on the recommendation from the Croatian Institute of Public Health (CIPH). The declaration of mandatory vaccination or the provision of free-of-charge vaccines is regulated by health care legislation, primarily the Act on the Protection of the Population from Infectious Diseases, which is further elaborated by the implementing provisions that are issued by the Minister of Health[3,4]. Mandatory vaccinations that are covered by

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the Childhood Vaccination Programme are obligatory for all in the defined target population because it is in the interest of the community to have all its members / citizens vaccinated, as well as being in the interest of the individual vaccinee. All mandatory vaccinations according to the Programme are free of charge for the vaccinee, because the vaccines are purchased and vaccination is reimbursed to primary health care physicians by the Croatian Health Insurance Institute. The current Childhood Vaccination Programme is presented in Table 1.

TABLE 1
CHILDHOOD VACCINATION PROGRAMME IN CROATIA FOR YEAR 2007

Age	Vaccine
Birth*	BCG, Hepatitis B
2 months	Hib, Hepatitis B
3 months	DTP, IPV
4 months	DTP, OPV, Hib
6 months	DTP, OPV, Hib, Hepatitis B
12–18 months	MMR, DTP, OPV, Hib
3 years	DTP
6 years	MMR, Td, OPV
12 years	Hepatitis B **
13 years	PPD, BCG***
14 years	Td, OPV
19 years	TT
60 years	TT

BCG – Bacillus Calmette-Guerin, Hib – Haemophilus influenzae type B, DTP – Diphtheria, tetanus, pertusis, IPV – Inactivated polio vaccine, OPV – Oral polio vaccine, MMR – Measles, mumps, rubella, PPD – Purified protein derivative testing, Td – Tetanus, diphtheria, adult formulation, TT – Tetanus toxoid, * Newborns to HBsAg positive mothers receive HBIG at birth together with the first dose of hepatitis B vaccine, which is continued according to a 0, 1, 2, 12 scheme, ** Three doses of hepatitis B vaccine according to a 0, 1, 6 scheme. Vaccination at the age of 12 will be discontinued when children vaccinated as newborns reach this age, *** BCG revaccination is administered only to PPD negative children

Mandatory vaccination carries with it the responsibility of the government to secure safe and effective vaccines and to organise the health care system in such a way that it makes vaccination accessible to all within the designated target population. This is also regulated by law, primarily the Health Care Act, the Act on the Protection of the Population from Infectious Diseases (noted above), as well as various bylaws and ordinances arising from these acts[5]. According to these documents, it is the duty of primary health care providers to vaccinate preschool children according to the Programme. Newborns are vaccinated in the maternity wards, while elementary school pupils are vaccinated at school by the school medical service that is affiliated to the County Institutes of Public Health.

As you will see, mandatory vaccination involves an enormous commitment on the part of the community, institutions and individuals so the decision to include a vaccine into the Programme or not must be based on solid scientific evidence and the highest professional expertise.

The Croatian Institute of Public Health (CIPH) is the institution responsible for designing the Childhood Vaccination Programme in Croatia. In collaboration with the relevant partners (representatives of paediatric associations, school health associations, epidemiologist associations, family doctors, etc.), the CIPH analyses the epidemiological situation regarding vaccine-preventable diseases, the performance of the proposed vaccination programme and all suggestions relevant to the Programme to develop and present a recommendation to the Minister. In certain instances, public opinion is taken into account in the decision-making process even if it is at odds with the scientific evidence and professional knowledge.

There are also a number of vaccinations which are mandatory for specific groups, e.g. hepatitis B (HBV) vaccination for health-care workers and for sexual partners of HBV carriers, then prophylaxis for persons exposed to rabies; these vaccines are free of charge for individuals and reimbursed by the Croatian Health Insurance Institute.

Recommended Vaccination

Non-obligatory vaccination recommendations are those considered to be of interest for the individual but not to the whole community, and these can be either paid for by public Health Insurance or the individual. For example, influenza vaccination for health-care workers, the elderly and chronically-ill patients, pneumococcal vaccination for asplenic patients and the elderly in nursery homes, etc. are examples of vaccinations that are recommended and paid for by public Health Insurance. Then, all vaccinations recommended to travellers to prevent diseases that are endemic in the countries they are going to such as cholera, meningococcal meningitis, yellow fever or hepatitis A are paid for by the vaccinee. The same applies to tick-borne encephalitis vaccine, which is recommended for persons exposed to ticks in endemic areas, either professionally or recreationally.

HPV Vaccination

As far as Human papillomavirus (HPV) vaccine is concerned, as soon as the vaccine is licensed and the batch control is finished, the vaccine will receive marketing authorization and can be used in Croatia. Then, recommendations for the use of vaccination will be issued by professional medical associations, e.g. gynaecology, paediatrics or epidemiology associations, or other non-governmental associations. Whether it will be reimbursed or not depends on the Health Insurance Institute. Any of the associations which issue recommendations can suggest the reimbursement of vaccination by the Health Insurance Institute or request the Ministry of Health to initiate the process for covering expenses by the Health

Insurance Institute. In both cases, the Health Insurance Institute establishes a committee of the relevant medical experts and eventually decides whether the Health Insurance Institute will cover the expenses or not.

The vaccination strategy that is anticipated to have the greatest impact on the incidence of cervical carcinoma in the future would involve at least universal vaccination of preadolescent girls[6]. In Croatia, this means introducing the vaccine into the mandatory Childhood Vaccination Programme.

Although the public health burden of cervical carcinoma in Croatia is well-known, together with the general epidemiology of HPV infection and the general characteristics of available vaccines, there are still uncertainties which must be clarified before considering the introduction of HPV vaccine into the Programme.

The reduction of precancerous and invasive cervical lesions achieved by vaccination with reasonably high coverage rates is anticipated to be substantial, but not enough to eliminate the need for cervical screening[6,7]. In order to calculate how substantial this reduction of precancerous lesions and cancer will be, a systematic review of available information on the prevalence of HPV types in our population is necessary. Then, this will need to be compared to the impact that could be achieved through improvements to the cervical screening system. Nevertheless, the potential of HPV vaccines to prevent precancerous lesions caused by HPV types included in the vaccine would appear to favour vaccine introduction regardless of the reductions in cancer that could be achieved by improvements to screening.

A characteristic of the HPV vaccine that could be an obstacle to the wide acceptance of the vaccine in public as well as in the professional community is the fact that a significant impact on the burden of disease can not be expected within 20–30 years of the introduction of the vaccination into the Programme, provided always that high coverage rates would be achieved and maintained continuously. And finally, the price of introducing this vaccine into the Programme is an important issue. In case it would be established that vaccination would achieve better results in reducing cervical cancerous lesions than alternative interventions (e.g. enhanced screening), the cost-effectiveness would have to be assessed against other interventions in the health care system, waiting to be implemented or improved. Since the health budget is a single source, money for screening programmes, paper towels or antibiotics all comes from the same source as the funds for vaccination, there are numerous needs competing with each other for the funds.

So far, the introduction of vaccines into the Programme was never limited by the health care budget, mostly due to the fact that it was clear the vaccines (at their given prices) were the most cost-effective interventions. However, it is expected that one dose of HPV vaccine would cost about 100 Euros and therefore, introducing this vaccine into the Programme to vaccinate only one birth cohort of girls (e.g. 11-year-old girls) would raise the annual cost of the Programme by 130% (the HPV vaccine would cost about 6.7 million Euros which is 30% more than all the other vaccines used in the Programme at the moment 5.1 million Euros[8]).

Conclusion

The HPV vaccine will soon be licensed in Croatia and will have an important role in the prevention of cervical cancer in this country. Immediately after licensure physicians will be able to use it on individual, patient-pay basis. In order to ensure reimbursement of the vaccine by the Croatian Health Insurance Institute, a request would need to be issued and an expert committee would then decide if the request should be accepted.

Regardless of the reimbursement policy for recommended vaccinations, no effect on the public health burden of cervical cancer can be expected unless vaccination is implemented to the whole target population within a well designed vaccination programme. This could be achieved by introducing HPV vaccination into the mandatory Childhood Vaccination Programme.

Still, much information must be gathered in order to assess the potential public health benefit of this vaccine in Croatia (type specific epidemiology of HPV in Croatia, potential of improved screening for cervical cancer in Croatia, etc.). From the perspective of an institution responsible for the introduction of new vaccines into the Childhood Vaccination Programme and the allocation of considerable amounts of public funding into one specific intervention, we believe that these associations advocating introduction of a new vaccine should make every effort to demonstrate this is the best available option.

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ZAKONSKA REGULATIVA VEZANO UZ CJEPIVA U HRVATSKOJ

S A Ž E T A K

U ovom članku raspravlja se o legislativi koja mora biti udovoljena da bi se cjepiva koristila u Hrvatskoj. Korišteni su zakoni i pravilnici kojima se regulira registracija, puštanje lijeka u promet, te način korištenja cjepiva. Postupci za uključivanje cjepiva u Program obveznih cijepljenja su također prodiskutirani, s naglaskom na cjepivo protiv humanog papilomavirusa (HPV). Preporuke za cijepljenja koja nisu obvezna se izdaju na temelju stručnog stava da je cijepljenje korisno za pojedinca. Nema sumnje da se cijepljenje protiv HPV-a može preporučiti djevojčicama u preadolescentnoj dobi. Međutim, da bi se donijela odluka o uvođenju cjepiva u Program obveznih cijepljenja potrebno je sa različitih medicinskih stajališta razmotriti uvođenje cijepljenja u odnosu na druge javnozdravstvene prioritete koji se nadmeću za ista sredstva.

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Early Sexual Intercourse and Risk Factors in Croatian Adolescents

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A B S T R A C T

Sexual behaviour in adolescence is a sensitive issue and has possible immediate and long term medical and psychical consequences. The aim of the study was to examine whether early sexual intercourse varies by gender and how is associated with unhealthy behaviour and factors of psycho-social well-being. 773 boys and 857 girls of 15.5 years old, included in a representative national school-based survey, conducted in Croatia in 2006, were invited to fill in anonymous questionnaires. Sexual experience before the age of 16 years was reported by 28.6% of the boys and 16.5% of the girls. Early sexual experience in boys was associated with smoking, drinking of alcohol, marijuana taking, physical fighting, and bullying other. The odds ratio was highest for smoking. (OR:8.1; CI:5.4–12.1). For girls the same variables were associated with the early sexual intercourse, marijuana use being the strongest independent predictor (OR:8.0; CI:5.0–12.6). While controlled for other behaviours, daily smoking remained the strongest predictor for both genders. Girls who had early sexual experience were more prone to be dissatisfied with their health (OR:2.9; CI:2.0–4.2), with their life (OR:2.1; CI:1.4–3.0), communication with father and mother (OR:1.9; CI:1.2–2.8 and OR:1.7; CI:1.1–2.6) and reported more psychosomatic symptoms (OR:2.9; CI:2.0–4.3). For both genders odds were higher if they had good communication with the friend of the opposite gender. Evenings spent out with friends were associated to early sexual experience in boys and girls as well as poorer school achievement. Early menarche was associated with the probability of being engaged in the early sexual intercourse and with smoking, marijuana use and psychosomatic symptoms. Early sexual intercourse is associated with unhealthy behaviour such as smoking, substance abuse, aggressiveness and lower psychosocial well-being. Preventive educational programmes should follow multi-facet approaches and recognize differences between boys and girls. Human papillomavirus (HPV) vaccination could be part of a comprehensive approach and is not to be viewed as an isolated activity.

Key words: *early sexual intercourse, risk factors, menarche, Croatia*

Introduction

Human sexuality includes complexity of physical characteristics and capacities for specific sex behaviours, determined by psychosocial values, norms, attitudes, and contextual factors that influence these behaviours. The major risks associated with teenage sexual behaviour include pregnancy, with physical and psychological consequences; cervical dysplasia, in which early onset of sexual activity is an important risk factor; and sexually transmitted disease, where rates, when adjusted for sexual activity, are greater for adolescents than for any other group[1]. Adolescent sexuality is a sensitive issue, teenage sexual health outgrows the previously mentioned health risks and the factors influencing risk sexual behaviour, particularly sexual intercourse and its consequences are the matter of the permanent adults' concern[2]. Sexual behaviour in adolescence can pose the immediate health risk but is also an important factor of reproductive health in later life[3]. Major concerns related to teenage sexual activity as listed above are far more important from female prospective. However, sexual behaviour of teenage boys shouldn't be neglect, as teenage boys are not only more susceptible to initiating intercourse[4], but also are becoming fully sexually active at a younger age than the girls[5,6,7], and are taking risks in doing so[8].

Different researchers show that sexual activity in adolescents is not an isolated experience and is connected to other risk behaviours, leading to variety of short term and long term consequences. Thus, it was shown that alcohol misuse and use of other substances may place teen-

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agers at greater risk of initiating early and unprotected sexual intercourse[9,10]. In addition to early (< age 13) experimentation with cigarettes and alcohol, and being involved in fighting was associated with early initiation of sexual activity independent of race or gender[11]. In other study health compromising behaviours (early initiation of sexual intercourse, cigarette smoking, alcohol and marijuana use, involvement in violence and delinquency) clustered among young people with associations being stronger for females[12]. That having tried smoking was associated with early sexual activity in both genders, while having tried drinking was associated with early sexual activity only for girls, was found for Canadian students[13].

Sexual behaviour of the young person is not only reflection of his or her personal characteristics, but is also determined by the broader surroundings in which young person lives, emphasising importance of family, peers, and school[14]. It was argued that decisions about initiation were strongly bound to social context with peers playing an important role in creating a sense of normative behavior[15]. Furthermore, among identified predictors for intimate sexual behaviours were time alone with groups of peers, and time alone with a member of the opposite sex[16]. Earlier age at first intercourse and deviant activities of peers each predicted a significantly higher risk of subsequently developing of substance use disorders[17]. Identified protective factors for early initiation of sexual activity included family and school connectedness[18]. The adolescents' relationship with their mothers was underlined, as well[19]. Reporting high academic achievement was significantly correlated with virginity and appeared to be protective for boys[20]. In another study, higher school achievement was associated with not having sexual intercourse for both sexes[21]. Several studies focused on early maturation in girls, showing that early menarche was associated to early initiation of the sexual intercourse[22,23,13]. Mellanby found that teenagers who start with sexual activity before the age of 16 take more risks than those do who wait until after that age[24].

Although sexual risk-taking behaviours during adolescence embrace different risks as well (multiple partners, unprotected intercourse, intercourse under the influence of drugs[6]), in this article we concentrated on early onset of sexual activity and associated factors. The early onset of sexual activity was defined as having had the sexual intercourse at the age of 16 or earlier. In Croatia, according to available data, the mean age of first sexual intercourse has been 17[5,25,26], and at the age before 16 minority of young people has been found as sexually active (9.7% of girls, and 23.2% of boys)[27].

The aim of the study was to examine whether selected risk factors are gender specific for the early sexual intercourse and to emphasize the implications for prevention.

Subjects, Material and Methods

Study Sample

In the article the results of the Health Behaviour in School-aged Children survey carried out in Croatia in 2006 were used. The survey involved students aged 11.5, 13.5 and 15.5 years, which in Croatia means fifth and seventh grades in the primary and the first grades in the secondary schools. In total 4,968 of the respondents were embraced, out of the 2,442 boys and 2,526 girls. In the sampling procedure the sampling unit was class, using the list of classes at the national level classes were randomly selected. For the first grades of secondary schools stratification was done, so to preserve the structure of the secondary schooling (gymnasiums, 4-year technical and 3-year vocational schools). For this specific article only the answers from the 15 years old students were used, as only for the questions about sexual experience were asked. The used sub-sample consists of 1,601 students, 773 boys and 857 girls.

Data collection

Participating students completed anonymous, structured questionnaire in a regularly scheduled classroom period under the supervision of a school counsellor. The participation was voluntary, confidentiality guaranteed completely. All filled-in questionnaires were put in the sealed envelopes, packed together and delivered to the Croatian National Institute of Public Health.

Measurement Instrument

The questionnaire had 53 questions in total. For this analysis 17 questions were used. The sexual experience was assessed as the »yes« answer to the question »Have you ever had sexual intercourse«. For the girls additional question about menstruation and the age of the menarche was asked. As the other risk behaviours the experimentation with the other substances and aggressive behaviour was assessed. For smoking the measurement was daily smoking, risky drinking was rated as drinking any sort of alcoholic beverage every week and drunkenness as being drunk twice or more. Marijuana use was measured as any use in the lifetime. Being involved in physical fight in the past 12 months, being bullied or bullied others in the past couple of months was rated as »no« or »yes« regardless the frequency in the respected period. Life satisfaction measured at the Cantril ladder was rated as positive if scored 6 or more. Satisfaction with own health measured at the 4-point scale was rated as satisfying for the »excellent« and »very good categories«. Psychosomatic symptoms were assessed using 5 point scale for 8 symptoms (headache, stomach-ache, back-ache, feeling low, being irritated or in bad temper, being nervous, difficulties in getting to sleep and dizziness), as two or more symptoms being present more that once a week. Communication was rated using 5 point scale and answers to question »How easy is for you to talk about the things that really bother you« to mother, father, best friend, friend of the same/opposite gender, and answers »easy/very easy« were used as a good communication measure. Number of close friends was subdivided as having up to three and three or more close friends. Number of evenings spent out with friends was used as a mean value of the evenings in the week. School environment

was assessed using answers to the question »How do you feel about school« and likeness as answers »I like it a lot/a bit«. The academic achievement was measured as answer to the question »In your opinion, what does your class teacher think about you school performance compared to your classmates«, at the four-point scale and the answers »very good/good« were rated as good school achievement.

Statistical analysis

Binary logistic regression analysis was used to produce unadjusted and adjusted odds ratios with 95% confidence intervals and asymptotic, two-sided, statistical significance, which indicate the likelihood of having early sexual experience for boys and girls who reported other risk behaviours like smoking, drinking, etc. Through the article statistical significance was defined by the conventional level of $p<0.05$. Two-by-two contingency tables were analysed by Fisher's Exact test. Three by two and larger contingency tables like in analysis of prevalence of early sexual experience by the age of first menarche were analysed by χ^2 test with Monte Carlo testing of statistical significance. Correlation of early sexual experience and the average number of evenings spent with friends were analysed by Man-Whitney U test and Monte Carlo test of statistical significance. Homogeneity of variance was assessed by Levene's test. Association of ordinal variables like academic achievement and nominal variables like gender, when ordinal was treated as dependent, was analysed by Somers' d test. All the analyses were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) statistical software package.

Results

Early sexual experience reported 28.6% of the boys and 16.5% of the girls with statistically significant gender difference (OR:0.5, CI:.3–0.6). Boys were more often engaged in the experimentation with alcohol and in the aggressive behaviour. Although girls smoke more and

TABLE 1
EARLY SEXUAL EXPERIENCE, RISK BEHAVIOUR, SATISFACTION WITH HEALTH, FAMILY, FRIENDS AND SCHOOL DETERMINANTS BY GENDER

	Boys N (%)		Girls N (%)		$OR_{girls\ versus\ boys}$ (95% CI) p*
Total	744	(100.0)	852	(100.0)	
Early sexual experience (< 16 years)	213	(28.6)	141	(16.5)	0.5 (0.3–0.6) **
Other risk behaviour					
Smoking daily	138	(18.5)	181	(21.2)	1.2 (0.9–1.5)
Drinking weekly	324	(43.4)	236	(27.7)	0.5 (0.4–0.6) **
Been drunk two times or more	354	(47.5)	247	(29.0)	0.5 (0.4–0.6)**
Marijuana in the lifetime	124	(16.7)	94	(11.1)	0.6 (0.5–0.8)*
Physical fight in the last year	385	(51.7)	183	(21.5)	0.3 (0.2–0.3)**
Being bullied in the last couple of months	109	(14.7)	104	(12.3)	0.8 (0.6–1.1)
Bullied others in the last couple of months	221	(29.7)	124	(14.6)	0.4 (0.3–0.5) **
Health					
Satisfied with own health	666	(89.5)	644	(75.8)	0.4 (0.3–0.5)**
Satisfied with life	604	(81.4)	604	(70.9)	0.6 (0.4–0.7) **
Psychosomatic symptoms	185	(25.5)	388	(46.0)	2.5 (2.0–3.1)**
Family					
Easy talk to father	456	(68.3)	376	(48.5)	0.4 (0.4–0.5)**
Easy talk to mother	585	(81.9)	669	(81.3)	1.0 (0.7–1.2)
Friends					
Three or more close male friends	644	(86.7)	444	(52.2)	0.2 (0.1–0.2)**
Three or more close female friends	515	(70.2)	620	(73.1)	1.2 (0.9–1.4)
Easy talk to the best friend	572	(85.4)	786	(94.9)	3.2 (2.2–4.7) **
Easy talk to the friend of the opposite gender	445	(66.7)	491	(60.5)	0.8 (0.6–0.9)
Easy talk to the friend of the same gender	525	(77.5)	731	(88.6)	2.3 (1.7–3.0)**
Four or more evenings spent out with friends	209	(28.1)	184	(21.6)	0.7 (0.6–0.9)
School					
Liking school	345	(46.2)	428	(49.8)	1.1 (1.0–1.4)
Good academic achievement	537	(72.0)	627	(73.7)	1.1 (0.9–1.4)

OR – odds ratio for girls; CI – confidence interval; p – asymptotic 2-sided statistical significance, ** $p<0.001$, *$p<0.05$

boys have more experience with marijuana, the differences are not statistically significant (Table 1).

Girls rated their life satisfaction lower (OR:0.6, CI: 0.4–0.7), were less satisfied with their health (OR:0.4, CI:0.3–0.5), and were more inclined to report psychosomatic symptoms (two or more symptoms more than once a week; OR:2.5, CI:2.0–3.1). Girls expressed more difficulties in talking to the father (OR:0.4, CI:0.4–0.5). Although girls as well as boys had more friends of the same gender, the probability for girls to have better communication to the best friend was three times as high as for the boys (OR:3.2, CI:2.2–4.7).

Risk behaviours

Independent associations of other risk behaviours with the early onset of sexual life were assessed by binary logistic regression for the boys and for the girls separately. Odds of having early sexual experience were in boys statistically significantly higher in case of smoking, drinking, marijuana taking, and engagement in the physical fight and bullying others, while no statistically significant independent influence was noticed for being bullied (Table 2). The highest probability, independent from the other included indicators, was detected for smoking. Odds to have an early sexual experience are eight times higher (OR: 8.1, CI: 5.4–12.1) for daily smokers than for the pupils who smoke less often or who do not smoke at all. On this particular sample level, smoking is followed by marijuana use and weekly drinking. Boys who have engaged into such activity have four times larger odds (OR:4.8 and 4.6, CI:3.2–7.2 and 3.2–6.5) for having had an early sexual experience than boys who didn't report these behaviours.

Multivariate analysis revealed that even controlled for the other risk behaviour daily smoking remains the most influencing factor – boys who were daily smokers had three times higher probability to have early sexual experience (OR:3.8; CI:2.4–6.1). Next to daily smoking is weekly drinking followed by the involvement in the physical fight in the last year. Getting drunk and taking marijuana in the lifetime had no significant influence on odds of having early sexual intercourse while other behaviours have been taken into account.

Girls who used marijuana had eight times higher probability to experience early sexual intercourse than their peers who did not try marijuana at the time of being questioned (OR:8.0; CI:5.0–12.6). In the independent analysis girls who were daily smokers had 7.9 times higher probability of being engaged in the early sexual intercourse, and higher probability if they reported drunkenness, weekly drinking, involvement in the physical fight or bullying others (5.5 time, 3.9 times, 3.2 times and 2.5 times respectively). Being bullied in the past couple of months did not show statistically significant influence on the early sexual behaviour (Table 3). While controlled for other behaviours, daily smoking had the strongest influence to odds of early sexual experience – girls who smoke every day had three times higher probability for having had early sexual intercourse (OR:3.6; CI:2.2–5.8). The probability was higher for drunkenness and marijuana use (2.2 and 2.0 times)

Psychosocial factors

The probability for the early sexual intercourse was higher for those boys with lower academic achievement (OR:2.2; CI:1.6–3.1), for those who had more female friends (OR:1.5; CI:1.0–2.1) and had better communica-

TABLE 2
ASSOCIATION BETWEEN EARLY SEXUAL EXPERIENCE AND OTHER RISK BEHAVIOR IN BOYS

		Early sexual experience N (%) of pupils				OR (95% CI) p*	
		no		yes		Univariate	Multivariate
Smoking daily	no	487	(80.1)	121	(19.9)	1	1
	yes	46	(33.3)	92	(66.7)	8.1 (5.4–12.1) **	3.8 (2.4–6.1) **
Drinking weekly	no	357	(84.6)	65	(15.4)	1	1
	yes	176	(54.3)	148	(45.7)	4.6 (3.2–6.5) **1	2.6 (1.7–4.0) **
Been drunk two times or more	no	326	(83.4)	65	(16.6)	1	1
	yes	207	(58.5)	147	(41.5)	3.6 (2.5–5.0) **	1.1 (0.7–1.7)
Marijuana in the lifetime	no	480	(77.7)	138	(22.3)	1	1
	yes	52	(41.9)	72	(58.1)	4.8 (3.2–7.2)**	1.8 (1.1–2.9) *
Physical fight in the last year	no	303	(84.4)	56	(15.6)	1	1
	yes	228	(59.2)	157	(40.8)	3.7 (2.6–5.2)**	2.5 (1.6–3.6)**
Being bullied in the past couple of months	no	456	(72.4)	174	(27.6)	1	
	yes	73	(67.0)	36	(33.0)	1.3 (0.8–2.0)	
Bullied others in the past couple of months	no	399	(76.3)	124	(23.7)	1	1
	yes	132	(59.7)	89	(40.3)	2.1 (1.6–3.0) **	1.9 (1.3–2.7) *

OR – odds ratio for girls, CI – confidence interval, p – asymptotic 2-sided statistical significance, ** $p<0.001$, *$p<0.05$

TABLE 3
EARLY SEXUAL EXPERIENCE IN GIRLS REPORTING OTHER RISK BEHAVIOR

		Early sexual experience N (%) of pupils				OR (95% CI) p*	
		no		yes		Univariate	Multivariate
Smoking daily	no	610	(90.9)	61	(9.1)	1	1
	yes	101	(55.8)	80	(44.2)	7.9 (5.3–11.8) **	3.6 (2.2–5.8) **
Drinking weekly	no	550	(89.3)	66	(10.7)	1	1
	yes	161	(68.2)	75	(31.8)	3.9 (2.7–5.6)**	1.5 (0.9–2.4) *
Been drunk two times or more	no	551	(91.1)	54	(8.9)	1	1
	yes	160	(64.8)	87	(35.2)	5.5 (3.8–8.1) **	2.2 (1.4–3.6)*
Marijuana in the lifetime	no	664	(87.9)	91	(12.1)	1	1
	yes	45	(47.9)	49	(52.1)	8.0 (5.0–12.6) **	2.0 (1.1–3.6) *
Physical fight in the last year	no	584	(87.6)	83	(12.4)	1	1
	yes	126	(68.9)	57	(31.1)	3.2 (2.2–4.7) **	1.4 (0.9–2.3)
Being bullied in the past couple of months	no	626	(84.4)	116	(15.6)	1	
	yes	81	(77.9)	23	(22.1)	1.5 (0.9–2.5)	
Bullied others in the past couple of months	no	623	(85.7)	104	(14.3)	1	1
	yes	87	(70.2)	37	(29.8)	2.5 (1.6–3.9) **	1.6 (0.9–2.7)

OR – odds ratio for girls, CI – confidence interval, p – asymptotic 2-sided statistical significance, ** $p<0.001$, *$p<0.05$

tion with the friend of the opposite gender (OR:2.1; CI:1.4–3.1). For the boys who liked school more the probability of being engaged in early sexual intercourse was lower (OR:0.7; CI:0.5–1.0) (Table 4). The same variables remained statistically significant while controlled in multivariate analysis, the easy talk to friend of the opposite gender being the strongest predictor (OR2.1; CI:1.4–3.2).

The independent analysis revealed that girls who had early sexual experience were more prone to be dissatisfied with their health (OR:2.9; CI:2.0–4.2), with their life (OR:2.1; CI:1.4–3.0) and reported more psychosomatic symptoms (OR:2.9; CI:2.0–4.2). For the girls who had poorer communication with father and mother the probability of having had early sexual intercourse was higher (OR:1.9; CI:1.2–2.8 and OR:1.7; CI:1.1–2.6), as well as for those with lower academic achievement (OR:2.4; CI: 1.6–3.5). Girls who had good communication with friend of the opposite gender had higher probability of being engaged in the early sexual experience (OR:3.0; CI:1.9–4.7) (Table 5).

While controlled for the other psychosocial factors, communication to friend of the opposite gender was the strongest predictor (OR:2.7; CI:1.6–4.4), and other factors increasing probability for early sexual intercourse were poor academic achievement (OR:2.1; CI:1.4–3.4), health dissatisfaction (OR:1.9; CI:1.1–3.1) and psychosomatic symptoms (OR:1.7; CI:1.1–2.8).

In analysing the evenings spent out with friends and early sexual experience the mean values were used, while the gender differences in going out were too high for determine exact common cut-offs. Mean number of evenings spent out with friend was 2.82 for boys and 2.48 for girls, with statistically significant gender difference ($p<0.001$).

As Levene's test indicated statistically significantly heterogeneous variances, mean number of nights was analysed by non-parametric Man-Whitney U test and Monte Carlo test of statistical significance. Mean number of nights spent with friends was statistically significantly higher for students who had early sexual experience ($p<0.001$) (Table 6). When evenings spent out with friends were treated as interval scale variable, the odds for both genders were higher for those students who spent out more evenings (boys: OR:1.3; CI:1.3–1.4; girls OR:1.3; CI:1.2–1.5).

No statistically significant confounding effect of gender on early sexual experience correlation with number of evenings spent with friends was found. This research has not found the indication that the relationship of number of evenings spent with friends and early sexual experience are affected by gender.

Maturity and menarche

Odds that 15 years old girls who had the first menstruation on time (Table 7) will engage in early sexual intercourse are 50% (OR:0.5; CI: 0.3–0.9) less than the odds for the same behaviour for girls who had the first menstruation early that is at the age of 11 or earlier. These with late first menstruation have 70% smaller odds (OR:0.3; CI: 0.2–0.6) for early sexual experience than these with the early first menstruation.

For the girls who had their first menstruation early (≤11 yrs.) marijuana trial (OR:6.4;CI:1.5–28.4) and daily smoking (OR:3.8; CI:1.2–12.2) were statistically significant predictors of early sexual experience. For the girls who had their first menstruation on time, the strongest predictors were daily smoking (OR:4.5; CI:2.5–8.2),

TABLE 4
EARLY SEXUAL EXPERIENCE IN BOYS ACCORDING TO PSYCHOSOCIAL FACTORS

		Early sexual experience N (%) of pupils				OR (95% CI) p*	
		no		yes		Univariate	Multivariate
Health satisfaction	yes	477	(71.6)	189	(28.4)	1	
	no	55	(70.5)	23	(29.5)	1.1 (0.6–1.8)	
Life satisfaction	high	434	(71.9)	170	(28.1)	1	
	low	95	(68.8)	43	(31.2)	1.2 (0.8–1.7)	
Psychosomatic symptoms*	no	394	(73.0)	146	(27.0)	1	
	yes	127	(68.6)	58	(31.4)	1.2 (0.9–1.8)	
Talk to father‡	easy	326	(71.5)	130	(28.5)	1	
	difficult	153	(72.2)	59	(27.8)	1.0 (0.7–1.4)	
Talk to mother	easy	416	(71.1)	169	(28.9)	1	
	difficult	94	(72.9)	35	(27.1)	0.9 (0.6–1.4)	
Talk to best friend	easy	410	(71.7)	162	(28.3)	1	
	difficult	73	(74.5)	25	(25.5)	0.9 (0.5–1.4)	
Talk to friend of the same gender	easy	374	(71.2)	151	(28.8)	1	
	difficult	114	(75.0)	38	(25.0)	0.8 (0.6–1.2)	
Talk to friend of the opposite gender	difficult	180	(81.1)	42	(18.9)	1	1
	easy	297	(66.7)	148	(33.3)	2.1 (1.4–3.1)**	2.1 (1.4–3.2) **
Close male friends	none to two	68	(68.7)	31	(31.3)	1	1
	three or more	465	(72.2)	179	(27.8)	0.8 (0.5–1.3)	0.8 (0.4–1.2)
Close female friends	none to two	168	(76.7)	51	(23.3)	1	1
	three or more	357	(69.3)	158	(30.7)	1.5 (1.0–2.1) *	1.5 (0.9–2.3)
Liking school	dislike	273	(68.1)	128	(31.9)	1	1
	like	260	(75.4)	85	(24.6)	0.7 (0.5–1) *	0.8 (0.5–1.1) *
Academic achievement	good	409	(76.2)	128	(23.8)	1	1
	not good	124	(59.3)	85	(40.7)	2.2 (1.6–3.1)**	2.3 (1.6–3.4)**

OR – odds ratio for girls, CI – confidence interval, p – asymptotic 2-sided statistical significance, ** p<0.001, *p<0.05, ‡ Independent variables that were not shown univariate statistically significant influence were omitted from multivariate analysis

drunkenness two times or more (OR:2.2; CI:1.2–3.9) and bullying others in the past couple of months (OR:2.1; CI:1.1–4.2). For the girls who had their first menstruation late, that is in the age of 14 or later, the strongest predictor of an early sexual experience was being drunk two times or more (OR:4.6; CI:1.3–16.7).

Regarding psychosocial factors the only statistically significant predictor for the early sexual intercourse among early menstruating girls (≤11 yrs.), was having had more psychosomatic symptoms (OR:4.4; CI:1.6–12.3). Within the sample of those with the first menstruation on time (12–13 yrs.) dissatisfaction with health (OR:2.7, CI:1.5–4.9), psychosomatic symptoms (OR:3.0, CI:1.9–4–8), and easy talk to the friend of the opposite gender (OR:3.4, CI:1.9–6.0) contributed statistically significantly to the prediction of early sexual intercourse. In the sample of girls with late onset of menstruation (14+ yrs.) statistically significant predictor of early sexual behaviour was difficult communication with father (OR:8.3, CI:1.5–47.3).

Discussion

The percentage of 15 year old students who reported to have had sexual experience had increased in Croatia between 2002 and 2006 for the boys 23.8% and for the girls 73.1%, indicating that the possibility of having an early sexual intercourse became higher for the girls[28]. The age of 15 was still for Croatia below the average age of the first sexual intercourse, which was found in majority of studies to be 17 for both genders[3,25,26], or for the boys a year earlier[5,6,7]. The prevalence of smoking, alcohol and marijuana use, physical fight and being bullied have been more frequent than in 2002, and the only variable decreased in the 4 year period was bullying others[28]. The other recent studies indicated that risk behaviours like smoking, alcohol drinking and marijuana use show quicker trends for girls than for boys, thus suggesting that girls are at a greater risk for being engaged in the clustered risk behaviour[29]. The gender differences indicated that girls were less satisfied with the health, life and more prone to the psychosomatic symptoms. In the

TABLE 5
EARLY SEXUAL EXPERIENCE IN GIRLS ACCORDING TO PSYCHOSOCIAL FACTORS

		Early sexual experience N (%) of pupils				OR (95% CI) p*	
		no		yes		Univariate	Multivariate
Health satisfaction	yes	564	(87.6)	80	(12.4)	1	1
	no	146	(70.9)	60	(29.1)	2.9 (2.0–4.2)**	1.9 (1.1–3.1)*
Life satisfaction	high	523	(86.6)	81	(13.4)	1	1
	low	188	(75.8)	60	(24.2)	2.1 (1.4–3.0)**	1.3 (0.8–2.1)
Psychosomatic symptoms*	no	410	(90.1)	45	(9.9)	1	1
	yes	294	(75.8)	94	(24.2)	2.9 (2.0–4.3)**	1.7 (1.1–2.8)*
Talk to father†	easy	333	(88.6)	43	(11.4)	1	1
	difficult	323	(80.8)	77	(19.3)	1.9 (1.2–2.8) *	1.3(0.8–2.1)
Talk to mother	easy	573	(85.7)	96	(14.3)	1	1
	difficult	120	(77.9)	34	(21.2)	1.7 (1.1–2.6)*	1.10 (0.6–1.8)
Talk to best friend	easy	657	(83.6)	129	(16.4)	1	
	difficult	37	(88.1)	5	(11.9)	0.7 (0.3–1.8)	
Talk to friend of the same gender	easy	612	(83.7)	119	(16.3)	1	
	difficult	79	(84.0)	15	(16.0)	1.0 (0.6–1.8)	
Talk to friend of the opposite gender	difficult	293	(91.6)	27	(8.4)	1	1
	easy	384	(78.2)	107	(21.8)	3.0 (1.9–4.7)**	2.7(1.6–4.4)**
Close male friends	none to two	343	(85.5)	58	(14.5)	1	
	three or more	362	(81.5)	82	(18.5)	1.3 (0.9–1.9)	
Close female friends	none to two	188	(82.5)	40	(17.5)	1	
	three or more	520	(83.9)	100	(16.1)	0.9 (0.6–1.4)	
Liking school	dislike	344	(81.1)	80	(18.9)	1	
	like	367	(85.7)	61	(14.3)	0.7 (0.5–1.0)	
Academic achievement	good	545	(86.9)	82	(13.1)	1	1
	not good	165	(73.7)	59	(26.3)	2.4 (1.6–3.5)**	2.1 (1.4–3.4)**

OR – odds ratio for girls, CI – confidence interval, p – asymptotic 2-sided statistical significance, ** p<0.001, *p<0.05, ‡ Independent variables that were not shown univariate statistically significant influence were omitted from multivariate analysis

TABLE 6
AVERAGE NUMBER OF EVENINGS SPENT OUT WITH FRIENDS WEEKLY ACCORDING TO EARLY SEXUAL EXPERIENCE

Early sexual experience	N	Mean	95% Confidence Interval for mean		Minimum	Maximum
			Lower Bound	Upper Bound		
No	1241	2.37	2.27	2.47	0	7
Yes	354	3.59	3.37	3.82	0	7

family communication girls are less satisfied with the communication to the father, which is a finding supported by other research[30] and could be considered as a risk factor for substance abuse and being bullied. Young people had more friends of the same gender, girls being more communicative and close to their best friends and to the friends of the same gender.

Dickson[31] found that many women regretted having sexual intercourse before age of 16 and that first inter-

TABLE 7
PREVALENCE OF EARLY SEXUAL EXPERIENCE BY THE AGE OF FIRST MENSTRUATION

	Early sexual experience N (%) of pupils				Total		OR (95% CI) p*
	no		yes				
Early (≤11 yrs.)	68	(72.3)	26	(27.7)	94	(100.0)	1
On time (12–13 yrs.)	467	(83.1)	95	(16.9)	562	(100.0)	0.5 (0.3–0.9) *
Late (14+ yrs.)	170	(89.5)	20	(10.5)	190	(100.0)	0.3 (0.2–0.6) **
Total	705	(83.3)	141	(16.7)	846	(100.0)	

Abbreviations: OR – odds ratio for girls; CI – confidence interval, χ^2, p=0.001, *p<0.05, ** p<0.001

course at a younger age was associated with risks that are shared equally between men and women. Coker[11] investigated the circumstances of the first sexual intercourse and later regrets, finding that at the age of 13 more black than white adolescents of both genders were sexually active, fighting, cigarette smoking and alcohol being connected with the early initiation of the sexual activity. Burack found that 20% of 13 years old reported that they already had either full or oral sexual intercourse[8]. In Swedish study 16% of the surveyed national sample reported to have had sexual intercourse before the age of 15, with more risky sexual behaviour in early than later starters (pregnancy, number of partners, oral or anal sex)[23]. In HBSC2002 Croatian adolescents were among the less sexually active 15 year old students being 34th out of 35 countries, but substantial increase in the proportion of female students already experienced early sexual intercourse indicated that the behaviour has been changing rapidly.

Risk behaviours

Daily smoking, weekly drinking of any alcohol beverage, getting drunk two times or more, taking marijuana, being involved in the physical fight and being bullied or bullied others were analysed for both genders. All variables increased odds for having had early sexual behaviour, smoking being the strongest predictor in univariate analysis for boys and marijuana use for girls. As girls in Croatia smoke generally more at that age than boys, and boys more often use marijuana the early sexual intercourse is strongly connected to the behaviour which is less socially common for the respective gender. When controlled for other behaviours, in boys daily smoking, weekly drinking and aggressive behaviour as being involved in physical fight remained statistically significant. In multivariate analysis for girls daily smoking was the strongest predictor, followed by drunkenness and marijuana use at the lower level of significance. When analysed separately, all risk behaviours were connected to early sexual experience, but in complex situation in the adolescent maturation the interception of the factors were different according to gender. Smoking, alcohol and drug misuse was found to be important for early sexual intercourse by other authors as well. Fergusson found that adolescents who reported misusing alcohol had odds of early onset sexual activity, multiple partners, and unprotected intercourse that were 6.1 to 23.0 times higher than for young people who did not misuse alcohol[9]. Alcohol and khat consumption was significantly and independently associated with risky sexual behaviour among Ethiopian youts[10] and Robinson found that for six-graders smoking was the highest predictor of engaging in sexual intercourse for all categories of race and gender[32]. Cornelius, using a prospective longitudinal study design, argued that early sexual intercourse predicts the development of substance abuse disorder[17]. Garriguet in a longitudinal study analysed characteristics of the 12 and 13 year olds, who at the age of 14 or 15 have been engaged in the sexual activity, stating that having tried smoking and drinking for the girls and smoking for the boys were significantly associated with early sexual intercourse[13]. Valois found that substance abuse and physical fighting were the strongest predictors for risk sexual behaviour for boys and girls respectively[33], and in Shrier's study students reporting they had ever used alcohol or marijuana and those reporting recent fighting were 2.7 and 1.6 times more likely to report sexual experience[27].

Health, family, friends and school

Health satisfaction, family and friend communication variables revealed that differences between genders in the respective behaviours remained significant when analysed in accordance to the early sexual intercourse. While girls were generally less satisfied with the life, health and reporting more psychosomatic symptoms, these factors in the independent analysis were found to be significant predictors for the early sexual experience for the girls, but not for the boys. In addition to that, in the univariate analysis difficult communication to father and mother and easy talk to the friend of the opposite gender were for the girls the additional factors relevant for the early sexual experience, while for the boys easy talk to the friend of the opposite gender was the only factor influencing probability for the higher odds. In multivariate analysis easy talk to the friend of the opposite gender remained the only significant factor for the both genders. Girls are in most European countries less satisfied with their life, health and reported more psychosomatic symptoms, so these variables could be considered as gender specific. Nevertheless, the feeling of dissatisfaction and psychosomatic symptoms seems to influence early sexual experience in girls. Thus, although not significant if controlled for other variables regarding communication in family and friends the gender differences and girls' susceptibility to psychosocial influences should be taken into account in planning the preventive programmes and activities. Poor communication with father had been found as a risk factor for girls' substance abuse and bullying[30,34,35]. That adolescents who were susceptible to early sexual intercourse had fewer positive connections with parents found L'Engle[4]. For girls association was found for having weak self concept (Guarriguet) and lacking a family member as a confidant (Liu)[13,36]. Boys and girls respectively who can easily talk to the friend of the opposite gender have after controlling for satisfaction, symptoms and family communication 2.1 and 2.7 times higher probability to be involved in the early sexual intercourse. The popularity self-concept was found by Dilorio as statistically significant predictor for intimate sexual behaviour[16]. The good communication with peers might reflect popularity but is also important because decisions about initiation are strongly bound to social context with peers playing an important role in creating sense of normative behaviour[15]. The number of evenings spent out with friends was the predictor of initiation of the sexual intercourse for boys and for girls respectively. Although boys go out more often, the same pattern was observed, meaning that more evenings spent

out meant higher probability of being engaged in the early intercourse, the very exact number of evenings depending on gender and on cultural norms. That time alone spent with group of peers was significantly associated with early sexual intercourse was found in Dilorio's study[16].

Liking school was not associated with the early sexual experience for girls, but boys who were engaged in the early sexual experience liked school less. Poorer academic achievement was a strong predictor of the probability for early sexual intercourse for both genders. That students engaged in the early sexual intercourse had lower school achievement was found by Kipke for the Croatian students[11], and relationships between intelligence and the coital status was demonstrated by Halpern[37].

Menarche

The early maturation poses the important health risk for female adolescent and predicts deleterious outcomes for young girls, including substance abuse, risky sexual behaviour and pregnancy[7]. Girls who mature early are more likely not to be engaged only in the early sexual intercourse, but to be exposed to other risk behaviours. In our sample marijuana tral and daily smoking were for early maturing girls the strongest predictors for early sexual intercourse, meaning that early maturation is an added risk for earlier observed risk behaviour. For the girls who had their menarche late getting drunk was strongly associated with the early sexual intercourse, which suggesting that pattern of substance abuse might be influenced by the physical maturity. Girls who had menarche on the average age were regarding satisfaction, communication with family and friends closest to the pattern in the whole sample. Early maturing girls expressed more psychosomatic symptoms which might be of a great importance for parents and professionals, because it might reflect the uncertainty and seeking for help or advice without being able to express it. In the late maturing girls difficult talk to father was the only predictor associated with the early sexual intercourse, which might reflect that poor communication has no connection to the physical maturity but to the age and stage of adolescence. In Croatian adolescents Džepina found that the average period between menarche and first sexual intercourse was four years, meaning that earlier menarche might influence earlier sexual experience[38]. That coitarche before the age of 15 is related to early menarche and that early starters reported menarche at the age of 11 or earlier more often stated Edghardh[23], and that onset of puberty was significantly associated with early sexual activity found Guarriguet[13]. It is important that health workers as well as school counsellors are sensitive to the risk associated with the early maturation among young girls adapting education and counselling related to other risk behaviour and self concept for this group. In should be taken into account that with now available Human papillomavirus (HPV) vaccine[39] one of consequences could be prevented especially when applied in girls who had not been sexually active and that with the prevention of the early onset of sexual intercourse the possibilities of medical prevention will definitely have a better compliance and outcomes.

Conclusion

Early sexual intercourse at the age of 16 or younger is associated with the complex risk and contextual factors, some of them being gender specific. Preventing early sexual intercourse means to prevent not only possible medical consequences, like adolescent pregnancy, sexual transmitted diseases or cervical dysplasia, but to prevent psychosocial consequences originated from feeling guilty, unsatisfied or regretful. Effective preventive activities need to be multi-faceted and take into consideration other components of the risk behaviour among youth. The possibility of the prevention of the long-term consequences being offered by the HPV vaccine has to be considered taking into account optimal schedule, gender differences, costs and efficiency. It was argued that the most successful vaccination programme have to be community-wide and avoid any stigma as associated with single sex vaccination. The costs may, however, restrict HPV vaccination to the girls, especially since clinical data on efficacy in boys are still being gathered[40]. Routine vaccination before sexual debut or shortly thereafter is important to achieve optimal effectiveness[41]. However, vaccination should not be isolated activity. From a public health perspective the first stage is education. Promoting the vaccine and, at the same time, making it clear that HPV is a sexually transmitted infection will require joint efforts in the wording of policy, education and publicity materials. Comprehensive health-promoting approach including family and peers should aim at preventing complex risk behaviour rather than early sexual intercourse as an isolated event at the early age.

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RANI SEKSUALNI ODNOSI I RIZIČNI ČIMBENICI U HRVATSKIH ADOLESCENATA

S A Ž E T A K

Seksualno ponašanje adolescenata je zbog mogućih neposrednih i dugoročnih medicinskih i psiholoških posljedica vrlo osjetljivo područje. Svrha istraživanja bila je utvrditi jesu li rani seksualni odnosi spolno specifično povezani s rizičnim ponašanjima i psihococijalnim čimbenicima. U nacionalno reprezentativno istraživanje u školama korištenjem anonimnog upitnika bilo je uključeno 773 dječaka i 857 djevojčica dobi 15,5 godina. Spolno iskustvo u dobi do 16 godina imalo je 28.6% dječaka i 16.5% djevojčica. Vjerojatnost započimanja ranih spolnih odnosa bila je statistički značajno veća u dječaka koji više puše, piju, opijaju se, uzimaju marihuanu, sudjeluju u tučnjavama i zlostavljanju drugih, najveći utjecaj registriran je za svakodnevno pušenje (OR:8.1, CI:5.4–12.1). Iste su varijable s ranim seksualnim iskustvom bile povezane i u djevojaka, a najveći je nezavisni utjecaj imalo uzimanje marihuane (OR:8.0; CI:5.0–12.6). Multivarijatna analiza pokazala je da je najutjecajniji faktor za oba spola redovito pušenje. Za djevojčice koje su nezadovoljnije zdravljem (OR:2.9; CI:2.0–4.2), životom (OR:2.1; CI:1.4–3.0), odnosima s ocem i majkom (OR:1.9; CI:1.2–2.8 i OR:1.7; CI: 1.1–2.6) ili imaju više psihosomatskih simptoma (OR:2.9; CI:2.0–4.2) veća je vjerojatnost stupanja u rane seksualne odnose. Vjerojatnost ranog seksualnog iskustva veća je i u dječaka i u djevojčica za one koji imaju bolju komunikaciju s prijateljem suprotnog spola. S ranim seksualnim odnosima povezan je i veći broj večeri provođen vani s prijateljima te lošiji školski uspjeh za oba spola. Ranija dob menarhe je povezana s većom vjerojatnošću ranijeg započimanja seksualnih odnosa te s pušenjem, uzimanjem marihuane i više psihosomatskih simptoma. Rano seksualno iskustvo povezano je s rizičnim ponašanjima kao pušenje, pijenje alkohola, uzimanje marihuane, agresivnošću te psihosocijalnim čimbenicima. Preventivni programi stoga trebaju slijediti sveobuhvatni pristup uzimajući u obzir spolne specifičnosti. Cijepljenje protiv humanog papilomavirusa (HPV-a) treba biti dio cjelokupnog preventivnog programa i ne bi se smjelo smatrati samo izoliranom aktivnošću.

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Review

How Can the European Federation for Colposcopy Promote High Quality Colposcopy Throughout Europe?

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A B S T R A C T

Since its inception in 1998, the European Federation for Colposcopy (EFC) now comprises 26 member societies. Its principle aim is to promote high quality colposcopy throughout Europe with special emphasis on training, education and treatment. This review summarises EFC's activities and achievements to date.

Key words: *colposcopy, training, education*

Introduction

Colposcopic diagnostic performance depends upon a practitioner's proficiency as well as the clinical context in which it is used. It is subjective and the related management decisions require problem solving skills and experience. Both diagnosis and subsequent management require not only adequate training but also a sufficient workload to maintain those skills.

There is increasing concern that patients should receive high quality and cost-effective care throughout Europe. The need to protect against inadequate practise is particularly relevant to colposcopy because it is subjective and because most women who are examined are well. Performed correctly colposcopy minimises damage but if performed badly the scope for needless damage is great. Whereas the indications for colposcopy may vary throughout Europe, its objective is the same, namely to detect cervical disease, particularly pre-invasive changes.

It is against this background that the need for shared standards throughout Europe has become increasingly recognised. These standards are needed in two key areas, namely training and treatment.

The European Federation for Colposcopy

From its inception in Dublin in 1998, it was agreed that a priority for the European Federation for Colposcopy (EFC)[1] should be to work towards standardisation of training for colposcopy and agreement on audit methods and outcomes of treatment.

The EFC is a federation of colposcopic societies which now comprises 26 member societies throughout Europe. During its short history two large and successful triennial conferences have taken place in Greece (Rhodes, 2001) and France (Paris, 2004) as well as a third meeting to be held in Serbia (Belgrade) this year. The Federation's main educational aim has been to create a framework that should promote high quality and uniform training throughout Europe and a principle goal has been for all member societies to be able to offer a training programme that shares common aims and objectives.

Core Curriculum

The primary aim of the training programme was to produce competent diagnostic colposcopists because diagnosis is the foundation for clinical management. Despite the fact the colposcopy can be performed in different settings and for different indications throughout Europe, nonetheless, a common set of competences is required. It was decided to identify and use these necessary core competences as the basis for curriculum design. Each identified competence would then act as a learning

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objective for the programme. In other words the training programme was more concerned with what a competent trained colposcopist should be able to do rather than producing a list of things they need to know[2].

In 2001 a consensus exercise was done to identify what these essential core competencies were, using a consultative technique called the Delphi technique, which sought the views of a number of expert colposcopists throughout Europe[3]. In all, 28 participants from 21 countries took part. The list of identified core competencies was presented to the EFC at its scientific meeting in Rhodes in 2001 and they were accepted as the basis for designing colposcopy-training programmes in each of the member societies. Already a number of societies (UK, Germany and Spain) have now incorporated this core curriculum into their training programmes.

Programme Structure

In addition to deciding what needs to be taught, some agreement is necessary on how the training should occur. In part, this concerns the structure of European colposcopy training programmes and in 2004 a consensus agreement was been reached on what the recommendations should be. It is recognised that training must involve actual colposcopic experience that is supervised by a trainer in a recognised centre. The trainer and the experience received must meet certain standards in terms of workload and case-mix. It has been agreed that in EFC-recognised training programmes trainees would see a minimum of 100 patients, half of which should be new presentations and at least a third should have confirmed histological abnormality.

Treatment Standards

In conjunction with the work on training, in 2004 EFC initiated a modified Delphi survey concerning treatment[3]. The term 'treatment' encompasses a wide range of considerations but we initially focused solely on treatment itself. Identifying standards is an important initial step in quality assurance which has again involved using a modified Delphi survey. The agreed standards can then be used as the basis for audit of treatment throughout Europe. It is possible that successful participation in this audit will be one of the criteria required for recognition as a training centre.

Education

Since 2006 the EFC has convened a number of educational meetings and courses on colposcopy in order to promote the principles embedded in the training and treatment initiatives. Courses were held in Italy (April 2006 in Turin as part of the EBCOG conference) and Greece (June 2006 in Athens). This year, further courses are planned in Croatia (April 2007 as part of the International Workshop on Human Papillomavirus and Consensus Recommendations for Cervical Cancer Prevention & Colposcopy Training), Romania and Serbia. The educational emphasis is on interactive teaching, focusing on image recognition and case discussion.

Where do We Go From Here?

The work to date has started to lay down a foundation on which to build a quality assurance structure. There is now an agreed core curriculum and training structure and quality standards for practise are now being developed. These are early but important steps. So far this partnership has been fruitful but continued success largely depends on how far each member state is prepared to realise the shared objectives of the EFC. It is clear that the role of the EFC is to co-ordinate the activities of its member societies and its goals will only be realised by the achievements of those societies.

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KAKO DA EUROPSKA UDRUGA ZA KOLPOSKOPIJU PROMOVIRA VISOKO-KVALITETNU KOLPOSKOPIJU DILJEM EUROPE?

S A Ž E T A K

Od samih početaka, u 1998. g., Europska udruga za kolposkopiju (EFC) obuhvaća 26 društava članova. Njezin ključni cilj je promovirati visoko-kvalitetnu kolposkopiju diljem Europe s posebnim naglaskom na usavršavanje, obrazovanju i liječenja. Ovaj članak sažima aktivnosti i postignute uspjehe EFC-e.

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Short communication

Limitations of Colposcopy in Early Invasive Cervical Cancer Detection

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A B S T R A C T

Colposcopy is a key element in the diagnostic chain required to reduce cervical cancer mortality but it has limitations in the diagnosis of malignant disease. In the Republic of Croatia the Croatian Society for Colposcopy and Cervical Pathology started constructing guidelines for early detection, therapy and follow-up of patients with early invasive cervical cancer in order to achieve the best possible results in diagnosis, therapy and follow-up. From 2001 to 2006 Croatian society for colposcopy and cervical pathology organised six courses »Role of colposcopy in early diagnosis and prevention of premalignant lesions of the uterine cervix« in cooperation with Medical faculty, University of Zagreb and the Croatian medical chamber. Leading presentations were focused on the epidemiology of cervical cancer, cytologic, colposcopic and pathohistologic classification, HPV testing and role of male partner. After the theoretical part, a series of colposcopic pictures were presented as a practical part of the course where attendees participated in colposcopic images description and estimation of what could be the underlying pathological process. Such, courses are needed for continued medical education and quality practice of colposcopy.

Key words: *early invasive cervical cancer, role of colposcopy*

Introduction

Worldwide, invasive cervical cancer is one of the most frequent causes of death from gynaecological malign diseases, with almost 500,000 new cases per year[1]. Half of cancer cases have a fatal outcome in the first five years following diagnosis. Its incidence is much higher in developing countries; it seems that both the incidence and the mortality rate are rather underestimated in these countries.

In Croatia, according to the 2003 yearbook, 316 new cases of invasive cervical cancer (IC) (13.7/100 000) and 493 cases of carcinoma *in situ* (CIS) were diagnosed. Although the cervical cancer mortality rate is decreasing, 100 women die from this disease every year[2].

Colposcopy is a key element in the diagnostic chain required to reduce cervical cancer mortality but it has limitations in the diagnosis of malignant disease. Herein, these limitations and the way of improvement of colposcopy are presented.

Limitations of Colposcopy

Colposcopy is necessary if the gynaecologist finds no unusual features of the cervix in the patient with symptoms or an abnormal Pap smear (Figure 1 and 2). Colposcopic detection of microinvasive cancer depends on its size and location (Figure 3). Smaller lesions can be missed, but the probability of stromal invasion increases with the size of lesion on the surface of the cervix. Microinvasive cancers on the ectocervix are characterised by atypical blood vessels that are prone to bleeding. These atypical blood vessels are located unusually, distributed randomly, vary in diameter, and often change direction forming sharp angles. The intercapillary distance is also larger than normal and variable.

Invasive carcinomas are visible to the naked eye (Figure 4), but the colposcope enlarges the image showing the surface and the atypical blood vessels more clearly. For example, endophytic tumors often look like erosions but when enlarged, the papillary surface and atypical blood vessels are visible allowing a more accurate diagno-

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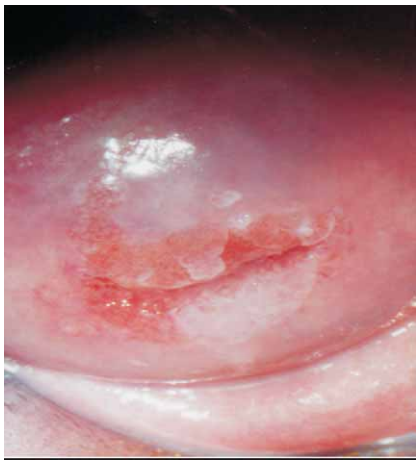

Fig. 1. Slight acetowhitening on the anterior and posterior lip of the uterine cervix – mild HPV change.

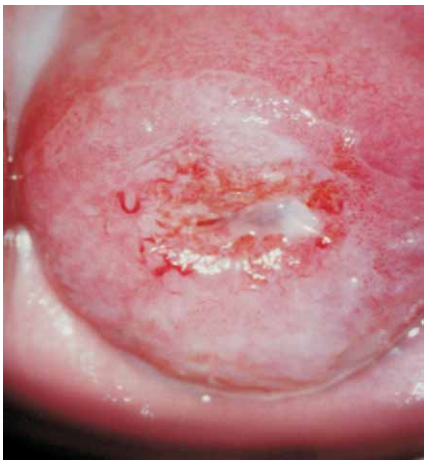

Fig. 2. Coarse acetowhitening – as sign of HPV induced cervical intraepithelial neoplasia.

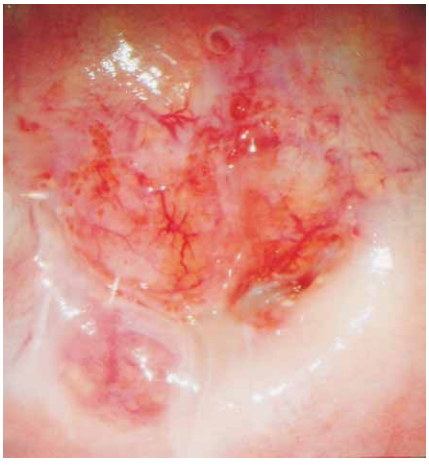

Fig. 3. Microinvasive cervical cancer.

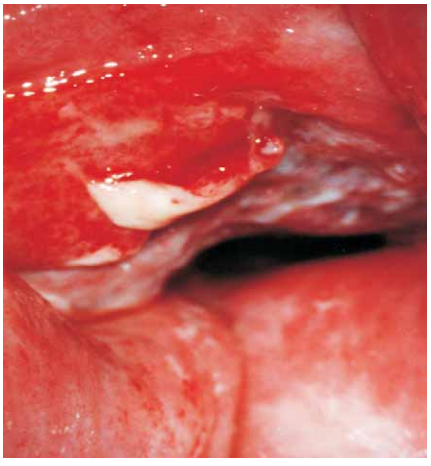

Fig. 4. Invasive cancer of the uterine cervix – pathohistological verification is needed.

sis. Keratosis can mask the colposcopic finding of an endophytic lesion, which is the reason, why biopsy is necessary. Adenocarcinoma has no grossly visible to distinguish it from squamous cancers with all of the vascular changes that have been described so far and a biopsy is required for its diagnosis. If a firm diagnosis cannot be made after the biopsy, diagnostic cone biopsy is recommended.

Basic Directives

Colposcopy classifications and achievements evolved with our understanding of cervical disease and the role of Human papillomavirus (HPV) infection from the Graz classification in 1975 to the Rome classification and finally the Barcelona classification in 2002[3–5]. The Graz classification[3] stated that acetowhite epithelium is a sufficient as an abnormal colposcopic finding. The Rome classification[4] then stated that such epithelium is almost pathognomonic for HPV infection, and can be more or less visible, with or without a borderline. Then, the Barcelona classification[5] added that the acetowhitening can appear quickly and disappear slowly, or appear slowly and disappear quickly and this is related to the intensity of the disruption of intracellular chromatine in the HPV infected cells.

It is important to recognise that colposcopic classification based on the grossly visible features of the cervix is complemented by both cytological classification[6] and pathohistological classification[7]. This necessitates collaboration between gynaecologists, cytologists and the pathologists, to establish protocols for diagnostics and treatment to achieve the best results. Such a protocol established at the conference celebrating 75 years of colposcopy in clinical practice, is a protocol for diagnostics and treatment of premalignant lesions of the cervix and lower genital tract and this has become a landmark for all of us who deal with the subject of preinvasive cervical lesions[8,9].

At clinical training courses in colposcopy and early diagnostics and prevention of neoplastic changes in the lower genital tract the theoretical practice ends with the protocol, and then we present a series of colposcopic images in order to entice discussion on possible gravity of lesions and pathohistological verification. Our great interest in the issue led us to the introduction of those ablation and destruction procedures that will spare the cervix and therefore the reproductive system of our patients, and their health on the whole.

The cytologic and colposcopic protocol that we established for the follow-up after classic probatory excision by forceps (Kevorkian, Thomas Gaylor) or after probatory excision by loop decreases the number of unnecessary cone biopsies by scalpel and diathermic cone biopsies by loop.

In cases of persistent cytologic abnormalities, after biopsy, and a second colposcopic examination, it could be easier to decide on one of such procedures in order to preserve the gynaecological and reproductive health. Although it is necessary to inform the patient that diathermic cone biopsy (LETZ) and classic cone biopsy with scalpel will remove the site of a potentially serious lesion the responsibility lies in the hands of the woman (and her partner) to make sure that such situation does not repeat in the future.

Common Questions in Colposcopic Practice

What happens when disease progresses? The collaboration of the gynaecologist, the cytologist, and the pathologist implies that the current state of a patient needs to be discussed on several levels: the stage of the initial invasion, the sample on which the initial invasion was discovered, age and parity of the patient. After we consider these levels, we can decide on further treatment of the patient.

The earlier the cervical cancer is diagnosed, the better the chances are of successful treatment. However, common issues that arise in clinical practice are:

- Treatment of microinvasive planocellular cervical cancer IA1 diagnosed after LETZ biopsy or classic test excision by forceps when the patient is a nulliparus, or has had one or more full term pregnancies?
- Treatment of microinvasive planocellular cervical cancer IA2 diagnosed after LETZ biopsy, or after classic test excision by forceps when the patient is a nulliparus, or has had one or more full term pregnancies?
- Treatment of microinvasive planocellular cervical cancer IA1 diagnosed after LETZ biopsy or after classic cone biopsy by scalpel when the patient is a nulliparus or has had one or more full term pregnancies?
- Treatment of microinvasive planocellular cervical cancer IA2 diagnosed after LETZ cone biopsy or after classic cone biopsy by scalpel. What if the patient is a nullipara, or if she had a baby once, or more times?

What about the »grey area«? How to treat patients with stromal invasion of more than 5 mm, the spread larger than 7 mm, and the change still not visible to the naked eye? Do they already belong to the IB1 group or not? What about reproduction? What if the finding is an incidental finding at hysterectomy, or at a diagnostic cone biopsy, or at one of the mentioned excohleation of the cervical canal or probatory excisions? All these issues require thorough study keeping in mind FIGO classification which is one of the more acceptable classification systems for all those involved in cervical cancer diagnosis and treatment[10,11] (Table 1).

Training Courses in Colposcopy

From 2001 to 2006, the Croatian Society for Colposcopy and Cervical Pathology organised six courses on the theme of the »Role of colposcopy in early diagnosis and prevention of premalignant lesions of the uterine cervix«, in cooperation with Medical Faculty of the University of Zagreb and the Croatian Medical Chamber[12]. Leading presentations were the epidemiology of cervical cancer, cytologic, colposcopic and pathohistologic classification, HPV testing and role of male partner. After the

TABLE 1
EXISTING PROTOCOL ON TREATMENT OF PATIENTS WITH ABNORMAL PAP SMEAR AND UNSATISFACTORY COLPOSCOPIC FINDING OR WITH MICROINVASIVE CARCINOMA OF SQUAMOUS CELLS ON PROBATORY EXCISION

Status of the excision of microinvasion up to 5 mm or less	Recommendations
Margins clear with negative ECC, stage IA1 with no spread to lymphovascular area	▪ cone biopsy if the patient wants to preserve fertility
Margins and/or ECC positive dysplasia	▪ repeat cone biopsy ▪ modified radical hysterectomy ▪ If conisation not appropriate ▪ Hysterectomy +/– pelvic lymphadenectomy
Stage IA1 with invasion into lymphovascular area	▪ pelvic lymphadenectomy + conisation, or ▪ radical trachelectomy (for fertility reasons) ▪ modified radical hysterectomy and pelvic lymphadenectomy

Adapted from Hacker, 2000[11], ECC – endocervical curettage, LVSI – lympho vascular space invasion, RH – radical hysterectomy

theoretical lectures there was the practical course based on series of colposcopic pictures as practical part of course where attendees participated in colposcopic description and prediction of the underlying pathological process. Among 500 gynecologists from Croatia, 60 of them attended the course and gave the following ratings: the average grade for quality of presentation was 3.98 points (maximum 5.2) and for quality in everyday practice was 3.95 points with the highest grading 5.2 points.

Numerous questions and answers due to diagnostic protocol of early invasive cervical cancer were brought up during the postgraduate clinical training course »Diagnostics, Treatment and Prognosis for Preinvasive Lesions and Carcinoma of the Cervix« held at the Obstetrics and Gynecology Clinic in Petrova in Zagreb, on 7th and 8th April 2006. Critical scientific and practical thinking and the participation of gynaecologists, pathologists, cytologists, radiologists and epidemiologists established firm foundations for a comprehensive insight into this issue.

Conclusion

These foundations created leeway both for experts and young colleagues in these areas of medicine to work on setting up a national programme for cervical cancer screening. Necessary collaboration among complementary specialities, consulting with more experienced colleagues and regular publication of results will improve women's gynecologic health care.

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OGRANIČENJA KOLPOSKOPIJE U OTKRIVANJU POČETNOG INVAZIVNOG RAKA VRATA MATERNICE

SAŽETAK

Kolposkopija je ključni korak u nizu dijagnostičkih postupaka potrebnih u sprečavanju smrti od raka vrata maternice, međutim metoda ima svoje nedostatke u dijagnostici zloćudne bolesti. Uvidom u kretanje pojavnosti raka vrata maternice u Republici Hrvatskoj iskrsava potreba za sveobuhvatnim dijagnostičko terapijskom pristupom u što ranijem otkrivanju, liječenju i praćenju pacijentica s početnim invazivnim rakom vrata maternice. To je prioritetni zadatak Hrvatskog društva za kolposkopiju i bolesti vrata maternice. U okviru postupaka sekundarne prevencije istaknuta je uloga kolposkopije. U razdoblju do 2001. do 2006. godine Hrvatsko društvo za kolposkopiju i bolesti vrata maternice Hrvatskog liječničkog zbora organiziralo je u suradnji s Medicinskim fakultetom Sveučilišta u Zagrebu i Kliničkom bolnicom Sestre milosrdnice šest tečajeva stalnog medicinskog usavršavanja prve kategorije »Mjesto i uloga kolposkopije u ranoj dijagnozi i prevenciji preinvazivnih promjena vrata maternice i donjeg genitalnog trakta. Vodeća poglavlja su bila o pojavnosti raka vrata maternice, zatim citološka, kolposkopska i patohistološka klasifikacija, testiranje na humani papilomavirus (HPV) te uloga muškog partnera u pojavnosti HPV-a. Nakon teorijskog dijela nastavljen je praktički dio u kojem su polaznici sudjelovali u opisivanju kolposkopskih slika i očekivanju kakva je bila patohistološka pozadina kolposkopskog ispoljavanja. Ovakav pristup, nužan je za trajnu edukaciju liječnika i održavanje kvalitete rada kolposkopije.

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Original scientific paper

Treatment of Invasive Cervical Cancer: Rijeka Experience

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A B S T R A C T

The aim of this retrospective analysis was to evaluate the survival rate in 661 patients with cervical cancer regarding two time periods 1990–1996 and 1997–2003 and the specific stage related risk factors. The respective five-year survival was 71.7% and 80.0%. Analyzing the risk factors in the univariate and multivariate regression modalities ultimately only two parameters, the two time periods and FIGO staging were found to be independent prognostic factors. The observed total improvement in the survival rate of the second time period is followed by an increase in conservative surgery in stage T1A1, a reduction in the use of adjuvant radiotherapy among operable stages T1b1, T1b2 and T2A, while the treatment of locally advanced cervical cancer did not differ significantly.

Key words: *cervical cancer, treatment, staging, FIGO, TNM, survival*

Introduction

Cervical cancer is the second most common malignancy in women worldwide. Although it has been considered a preventable cancer because of cervical cytological screening programs and effective treatment of preinvasive lesions, the mortality rate is still high.

In Croatia during last decade the incidence of cervical cancer was about 16 patients per 100,000 women a year, reaching an incidence of 13.7 patients per 100,000 women in 2003[1].

Risk factors in developing cervical cancer include young age at first intercourse, multiple sexual partners, cigarette smoking, high parity and low socioeconomic status. There is some relationship to oral contraceptives as risk factors in cervical cancer development with a possible small increase[2]. However, infection with the human papillomavirus (HPV) has been detected in up to 99% of women with squamous cervical cancer and is defined as the principal risk factor in cervical cancer development[3].

Until recently, major breakthroughs in reducing the incidence and mortality of cervical cancer have occurred because of the widely used screening programs. The Papanicolaou test, known as the Pap test, has been the most cost- effective cancer-screening test ever developed. In Rijeka, Croatia, the Pap-test was introduced as a routine test in gynecologic examination since 1960[4].

Nevertheless, cervical cancer is still present in our population and affected patients require diagnosing and treatment that consists of four steps: establishing the diagnosis, defining the extent of the disease, determining and conducting the optimal treatment and follow-up of patients for evidence of recurrence and/or treatment related complications.

The diagnosis of cervical cancer is made exclusively by histological analysis of a biopsy specimen or by conization. Once histological diagnosis is arrived at, based on the International Federation of Gynecology and Obstetrics (FIGO) classification, clinical (preoperative) staging has to be defined. Conization may be part of diagnostic workup, however, its role in definitive treatment will be discussed later. Diagnostic workup is necessary to de-

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fine preoperative staging using only: physical examination, colposcopy, cervical or cone biopsy, cystoscopy, lower gastrointestinal endoscopy or barium enema, intravenous pyelography and chest radiography. Computed tomography (CT) and magnetic resonance (MRI) and positron emission tomography with computerized tomography (PET-CT scan) are very useful tools for better definition of the disease presence, but FIGO is not taken into account as a staging modality. The treatment strategies for cervical cancer are related to the diagnosis and the clinical staging system.

The aim of this retrospective analysis was to evaluate the survival rate regarding two time periods 1990–1996 and 1997–2003 and the specific stage related risk factors.

Subjects and Methods

Medical records of all patients with cervical cancer primarily treated at the Clinical Hospital Center Rijeka between 1990 and 2003 were retrospectively reviewed. The hospital is a tertiary referral center and educational base of the University of Rijeka, School of Medicine for the surrounding area of three counties including about 550,000 inhabitants. Six hundred and sixty one patients with primary cervical cancer were identified.

The prognostic variables investigated for this study included two time periods, the first from 1990 to 1996, and the second from 1997 to 2003. December 31, 2006 was the cut off date for patient follow-up

In both time periods we analyzed the following prognostic variables: T stage[5], FIGO stage according to the last revision of cervical cancer staging[6] and compared the 5-year survival rate between each subgroup of patients and the entire group. The stage indicated in this study referred to pathologic examination after primary surgery and clinically in cases where radiotherapy or chemo-irradiation was the first therapeutic option. During the observed period, cervical cancer treatment was based on guidelines agreed at the national and hospital level. The surgical approach was primarily applied to clinical FIGO stages IA1 to IIA. In stage IIB the primary treatment approach depended on the clinician. In higher stages radiotherapy was the treatment of choice. The guidelines on adjuvant radiotherapy after surgery changed during the two time periods as well as those on the conservative option in the treatment of the early stage.

The groups of patient with histology defined as FIGO Ia1 stage (stromal invasion of not >3.0 mm in depth and extension of not >7.0 mm) were analyzed separately to compare the type of treatment and the 5-year survival rate between the two time periods. Stage IA1 and stage IA2 cervical cancer were diagnosed either on cone or hysterectomy (simple or radical) specimen.

All cone biopsies were bisected and each half was embedded completely and serially processed into 40–90 individual sections. The cervices of the extirpated uteri were treated as a cone and sampled by conventional methods. Groups of patients with stage disease of IB1 and higher underwent clinical and instrumental staging. In particular, stage T1b1, T1b2 and T2a tumor size was assessed by pelvic examination and under anesthesia at the time of biopsy or surgery. The definitive tumor dimension in patients undergoing surgery was determined by measuring the tumor after uterus removal, while in patients without surgery the definitive dimension was determined clinically. All patients treated before the last revision of FIGO staging in 1994 were restaged according to the new recommendations[6].

In the group of patients with pathologic T stages T1b1, T1b2 and T2a the following categorical variables were evaluated and compared in the two time periods (1990–1996 vs. 1997 to 2003): the tumor diameter (less than 2 cm, 2 to 4 cm, more than 4 cm), age (under 40, 40 to 59, 60 years and over), histology (squamous and adenocarcinoma), tumor differentiation (G1 – well, G2 – moderate and G3 – poor), T stage, FIGO stage, lymph node involvement (Nx – not assessed, No – negative node, N1 – positive node), lymphovascular space involvement (No – Yes) and the mode of treatment (assigned in each table).

Statistics

Absolute numbers with percentages were used to show the number of patients per group. The Chi-square test was used where appropriate. The Kaplan-Meier method was used to estimate the survival curves. Survival time was calculated in months from the date of surgery or therapy beginning at either the date of death, or the date of last follow-up visit for surviving patients. Univariate analysis of categorical variables was performed for prognostic significance using the Cox proportional hazard model and the log-rank test for significance, respectively. Variables with $p<0.10$ on univariate analysis were then included in a multivariate Cox proportional hazard regression analysis. Statistical analyses were performed using MedCalc for Windows, version 9.2.0.2 (MedCalc Software, Mariakerke, Belgium).

Results

The distribution of »T stage« among cervical cancer patients in the two time periods, the survival in each stage and the survival per entire group are presented (Table 1). Staging is the most important predictor of survival, reflecting the extent of the disease with the risk of death being higher as a stage increases. Comparing total survival between the two periods, there is a clear statistically significant higher 5-year survival rate among patients treated in the second time period. The group of patients in T1B1 stage treated in the second period had a significantly better survival rate than the same staged patients treated in the first period. However, there was no difference in patient distribution per stage (Chi-square=0.9, p=0.34; not shown). The evaluated patient age is equal in both time periods (48.7 vs. 48.6 years).

TABLE 1
DISTRIBUTION AND SURVIVAL OF CERVICAL CANCER PATIENTS (n=661) ACCORDING TO THE »T« STAGE AND TIME PERIOD

Stage	Period 1990–1996				Period 1997–2003				Statistical significance for survival
	n	(%)	–	5-years survival	n	(%)	–	5-years survival	
T1A1	115	(37.6)	–	100%	145	(40.8)	–	98.4%	NS
T1A2	9	(2.9)	–	100%	5	(1.4)	–	100%	NS
T1B1	69	(22.5)	–	80.2%	85	(23.9)	–	94.2%	p=0.017
T1B2	14	(4.6)	–	60.9%	25	(7.0)	–	77.6%	NS
T2A	8	(2.6)	–	50.0%	6	(1.7)	–	100%	NS
T2B	43	(14.1)	–	40.8%	26	(7.3)	–	53.4%	NS
T3A	2	(0.7)	–	0.0%	2	(0.6)	–	0.0%	NS
T3B	43	(14.1)	–	21.3%	56	(15.8)	–	23.2%	NS
T4	3	(1.0)	–	0.0%	5	(1.4)	–	0.0%	NS
Total	306		–	71.7%	355		–	80.0%	p=0.02

Distribution of patients in the FIGO stage shows no difference (Chi-square=14.8, p=0.098; not shown) between the two time periods (Table 2). The 5-year survival rate in stages IA1 and IA2 is excellent in both time periods. Stages IB1, IB2, IIA, IIB and IIIB in the second time period had better survival rates but without statistical significance. Nevertheless, a total 5-year survival rate is significantly higher in the second time period (Table 2).

In our study, FIGO stage IA1 was present in 260 (39.3%) out of 661 patients (Table 3). In the second time period we found an increase from 37.6% to 40.8% without statistical significance (Chi-square=0.60, p=0.4; not shown) of patients with stage IA1 (Table 2). The types of patient treatment in stage IA1 are presented in the Table 3. The distribution of patients regarding the mode of

TABLE 3
TREATMENT OF CERVICAL CANCER PATIENTS IN T STAGE T1A1 (n=260)

Mode of treatment	Period 1990–1996 n=115		Period 1997–2003 n=145	
	n	(%)	n	(%)
Conisation	54	(47.0%)	84	(58.0%)
Hysterectomy	35	(30.4%)	35	(24.1%)
Hysterectomy & Lymphadenectomy	23	(20.0%)	16	(11.0%)
Radical hysterectomy & Lymphadenectomy	*3	(2.6%)	10	(6.9%)

*2 patients treated with adjuvant radiotherapy

TABLE 2
DISTRIBUTION AND SURVIVAL OF CERVICAL CANCER PATIENTS (n=661) ACCORDING TO THE FIGO STAGE AND TIME PERIOD

Stage	Period 1990–1996				Period 1997–2003				Statistical significance for survival
	n	(%)	–	5-years survival	n	(%)	–	5-years survival	
IA1	115	(37.6)	–	100%	145	(40.8)	–	98.4%	NS
IA2	9	(2.9)	–	100%	5	(1.4)	–	100%	NS
IB1	59	(19.3)	–	87.5%	76	(21.4)	–	93.5%	NS
IB2	13	(4.2)	–	66.1%	18	(5.1)	–	81.6%	NS
IIA	7	(2.3)	–	57.1%	6	(1.7)	–	100%	NS
IIB	36	(11.8)	–	43.2%	18	(5.1)	–	61.4%	NS
IIIA	2	(0.7)	–	0.0%	2	(0.6)	–	0.0%	NS
IIIB	61	(19.9)	–	24.8%	74	(20.8)	–	40.8%	NS
IVA	2	(0.7)	–	0.0%	4	(1.1)	–	0.0%	NS
IVB	2	(0.7)	–	0.0%	7	(2.0)	–	0.0%	NS
Total	306		–	71.7%	355	–	80.0%	p=0.02	

treatment and the time periods shows high significance (Chi-square=13.87, p=0.008). An increase in conservative surgical treatment requiring only conization is identified in 58% of cases in the second time period, while hysterectomy decreased from 30.4% to 24.1%, also in the second period. Radical procedures, including lymph node assessment, mainly due to lymph-vascular space involvement, were present in a similar percent in both observed periods. The apparent increase in the use of radical hysterectomies in the second time period is due to dubious biopsy material in some patients. However, in the second time period lymph node staging and/or radical hysterectomy dropped from 22.6% to 17.9% (not shown). During the first time period two patients received adjuvant pelvic irradiation.

»T« stage was used to evaluate the dimension of primary tumor in women with cervical cancer localized in the cervix (stage T1b1 and T1b2) and with only vaginal involvement (T2a), excluding parametrial involvement. In this group all patients with a concomitant serious medical condition were not treated or were treated with palliative radiotherapy. In the medically uncompromised patients older than 75 years primary treatment included primary radiotherapy. Patients with primary surgery were divided into two groups with respect to the use of adjuvant pelvic irradiation. The indications for adjuvant pelvic irradiation were changed during the observed time. The first group of patients consisted of those with stage from T1B1 to T2A and a dimension of cervical cancer less than 2 cm in diameter. The next group of patients included tumor diameters of 2 to 4 cm and the third group of patients included tumors of a diameter larger than 4 cm. Patients staged T2b and T3b were not divided in the subgroups. Results are presented as a total number of patients while percents include the proportion of the entire group within the stage categories (Table 4).

In the group of patients with the tumor diameter less than 2 cm we observed an obvious inversion in the use of

TABLE 4
TREATMENT OF CERVICAL CANCER PATIENTS REGARDING T STAGE AND TUMOR DIMENSION

T stage & tumor dimension	Type of treatment	Period 1990–1996		Period 1997–2003	
		n	(%)	n	(%)
T1b1-T2a (< 2cm)	Without therapy	0		0	
	Radiotherapy	2	(7.4%)	1	(2.1%)
	Radical surgery	7	(25.9%)	36	(75.0%)
	Radical surgery & adjuvant radiotherapy	18	(66.7%)	11	(22.9%)
	Overall survival	96.3%		96.7%	
T1b1-T2a (2–4 cm)	Without therapy	0		1	(2.4%)
	Radiotherapy	2	(4.4%)	1	(2.4%)
	Radical surgery	2	(4.4%)	18	(43.9%)
	Radical surgery & adjuvant radiotherapy	41	(91.2%)	21	(51.3%)
	Overall survival	69.3%		87.1%	
T1b2-T2a (>4 cm)	Without therapy	1	(5.3%)	0	
	Radiotherapy	2	(10.5%)	3	(11.1%)
	Radical surgery	0		7	(25.9%)
	Radical surgery & adjuvant radiotherapy	16	(84.2%)	17	(63.0%)
	Overall survival	54.9%		87.7%	
T2b	Without therapy	2	(4.7%)	0	
	Radiotherapy	27	(62.8%)	12	(46.2%)
	Radical surgery	1	(2.3%)	0	
	Radical surgery & adjuvant radiotherapy	13		14	(53.8%)
	Overall survival	40.8%	(30.2%)	53.4%	
T3b	Without therapy	6	(14.0%)	10	(17.9%)
	Radiotherapy	37	(86.0%)	44	(78.6%)
	Radical surgery	0		0	
	Radical surgery & adjuvant radiotherapy	0		2	(3.5%)
	Overall survival	21.3%		23.2%	

adjuvant pelvic irradiation in the second time period (Chi-square=17.04, p=0.0002; not shown) with a similar five-year survival rate. In these groups of patients only one patient with positive pelvic lymph node was identified in the second time period.

In the group of patients with primary cervical tumor diameter of 2 to 4 cm an inversion of treatment modalities was identified (Table 4). Namely, the majority of cervical cancer patients in the first time period were treated with adjuvant pelvic irradiation with a 5-year survival rate of 69.3%. In the second time period there was a significant difference in patients distribution (Chi-square =20.44, p= 0.0001; not shown), with a decrease in the use of adjuvant pelvic irradiation after radical surgery and a total increase of the 5-year survival rate. In the first time period 10 (23.2%) patients had positive lymph nodes, while in the second time period positive nodes were found in 8 patients (20.5%).

In the group of patients with primary cervical tumor greater than 4 cm primary radiotherapy was applied in about 11% (Table 4). All patients who underwent surgery in the first time period were adjunctively treated with pelvic irradiation. In the second time period one fourth of patients were treated only with radical surgery. Although there is no significant change in the modality of treatment in the second time period, the 5-year survival rate is significantly higher (Chi-square=6.05, p=0.014; not shown). However, in the second time period a trend of decrease in the use of adjuvant radiotherapy was also observed. Analyzing the rate of positive lymph nodes we identified 10 out of 16 patients (62.5%) in the first time group and 8 out of 24 (33.3%) patients in second time period. The observed difference had no statistical significance (Chi-square=2.26, p=0.135; not shown).

In patients with T2B stage in the first time period the therapy mostly used was radiotherapy (Table 4). In the second time period radical surgery with adjuvant pelvic irradiation was encountered in a higher proportion. The difference of the treatment modality distribution is not significant. The 5-year survival rate, although higher in the second time period, did not reach a statistical significance.

Clinically staged patients in stage T3B in both time periods received similar treatment options (Table 4). The majority of patients were treated with primary radiotherapy, while only a small number of patients after the year 2001 received combined chemoirradiation. The rate of survival in both time period groups was similar.

The patient groups staged T1B1 to T2B and T3B with 177 and 198 patients had a 5-years survival rate of 54.6% and 68.1% in first and second time period, respectively (Logrank test p=0.0124; not shown).

Univariate analysis of variables was performed in the entire group of cervical cancer patients (n= 661). Variables, two time periods, histology, FIGO stage, degree of differentiation (G), T stage, lymph node status, type of treatment and patient age were categorized as shown (Table 5). All analyzed variables were significant, and were subsequently included in the multivariate model. Using Cox proportional hazard regression only two variables remained significant: two time periods and FIGO stage (Table 6).

Discussion

The distribution of cervical cancer patients regarding T stage and FIGO stage are similar during the two observed periods. Approximately 40% of patients with cervical cancer presented with a microinvasive disease limited to the invasion of 3 mm and 7 mm or less in width. The diagnosis of stage IA1 cervical cancer has to be established at least via cone specimen. Acceptable methods for diagnostic purposes are cold knife conization and loop electrosurgical excision. The prognosis is excellent, as shown in our series. A total 5-year survival for 260 patients presented with microinvasive cervical cancer in our analysis is 99.1% (not shown). There is no difference in the 5-year survival between the two time period groups. In the last FIGO analysis 829 (7.12%) patients with cervical cancer stage IA1 out of 11639 had a five-year survival rate of 97.5%[7]. There are a significantly smaller proportion of patients with microinvasive cervical cancer in world statistics compared to our patients (7.12% vs 39.3%). A relative increase in the total number as well as in the proportion of the entire cervical cancer group could be attributed firstly to the meticulous analysis of cervices with multiple serial sections per specimen.

Surgical treatment of patients with cervical cancer stage IA1 moved to conservative treatment is present in almost 60%. Hysterectomy is reserved primarily for women that are past childbearing. Lymphadenectomy is reserved for those with lymph space involvement, although there is little, if any risk of lymph node metastasis, recurrence and death[8–10]. Of the 52 patients (not shown) in our series treated with lymphadenectomy as part of treatment option firstly due to lymph vascular space involvement, none had lymph node metastasis.

The 5-year survival rate in our group of patients is rather high but without statistical significance (99.1% vs 97.5%). In one series with median follow-up of 45 months, 10% of patients developed cervical intraepithelial neoplasia 3 – CIN III[11]. In our series of 126 patients with cervical cancer stage IA1 treated with a conservative surgical procedure the cold knife conization and a median follow-up of 72 months, we detected local recurrence in form of cervical intraepithelial neoplasia irrespective of their severity in 7 (4%) patients (data not shown).

Squamous lesions are predominantly present in the early stage of cervical cancer stage IA1, while glandular lesions are rarely recognized in the early stage. This is mainly due to difficulties in measuring the glandular lesions invasion depth. In our series of 260 patients with cervical cancer stage IA1 we identified 6 (2.3%) patients with glandular lesions (data not shown). Currently, the options of treatment modalities based on retrospective data include the same procedures with the same indications as a squamous lesion[12,13].

TABLE 5
UNIVARIATE ANALYSIS OF CERVICAL CANCER PATIENTS (n=661)

Parameter		Number of patients	5-year survival	Chi-square	Significance level	Hazard ratio	(95% CI)
Period	1990–1996	(306)	72.0%	Reference			
	1997–2003	(355)	80.0%	5.4	p=0.02	1.46	(1.06–2.02)
Histology	Squamous	(583)	77.3%	Reference			
	Adeno	(78)	65.5%	7.2	p=0.0074	0.57	(0.29–0.83)
FIGO	IA1	(260)	99.1%	Reference			
	IA2	(14)	100.0%	0.1	p=0.74	1.01	(0.01–1571)
	IB1	(135)	91.0%	17.0	p<0.0001	0.08	(0.03–0.29)
	IB2	(31)	74.4%	50.8	p<0.0001	0.03	(0.00–0.003)
	IIA	(13)	76.9%	38.1	p<0.0001	0.03	(0.00–0.0001)
	IIB	(54)	46.8%	161.0	p<0.0001	0.01	(0.00–0.003)
	IIIA	(4)	0.0%	315.0	p<0.0001	0.004	(0.00–0.00001)
	IIIB	(135)	33.3%	243.0	p<0.0001	0.008	(0.01–0.03)
	IVA	(6)	0.0%	271.9	p<0.0001	0.005	(0.0000–0.0000)
	IVB	(9)	0.0%	337.7	p<0.0001	0.005	(0.0000–0.0000)
Gradus	G1	(42)	87.5%	Reference			
	G2	(260)	57.8%	10.9	p=0.001	0.25	(0.25–0.70)
	G3	(104)	59.5%	9.6	p=0.002	0.26	(0.20–0.69)
	Undetermined	(255)	99.1%	20.8	p<0.0001	0.06	(0.00–0.05)
T-stage	T1A1	(260)	99.1%	Reference			
	T1A2	(14)	100.0%	0.1	p=0.74	–	
	T1B1	(154)	88.1%	24.8	p<0.0001	0.06	(0.04–0.25)
	T1B2	(39)	70.5%	64.9	p<0.0001	0.03	(0.0001–0.004)
	T2A	(14)	70.7%	54.6	p<0.0001	0.02	(0.0000–0.0000)
	T2B	(69)	45.2%	180.5	p<0.0001	0.01	(0.001–0.007)
	T3A	(4)	0.0%	315.9	p<0.0001	0.004	(0.0000–0.0000)
	T3B	(99)	21.7%	307.5	p<0.0001	0.007	(0.002–0.007)
	T4	(8)	0.0%	327.6	p<0.0001	0.005	(0.0000–0.0000)
Lymph	Nx	(389)	69.9%	Reference			
node	No	(224)	92.3%	40.2	p<0.0001	4.6	(2.22–4.52)
status	N1	(48)	49.0%	7.0	p=0.008	0.55	(0.27–0.82)
Therapy							
Wertheim (W)	(85)	94.5%	Reference				
Without therapy	(21)	0.0%	138.0	p<0.0001	41.5	(1416–25897)	
Radiotherapy	(138)	33.8%	74.0	p<0.0001	20.6	(4.19–9.77)	
Conisation	(138)	98.4%	2.2	p=0.1	0.3	(0.05–1.5)	
Hysterectomy	(71)	100.0%	3.5	p=0.06	0.0	(0.02–1.1)	
Hysterect. & adj. radiotherapy (7)	68.6%	7.5	p=0.006	7.5	(3.7–2707)		
Hysterect. & lymhadenect.	(40)	100.0%	2.0	p=0.16	0.0	(0.03–1.79)	
Hysterect. & lymhadenect. & adjuvant radiotherapy	(13)	44.0%	34.6	p<0.0001	15.4	(50.8–2573)	
Wertheim & adj. radiotherapy	(148)	77.3%	11.7	p=0.0006	0.2	(0.16–0.61)	
Age	< 40	(229)	93.8%	Reference			
(years)	40–59	(260)	79.2%	21.6	p<0.0001	0.26	(0.19–0.51)
	≥ 60	(172)	48.1%	111.2	p<0.0001	0.09	(0.07–0.18)

TABLE 6
COX PROPORTIONAL HAZARDS REGRESSION OF CERVICAL CANCER PATIENTS (n=661)

Parameter	Significance level	Hazard ratio	(95% CI)
Period (1990–1996 and 1997–2003)	p=0.012	0.66	(0.48–0.91)
Histology	p=0.08 (NS)	1.47	(0.96–2.25)
FIGO	p=0.0001	1.5	(1.23–1.83)
Tumor differentiation (G)	p=0.21 (NS)	1.2	(0.91–1.57)
Lymph node status	p=0.47 (NS)	0.8	(0.41–1.50)
T stage	p=0.16 (NS)	1.16	(0.94–1.41)
Therapy	p=0.92 (NS)	0.99	(0.85–1.15)
Patients age	p=0.47 (NS)	1.11	(0.84–1.46)

Categories of variables as shown in Table 5, NS – non-significant

The patients with cervical cancer stage IA2 are present in a small percentage (2.9%). The rate (2.4%) of this stage is similar to that in the last FIGO statistics[7]. The diagnosis of stage IA2 cervical cancer should also be established via cone biopsy. Although the prognosis for these patients is also excellent (in our statistics 14 patients had a 5-year survival rate of 100%, while in previously mentioned statistics the survival rate in 275 patients was 94.8%) they are at higher risk for lymph node metastasis and treatment failure. In one series a 6.8% incidence of lymph node metastasis in patients with cervical cancer stage IA2 is reported[10]. Currently, there is a shortage of plausible recommendations for the management of cervical cancer in stage IA2 based on reliable and prospective studies. Until more data become available pelvic lymph node assessment is necessary. Hysterectomy could be performed as a simple procedure, while radical hysterectomy represents surgical trauma without confirmed survival advantage. In patients with desired childbearing, large conization with absolute clear margins and negative lymph node could be the treatment of choice[14].

The treatment of choice in cervical cancer stages IB1 to IIA can be influenced by patient age, coexisting medical conditions and physician bias. Retrospective as well as prospective studies comparing radical surgery with pelvic radiation therapy showed similar survival rates[15,16]. Radical surgery offers few advantages in respect to preservation of ovaries with fewer detrimental effects on vaginal function. The advantages of radical surgery over radiotherapy can be eliminated in patients receiving postoperative adjuvant radiotherapy. Indication for the use of adjuvant radiotherapy includes positive lymph node, positive margins and parametrial involvement. Other indications for the use of postoperative radiotherapy in cases without evidence of disease outside the cervix include the presence of high risk factors in the hysterectomy specimen like large tumor diameter, deep cervical stromal invasion and the invasion of lymph vascular space involvement[17]. As tumor diameter increases, the risk of treatment failure is greater. Tumor diameter greater than 4 cm leads to the use of various treatment regimens. The T stage T1B2 includes radical hysterectomy with pelvic and paraaortic lymphadenectomy followed by tailored chemoradiation therapy for high-risk patients, radiation therapy followed by extrafascial hysterectomy, radiation therapy plus concurrent chemotherapy and neoadjuvant chemotherapy followed by radical pelvic surgery[18,19]. Although some authors recommend the use of concurrent radiotherapy and chemotherapy as an optimal treatment option[19], radical hysterectomy with tailored chemoirradiation for high-risk patients is the most cost-effective strategy to manage Stage IB2 cervical cancer, resulting in a 5-year survival of approximately 70%. In our series we present only 3 patients treated with primary radiotherapy, 7 treated with radical hysterectomy and 17 patients treated with radical hysterectomy and adjuvant radiotherapy with a total 5-year survival of 87.7%. Radical surgery followed by tailored radiotherapy is especially suitable in settings where resources are limited. Adding radiotherapy and chemotherapy in form of neoadjuvant chemotherapy unnecessarily increases the total cost without clear advantage in a 5-year survival[18].

Comparing the use of adjuvant radiotherapy between the two time periods in our series we found that in cases with a tumor diameter less than 2 cm reduction in the use of radiotherapy did not influence the 5-year survival rate. In the group of patients with cervical cancer of diameters from 2 to 4 cm the observed reduction in the use of adjuvant radiotherapy lead to a better 5-year survival. In the group of patients with cervical cancer stage IB2 (tumor diameter greater than 4 cm) adjuvant radiotherapy in the second time period was in part reduced with a higher 5-year survival.

From our results, the philosophy of treatment in the second time period is one of the significant points in the treatment acceptance and success measured through a 5-year survival. The lack of this analysis precludes the precise definition of all elements used in building the indications for adjuvant radiotherapy during the observed period. Moreover, the indications for adjuvant radiotherapy after radical surgery changed several times, especially in the second time period. Conclusively, from the retrospective results and the results of others[20,21] adjuvant radiotherapy seems to be useful only in the group of patients with the disease beyond the cervix, i.e., lymph node metastasis and parametrial involvement.

The standard accepted therapy in the last 15 years for locally advanced cervical cancer includes radiotherapy present in the majority of cases in our series. The addition of cisplatin or a cisplatin containing regimen to a radiation therapy results in better disease control and a 5-year survival[22,23]. It is part of a standard recommended therapy. A subsequent meta-analysis of 19 randomized controlled trials including 4560 patients confirmed the effectiveness of concurrent chemotherapy and radiation therapy, which improved the overall survival of patients with a locally advanced disease[24]. The new therapeutic option in patients with locally advanced cervical cancer promoted by Croatian authors[25] seems to produce higher survival rates, but the preliminary results have to be confirmed in large multicenter trials.

In our series of patients in stage T2B or greater even though the 5-year survival rate is better, the difference has not reached statistical significance. In stage T2B a more extensive use of radical surgery with adjuvant radiotherapy and better survival had also no statistical significance. A minor number of patients in stage T3B were treated with the use of chemoirradiation, which was not taken into account in the actual analysis, and we have not performed statistical evaluation regarding the application of concurrent chemotherapy and radiation therapy in patients with locally advanced cervical cancer.

Conclusions

The comparison of two time periods, 1990–1996 and 1997–2003, showed an overall higher five-year survival rate in the second time period denoting a significant independent prognostic parameter. The observed total improvement in survival rates was followed by an increase in conservative surgery in stage T1A1, reducing the application of adjuvant radiotherapy in operable stages T1B1, T1B2 and T2A, while the treatment of locally advanced cervical cancer has not differed significantly.

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LIJEČENJE INVAZIVNOG RAKA VRATA MATERNICE: ISKUSTVO RIJEKE

SAŽETAK

Cilj ovoga retrospektivnog rada uključuje analizu preživljavanja u 661 bolesnice s rakom vrata maternice obzirom na dva razdoblja, 1990–1996 i 1997–2003 kao i specifičnih čimbenika rizika obzirom na stadije bolesti. Petogodišnje preživljavanje prve skupine iznosilo je 71,7%, dok je u drugoj skupini ono iznosilo 80,0%. Analizirajući čimbenike rizika u univarijatnoj, te u multivarijatnom regresijskom modelu naposljetku samo dva parametra, vremenska razdoblja i FIGO stadij imaju nezavisni prognostički značaj. Opaženo bolje preživljavanje u drugom vremenskom razdoblju praćeno je povećanjem konzervativnih operativnih zahvata u FIGO stadiju IA1, smanjenjem primjene adjuvantne radioterapije među bolesnicama operabilnog stadija T1b1m T1b2 i T2A, dok se liječenje lokalno uznapredovanog raka vrata maternice nije bitno razlikovalo obzirom na vremenska razdoblja.

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Original scientific paper

Laporovaginal Surgery in Cervical Cancer: A Croatian experience

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A B S T R A C T

With correct staging a large number of patients with cervical cancer FIGO stages IA2 and IB can be spared of unnecessary radiation therapy by laparoscopic assisted vaginal radical hysterectomy (LAVRH) as an option of radical surgical treatment in such patients. The development of laparovaginal surgery, indication and contraindication were presented. Also, the surgical technique was described in detail. Fifty-two patients were followed up in 2003 after LAVRH or open surgery, performed in our single center. Only 5 (14%) patients died from cervical cancer within 3 years following the treatment. They were all clinical stage IB treated with open surgery. There were 4 (11%) complications following treatment and they were all in patients with clinical stage IB, also treated with open surgery. There was no complication in LAVRH treated patients. The results and complications of the sole Croatian center performing LAVRH or open surgery in patients with cervical cancer FIGO stages IA and IB were similar to those in centers across the world.

Key words: *cervical cancer, FIGO stage IA2 and IB, laparoscopic assisted vaginal radical hysterectomy, Schauta operation*

Introduction

Development of vaginal surgery

The first written traces on surgical treatment of cervical carcinoma reach back to the 16th and 17th centuries[1] when amputation of cervical tumors was performed. At the end of the 18th century, Marschall and Schroder as well as Osiander, at the beginning of the 19th century, recommend »partial trachelectomy« in patients with prolapsing cervical carcinoma[1]. In 1813, Langenback performed »subperitoneal removal« of prolapsed cervical carcinoma[1,2]. He used tying of blood vessels as hemostasis. In 1821, Sauter operated a patient with cervical carcinoma through the vagina, by opening the peritoneum and removing the uterus without the use of ligatures in bleeding control. The patient developed vasicovaginal fistula and died four weeks following the surgery for unknown reasons, even though the author did not mention perioperative bleeding[3]. This type of surgical technique was developed for the following twenty years. The five-year survival rate for all stages of the disease was around 10%[1]. By developing surgical technique (haemostatic clip) and introducing Lister's aseptic principles, Czerny reduced perioperational mortality to 32% [1,4]. In 1880, the Czech gynecologist Pawlik performed extended vaginal hysterectomy, in a patient with advanced cervical carcinoma[4]. Thirteen years later, Schuhardt described radical vaginal hysterectomy with help of »paravaginal help incision«[4].

Staude also performed extended vaginal hysterectomy in 1894, using two Schuhardt's incisions to improve the operating approach[5]. At this time, Reis, Rumpf, Kelly and Wertheim developed abdominal radical hysterectomy as a method of choice in treating cervical carcinomas. They emphasize that the mere removal of the organ is not sufficient but it is necessary to remove the regional lymph nodes as places of supposed spreading of the disease[1,4,6,7]. However, high perioperative mortality (18.6%) in that period distanced most gynecologists from the laparotomy approach. In June 1901, in Vienna, Schauta performed the first radical hysterectomy through the vagina, and published his experience in treating cervical carcinoma in 258 cases in 1908[8]. Since that time, radical vaginal hysterectomy has mostly been called Schauta's operation, particularly emphasizing preparation of the urinary bladder and preserving the ureter. Amreich fur-

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ther improved the original Schauta's operation. His experiences in 1505 cases were published in 1943[9]. In comparison to Schauta's operation, the Amreich's approach involves more radical resection of the dorsal part of lateral parametria. In Germany, Stoeckel modified Schauta's operation and introduced the principle of mandatory preparation of the ureter. While Schauta only inserted the ureter proximally, Stoeckel introduced systematic anatomical preparation of the ureter [10].

Bearing in mind the radical nature of removing the parametria, Wertheim and Schauta's operations are complementary. Both approaches, according to the Routledge classification, belong to the type II of radicalness, i.e. the medial half of cardinal and sacrouterine ligaments is being removed. The Mackenrodt-Latzko-Meigs operation is as radical as the Schauta-Amreich operation and, according to the Routledge classification, falls into the type II-II of radicalness[11,12]. Complete resection of the parametria by vaginal approach is not possible.

Development of laparoscopy

Lymphogenic spreading of cervical carcinoma is the primary route of transmission of the disease. In approximately 15% of stage I cervical carcinoma cases metastases into pelvic lymph nodes were detected. As far back as 1895, Ries supposed that the removal of iliacal lymph nodes should be the key part of surgical treatment of cervical carcinomas[1,4].

With time, individual authors combined lymphadenectomy with radical vaginal hysterectomy. Stoeckel combined radical vaginal hysterectomy with transperitoneal pelvic lymphadenectomy in individual cases[10,13]. Mitra in India and Navratil in Austria, combined extraperitoneal lymphadenectomy according to Nathanson, with radical vaginal hysterectomy[13–15]. Mitra performed extraperitoneal lymphadenectomy three weeks after the vaginal operation. In Europe this method was accepted by van Bastiianse and Inquilla[1,13,16]. However, these operative techniques were not widely accepted because, at that time, the Wertheim's operation was the »golden standard« in surgical treatment of cervical carcinomas. Due to this fact, radical vaginal operation was »neglected« for a long time in treating cervical carcinomas. Development of laparoscopy enabled the reaffirmation of radical vaginal hysterectomy. In 1969, Bartel described pelvic retroperitoneoscopy. By using the Carlens' mediastinoscope, modified according to Maasen, through an incision cut along the spina iliaca anterior, he explored the retroperitoneal region ventrally from spinae[17]. In 1973, Wittmoser insufflated CO_2 into the retroperitoneum to have a better view when performing lumbar sympathectomy[18]. With time, the retroperitoneoscopic technique was improved. In 1987 Dargent, using the pelviscope (laparoscope), was the first to perform retroperitoneal interiliacal lymphadenectomy in the same session as radical vaginal hysterectomy[19]. He was the first to actualize the extended vaginal hysterectomy. Two years later, Querleu introduced transperitoneal pelvic lymphadenectomy laparoscopically. He removed the »interiliacal lymph node packet« between the arteria umbilicalis and the vena iliaca externa laparoscopically[20]. Canis was the first to publish »endoscopic radical hysterectomy type II« in 1990[21]. A year later, Querleu published the »laparoscopically assisted vaginal radical hysterectomy according to Schauta«[22]. At that time Dargent improved the operational technique. He laparoscopically resected the proximal and dorsal part of the lateral parametria, prepared the ureter vaginally and standardized this surgical operation in 1992 as the laparoscopically assisted radical vaginal hysterectomy (LAVRH)[23–25].

Operative Procedures

Laparoscopic lymphadenectomy and radical vaginal hysterectomy – LAVRH

Two surgeons operate, each operating a contralateral side, the patient is in the lithotmic position (»supine position«). The operation is performed under general anesthesia and indwelling of the urinary catheter is mandatory. Through an infraumbilical incision, a 10 mm trocar is inserted and through it, a telescope. Under visual control we insert additional 5 mm of the trocar, one in the medial line, two transversal fingers above the symphysis and two lateral at around 3 cm, laterally and cranially from the *arteria epigastrica inferior*. Our approach and operative technique are based on the experiences of Possover and Schneider[26–30]. The operation begins with inspection of the whole abdomen. The patient is then put into the Trendelenburg's position at 30 degrees. Using the bipolar electrodes and scissors, we mobilize the caecum and the sigma in order to detach them from the lateral pelvic wall. The right side is operated first. After this, using the bipolar electrode and the scissors, we perform resection of the *ligamentum teres uteri* (Figure 1). With the help of the blunt preparation procedure, we open the Wagner's pit and show the *musculus iliopsoas*. Then we identify the *ligamentum umbilicale laterale* (obliterated *arteria umbilicalis*) (Figure 2). Using firm grasp forceps, we grip that ligament and pull it medially. Medially and ventrally from it, where the urinary bladder is situated. Pushing the urinary bladder medially, we secure access to the interiliacal lymph nodes and the *fossa obturatoria*. Then we show the *arteria iliaca externa* and remove it from the muscle iliopsoas. The fat tissue with lymph nodes is situated between the muscle iliopsoas and the *arteria iliaca externa*. Laterally from this tissue, there is the *nervus genitofemoralis* requiring extra precautions. During this preparation caution is also required with numerous branches of *arteria iliaca externa*, which need to be coagulated. The lower limit of dissection of the lymph nodes along the *arteria iliaca externa* is the *vena circumflexa ilium profunda*, which is situated dorsally from the *ligamentum inguinale*. By pulling the fat tissue with the lymph nodes dorsally, using blunt preparation we show the *vena iliaca externa*. Laterally and dorsally from it, there is the nervus obturatorius which is showed with blunt preparation (Figure 3). Medially from the nerve in the fat tissue, the surface

obturatory lymph nodes are situated, and laterally are the deep obturatory lymph nodes. The lateral dissection limit is the *fascia musculi obturatori internusa*. In the obturatory opening, along with the nerve, the arteria and vena obturatoria also enter. If these blood vessels are injured during preparation, they can be coagulated with no fear. During the preparation of the obturatory nerve, it should not be injured because its injury causes paresis of the adductor mucles of the upper leg. After »cleaning« the obturatory pit, the fat tissue with the lymph nodes »en block« is being pulled proximally and laterally. In the triangle between a. and v. *iliaca externa* and the a. and v. *iliaca interna*, caution is required because this region contains numerous branches of the v. *iliaca interna*. By pulling the fat tissue with the lymph nodes further proximally, by blunt preparation with coagulation with bipolar electrodes we come to bifurcation of the arteria iliaca communis. This is the upper limit of dissection. Thus, »en block« resection of the iliacal, interiliacal and obturatory lymph nodes has been performed (Figure 4).

The same procedure is repeated on the left side. Following this, we go on to the mobilization of the ureter and proximal resection of the parametria. By removing the obturatory lymph nodes, we show the *fossa paravesicalis* (Figure 5). Mobilization of the ureter shows the starting point of the *arteria iliaca interna*. Dorsally and caudally from the *arteria uterina* is the *fossa pararectalis*. The *fossa pararectalis* and the *fossa paravesicalis* are divided by the *ligamentum cardinale*. When the *ligamentum cardinale* is optimally shown using blunt preparation and coagulation with a bipolar electrode, the resection is performed »step by step« exclusively on the vascular part of the cardinal ligament. When performing the resection, we have to be careful about the *pars nervosa ligamenti cardinali* because there the *nervi splanchnici pelvini* are situated, which are very important for normal functioning of the rectum and urinary bladder. In case there are problems with bleeding, we can easily coagulate the *arteria iliaca interna*. The cardinal ligament is resected caudally to the level of the *arteria iliaca interna*. Then we coagulate and cut the *arteria uterina* on the level of the starting point of the *arteria iliaca interna*. Then, in several steps, we resect the proximal (supraureteral) part of the »bladder pillars« and after this, using bipolar electrodes and scissors, we detach the peritoneum of the urinary bladder from the uterus (*plica vesicouterina*)[28,29]. This finishes the laparoscopic part of the surgery and we proceed with the transvaginal part of the surgery.

The patient is put into the position for a vaginal operation (candy kane position). The vaginal membrane is clamped with six straight traumatic clamps and infiltrated with vasocontricting solution (2% mixture of xylocain and adrenaline). Using a monopolar electrode, we circumcise the whole circumference of the uterus to the dorsal and ventral vaginal fascia. We circumcise 1.5–2 cm of the uterine membrane from the vaginal-cervical point of connection. A »vaginal cuff« is closed with an extended stitch, then we remove the urinary catheter. By pulling the vaginal cuff ventrally, we show the septum rectovaginale.

By pulling the vaginal cuff caudally and dorsally using scissors, we detach the dorsal side of the urinary bladder from the uterus and the cervix (Figure 6). The preparation must be exactly in the medial line to the *plicae vesicouterinae* that was previously laparoscopically preparated. When we have completely detached the bladder from the vagina and the uterus a Breisky speculum is introduced, and the bladder is pushed ventrally. Then we proceed with opening of the *fossae paravesicale*. The medial side of the bladder pillars was shown during the preparation of the urinary bladder. Now preparation of the lateral side follows.

The vaginal membrane is clamped with Kocher clamps on the left side at »1 and 3 o'clock« and on the right side at »9 and 11 o'clock«. Using scissors, we make blunt preparation in the lateral-dorsal direction, thus detaching the lateral part of the bladder pillars from the endopelvic fascia. After this, identification of the ureter follows. The ureter is identified with help of the »click manoeuvre«[30]. Namely, the collateral index finger is set on the medial side of the bladder pillars and a Peham clamp on the lateral side in a way that top of the clamp is directed to the laterodorsal direction. Between the clamp and the finger, we can clearly feel the ureter. By moving the Peham clamp dorsomedially and using the finger to push the ureter cranially, we detach the ureter from the distal part of the bladder pillars. When we have showed the ureter, we resect and bind the distal part of the bladder pillars (Figure 7). Using the ureteral clamp, we pull the ureter caudally and see if there is some unresected supraureteral part of the bladder pillars which are then resected, using a clamp, and then bound. Then we introduce a Breisky speculum into the Douglas and push the rectum dorsally. At the same time, using another Breisky speculum, we push the bladder and the ureter ventrally. In this way we show the rectal pillars that are gripped by a strong clamp (Wertheim clamps), we cut and bind. In this way the preparation is completely removed (Figure 8).

The *sacrouterine ligamentum* is fixated to the back membrane of the vagina using a 2.0 vycril stitches and the uterus is closed using individual vycril 2.0 stitches.

In the end, the intraabdominal operation field is checked laparoscopically and drained using the drain 14Ch through a suprapubic cut.

Laparoscopically assisted radical vaginal trachelectomy – LAVRT

Cervical carcinomas are spread primarily by metastasing into the regional lymph nodes and infiltrating into the parametria. Vertical invasion into the uterus body is relatively rare and described only in cases when the primary tumor is > 4 cm in diameter. Patients suffering from cervical carcinoma of the FIGO stage of the disease IA2 and IB1 have a 5–15% chance of metastasing into the regional lymph nodes. In the IA2 stage of disease, risk of invasion into the parametria is negligible[31]. Therefore, the fertile ability of those patients can be preserved. It is sufficient to perform thorough conization

LAVRH – laparoscopic assisted vaginal radical hysterectomy

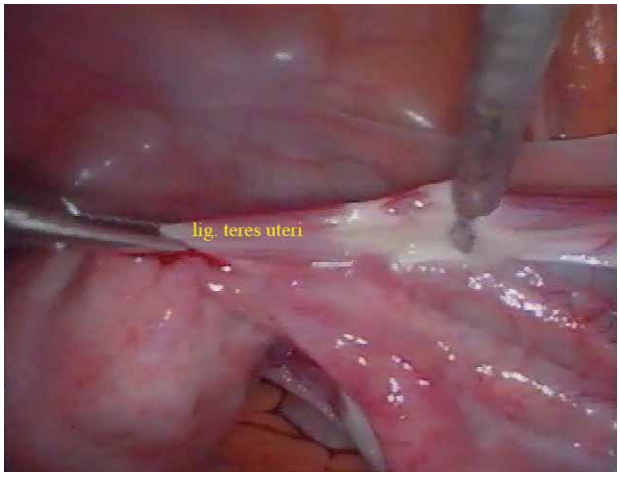

Fig. 1. Coagulation and resection of the ligamentum teres uteri during LAVRH.

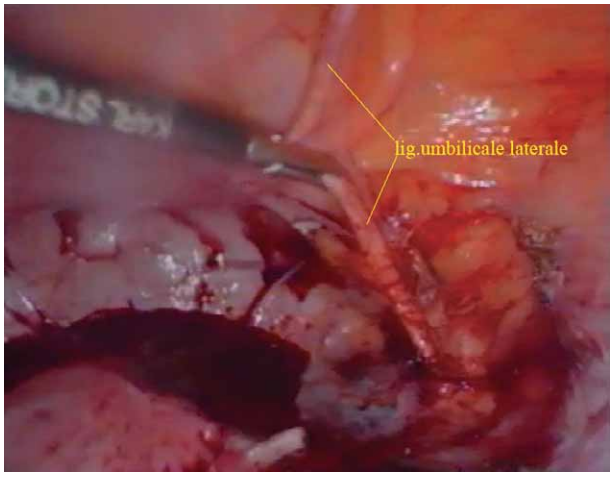

Fig. 2. Identifying of the ligamentum umbilcale laterale during LAVRH.

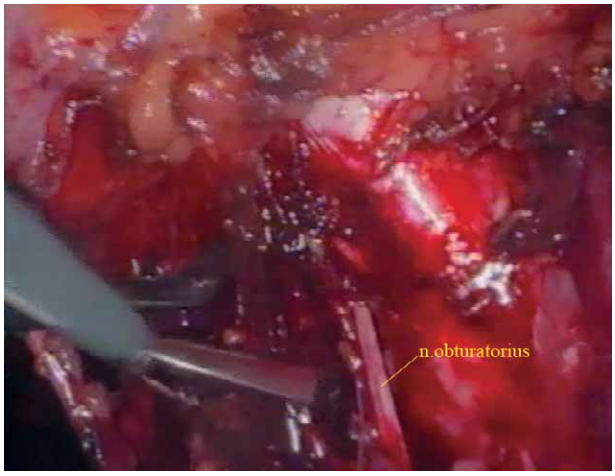

Fig. 3. Preparation of the nervus obturatorius during LAVRH.

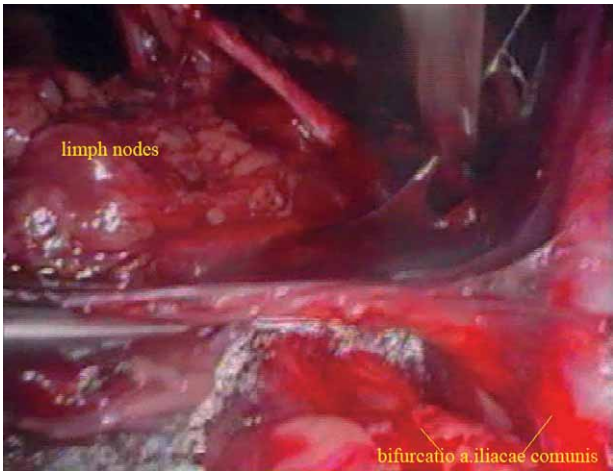

Fig. 4. Bifurcation of the arteria iliaca communis – the upper limit of dissection in LAVRH.

and laparoscopic pelvic lymphadenectomy in cases where edges and top of the conus are free, and the lymph nodes negative, for this stage of the disease[31].

At the IB1 stage of the disease risk of invasion into the parametria grows with size of the tumor and depth of penetration into the stroma[31]. However, fertility can be preserved in this stage of the disease as well. In 1995, Dargent was the first to report on a modified Schauta-Amreich radical vaginal surgery where the body of the uterus was preserved. He called this new operational technique radical vaginal trachelectomy[32]. For the past ten years several authors have reported on their experience with radical vaginal trachelectomy combined with laparoscopic pelvic lymphadenectomy[33–35]. Debates on which indications point to LAVRT are led as long as today. Burnett has analyzed results of four large series with a total of 152 patients with the follow-up period of 23–47 months. Four (2.5%) patients have developed a relapse within the follow-up period[35]. Similar results were published by Dargent [36].

Disease relapses occurred in patients where the primary tumor was > 2 cm. Hence, most authors consider that LAVRT is indicated for patients under the age of 40 with cervical tumor < 2 cm and who want to preserve their fertility. Presurgical procedures include the MRI for accurate assessment of the tumor size or possible invasion into the parametria.

The surgical technique is similar to that of the LAVRH. First, laparoscopically, we remove all pelvic lymph nodes. Frozen section is obligatory. In case the lymph node is positive, the operation is discontinued and the patient is referred to chemoirradiation therapy or LAVRH is performed. In case the removed lymph nodes are negative,

LAVRH – laparoscopic assisted vaginal radical hysterectomy

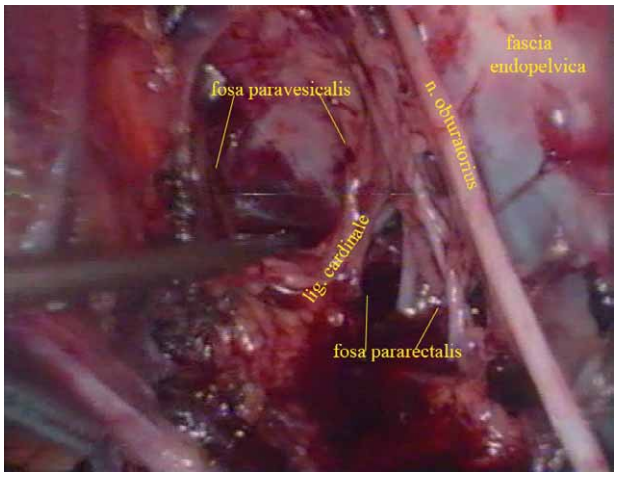

Fig. 5. Fossa paravesicalis and pararectalis after removing obturatory lymph nodes in LAVRH.

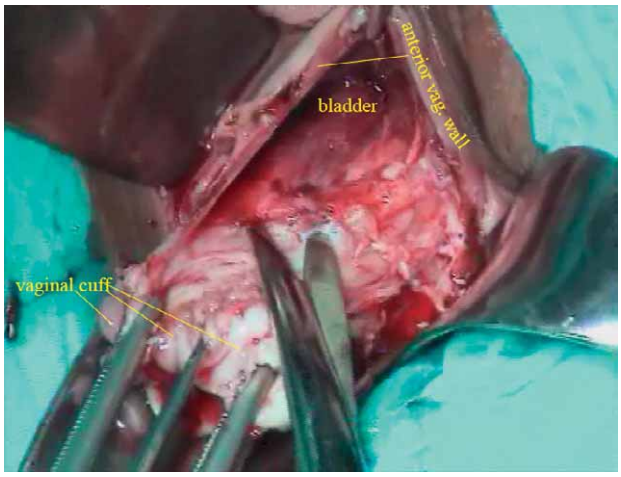

Fig. 6. Detaching the dorsal side of the

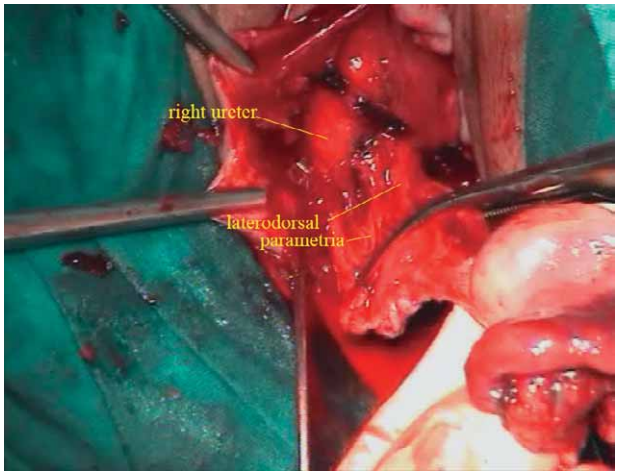

Fig. 7. Right ureter and resection of the laterodorsal parametria during LAVRH.

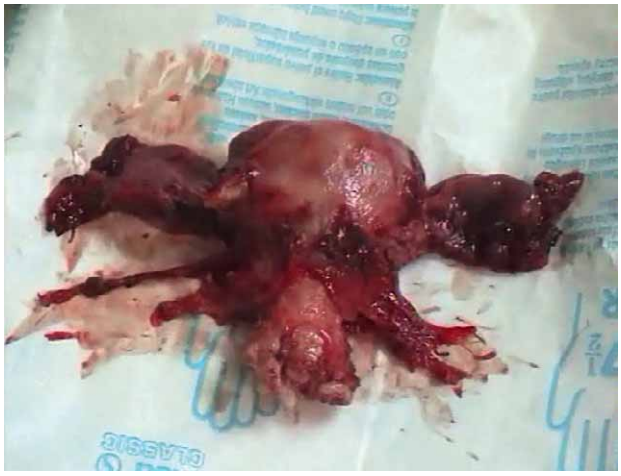

Fig. 8. Specimen of the resected uterus after

the vaginal part of the procedure is continued. The preparation should ideally contain 1–2 cm of the uterus, the whole cervix and 1–2 cm of the lateral parametria. After removing the preparation, frozen section is performed. In cases where edges of the preparation are free the procedure is finished by reconstructing the vagina and forming the »new exocervix«, using the classical Sturmdof technique.

In cases where edges of the preparation are positive to frozen section, LAVRH is to be performed. All risks involved must be carefully explained to the patient prior to the procedure.

Numerous pregnancies have been described in patients who underwent LAVRT but with a large number of spontaneous abortions (40%) and a significant number of premature births[37].

LARVT is a new surgical technique and represents an important step in the development of surgical policy on treating cervical carcinoma. It requires enormous experience of the surgeon, both in the area of vaginal surgery and in the area of invasive (radical) laparoscopic pelvic surgery. Therefore, LAVRT is to be performed exclusively in highly specialized institutions that have a well tuned-in surgical team and an expert pathology team when interpretation of the frozen section pathohistological results is concerned.

Results and Discussion

In our single center 52 patients with cervical cancer FIGO stage IA and IB underwent either open surgery or LVRH in the 2003 and were followed up for at least 3 years. The distribution of patients according to clinical

stage, infiltration of parametrium, type of operation, number of lymph nodes removed is presented (Table 1 and 2). Pathohistological findings include 10 (19%) patients with *adenocarcinoma*, 8 (16%) with *adenosquamous carcinoma* and 34 (65%) with *planocellular carcinoma.* The survival rate and time period to recurrence of the disease according to clinical stage is presented (Table 3). Only 5 (14%) patients died from cervical cancer *within 3 years following the treatment.* They were all FIGO stage IB and all underwent open surgery. There were 4 (11%) complications following treatment and they were all in patient FIGO stage IB, also only in open surgery treated patients. Two postirradiation ileitis and two hydronefrosis were reported. The distribution of complications following treatment according to the type of surgery is presented in Table 4. There were no complications in patients treated with LAVRH.

It was already mentioned that Wertheim performed the first radical surgery of cervical carcinoma in 1898. The same year, Marie Curie discovered radium, the application of which in medicine began four years later[1,37]. A whole century has passed in debates on which modality of treating cervical carcinomas (surgical or via radiation therapy) is more efficient. While gynecologists and oncologists agree that, for stages of the illness of IIB and above, chemoirradiation is the optimal choice, there has been continuous doubt about which modality of treatment is best for stages IB and IIA. Landoni tried to resolve these doubts. According to his research, the survival rate of patients with stages IB and IIA depends exclusively on the size of their tumor, not on the modality of treatment[38]. The results of our study which include patients treated by open surgery or LAVRH support this conclusion because we show that survival rate in 3 years for patients FIGO stage IA2 is 100% regardless the type of surgery and 91.7% in patients with FIGO stage IB1 treated with LAVRH (Table 3).

The benefits of surgical treatment need to be pointed out: preservation of the ovarian function and preservation of the vaginal function (lubrication, stenosis). Apart from this, together with correct staging, a large number of patients with disease stages IB and IIA can be spared of unnecessary radiation therapy[39]. Therefore, Dargent emphasizes: »Surgery in comparison with radiotherapy is a single step treatment: instant treatment with complete response obtained in a few hours«[37].

Most gynecologists including our team today consider the primary surgical treatment a method of choice for tumors < 4 cm. With respect to the fact that only 15% of cervical carcinomas of this size have regional metastases, this stage of disease is ideal for »surgical-pathological staging«. Regardless of the fact that FIGO has not accepted it so far. This approach includes postoperative radiation of patients whom are found to have: metastases into lymph nodes or infiltration of the parametria or infiltration > ½ of the cervix thickness or lymphovascular space involvement (LVSI). This information is obtained only after a radical surgery. This is the advantage of the laparo-vaginal approach. Namely, a surgeon starts the operation with laparoscopic pelvic lymphadenectomy. During the procedure »ex tempore« the removed lymph nodes are subject to pathohistologic examination. In cases where metastases in the lymph node are found, the op-

TABLE 1
THE DISTRIBUTION OF PATIENTS ACCORDING TO CLINICAL STAGE OF CERVICAL CANCER, MEDIAN AGE AND INFILTRATION OF PARAMETRIUM

Type of operation (N)	Clinical stage	Number of patients N (%)	Median age (year)	Infiltration of *parametrium* N (%)
Open surgery (38)	I A 1*	3 (8%)	37	0
	I A 2	12 (31%)	36	0
	I B	23 (61%)	43	3 (13%)
	Total	38	38.7	3 (8%)
LAVRH (14)	I A 2	2 (14%)	34	0
	I B1	12 (86%)	40.5	1 (8%)
	Total	14	37.4	1 (7%)

*radical surgery was performed only when lymphovascular space involvement (LVSI) was positive in cone biopsy

TABLE 2
THE DISTRIBUTION OF TYPE OF SURGERY, NUMBER OF REMOVED LYMPH NODES ACCORDING TO CLINICAL STAGE OF CERVICAL CANCER

Type of operation (N)	Clinical stage	Type of surgery		Number of removed lymphnodes	Mean number of lymph nodes per patient
		Rutledge II N (%)	Rutledge III N (%)		
Open surgery (38)	I A 1*	1 (7%)	2 (8%)	42	21
	I A 2	10 (71%)	2 (8%)	211	17.5
	I B	3 (21%)	20 (84%)	454	19.7
	Total	14 (37%)	24 (63%)	707	19.4
LAVRH (14)	I A 2	2 (100%)	0	35	17.5
	I B1	0	12 (100%)	228	19.0
	Total	2 (14.3%)	12 (85.7 %)	263	18.8

*radical surgery was performed only when lymphovascular space involvement (LVSI) was positive in cone biopsy

TABLE 3
THE DISTRIBUTION OF PATIENT ACCORDING TO NUMBER OF METASTASIS, RECURRENCE OF DISEASE FROM CERVICAL CANCER AND SURVIVAL RATE

Type of operation	Clinical stage (N)	Patients with metastasis N (%)	Total number of metastasis	Patients with recurrence disease (N)	Period (months)	Survival rate (3-years)
Open surgery	I A 1* (3)	0	0	0	0	100%
	I A 2 (12)	0	0	2	17	100%
	I B (23)	6 (26%)	16	7	19	78%
LAVRH	I A 2 (2)	0	0	0	0	100%
	I B (12)	2 (17%)	3	1	18	91.7%

LAVRH – laparoscopic assisted vaginal radical hysterectomy, *radical surgery was performed only when lymphovascular space involvement (LVSI) was positive in cone biopsy

TABLE 4
THE DISTRIBUTION OF COMPLICATION ACCORDING TO THE TYPE OF OPEN SURGERY OF CERVICAL CANCER

Type ofsurgery (N)	Postirradiat*ion ileitis* N (%)	*Hydronephrosis* N (%)	Total N (%)
Rutledge II (15)	0	1	1 (6.7%)
Rutledge III (23)	2	1	3 (13.0%)
Total (38)	2 (5%)	2 (5%)	4 (10.5%)

eration is discontinued and the patient is referred to chemo-irradiation treatment. In cases where there are no metastases in the lymph nodes, radical vaginal hysterectomy is continued. If the already mentioned risk factors are found on the removed preparation, the patient is referred to additional radiation treatment. There is no evidence that radio-therapy treatment following LAVRH has the higher morbidity rate than following open abdomen operations[38]. Patients with a tumor < 2 cm are ideal for this operation. Namely, regardless of histologic type of the tumor, if parametria, lymph nodes and LVSI are negative, there is no chance of a relapse.

In tumors > 2–4 cm we need to be more cautious in setting the indication for LAVRH. In such patients, in spite of the negative lymph nodes and negative LVSI, the relapse risk is around 15%. If metastases were detected in pelvic lymph nodes, or there is a case of histologically aggressive tumor (small cell cancer, neuroendocrine cancer), relapse of the disease is present in 30–50% of the cases[26].

Contraindications for LAVRH include a tumor > 4 cm or poor histologic type. When assessing the tumor size, only MRI is used. The presence of enlarged regional lymph nodes does not necessarily represent a contraindication for LAVRH. In case the MRI shows an enlarged lymph node, laparoscopy is indicated. During the procedure, the lymph node is removed and sent to urgent histologic examination. If the frozen section result is negative, laparoscopic lymphadenectomy is continued, followed by radical vaginal hysterectomy. If the lymph node is positive, the operation is discontinued and the patient is referred to an oncologist for chemoirradiation. In case during laparoscopy we find a »bulky« lymph node, it is recommended to remove it and mark its spot with metal clips to ease the radio-therapist's plan for radiotherapy.

Several key questions arise here: (1) Can the frozen section be given absolute trust? In case the lymph node is positive, (2) does the uterus need to be preserved for future brachytherapy? (3) Do we need to continue with lymphadenectomy because the removal of tumorous metastases makes the following radiation treatment more effective? (4) Do we need to continue the LAVRH or (5) go on with the open abdomen surgery?

So far, we have no clear answers to these questions but most gynecologists-oncologists believe that in such situations »pelvic clearance« needs to be performed, and then the patient referred to further chemoirradiation treatment.

The efficacy of LAVRH was shown in a series of 200 patients with FIGO stage IA1 to IIB cervical cancer. This is the largest reported study[40]. They reported follow up for at least 40 months and overall survival rate was 83 %. Comparing our overall survival rate in patients with cervical cancer FIGO stage IA2 and IB which was 92.8% show that we had the same success with the same complications rate. We did not perform this procedure in patients with cervical cancer FIGO stage IIB, so this was robably the reason why our survival rate is slightly higher than in the study by .[40] One possibility why we did not have complication in LAVRH treated patients is the small number of procedures (14), but the lack of complications in LAVRH treated patients supports the conclusion that with correct staging a large number of patients with cervical cancer FIGO stages IA2 and IB can be spared of unnecessary abdominal radical surgery; because the survival rate, number and severity of complications is almost the same[41,42]. Several other observational studies support these conclusions[40–46]. We had 4 (10.5%) complications in open surgery treated patients which were not different from other comparing studies between these two operative types.[41,42,46]

Conclusion

With correct staging a large number of patients with cervical cancer FIGO stages IA2 and IB can be spared of unnecessary abdominal radical surgery and treated with LAVRH because the survival rate, number and severity of complications is almost the same. Our results and experiences from the sole Croatian center performing LAVRH or open surgery in patients with cervical cancer FIGO stage IA2 and IB were similar to those in centers across the world. Primary surgical treatment is a method of choice for tumors of the cervix < 4 cm.

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ULOGA LAPAROVAGINALNE KIRURGIJE U KIRURŠKOM LIJEČENJU RAKA VRATA MATERNICE

SAŽETAK

Korektnim određivanjem stadija bolesti prema FIGO klasifikaciji veliki broj bolesnica s rakom vrata maternice stadija IA2 i IB mogao bi se poštediti nepotrebnog radikalnog abdominalnog kirurškog liječenja gdje je i laparoskopski asistirana vaginalna radikalna histerektomija (LAVRH) moguća. Prikazani su povijesni pregled, indikacije i kontraindikacije za LAVRH. Kirurška tehnika LAVRH je detaljno opisana. 52 bolesnice su praćene tijekom 2003. godine nakon provedenog liječenja LAVRH-om ili abdominalnom radikalnom kirurškom operacijom izvedenom u našem centru. U razdoblju praćenja 5 (14%) bolesnica je umrlo od raka vrata maternice i sve su bile FIGO stadija IB, liječene abdominalnom radikalnom operacijom. Prikazane su 4 (10.5%) komplikacije, sve u bolesnica s FIGO stadijem IB liječenih abdominalnom radikalnom operacijom. U bolesnica liječenih LAVRH-om nisu zabilježene komplikacije. Preživljenje, kao i broj komplikacija nakon LAVRH ili radikalne abdominalne operacije, u našem centru u Hrvatskoj, ne razlikuju se u usporedbi s drugim svjetskim centrima.

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Short communication

Adjuvant Therapy after Radical Surgery of Cervical Cancer: Zagreb Experience

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A B S T R A C T

The results of the analysis of the treatment of 72 patients with carcinoma of the uterine cervix are presented. Seventy-two patients with Stage IB1 carcinoma of the cervix underwent a radical hysterectomy and pelvic lymphadenectomy. The low-risk group includes the patients without unfavourable prognostic factors that were treated by surgery alone. The high-risk group included women with pelvic node metastases, clinical tumour size greater than 3.0 cm, depth of stromal invasion greater than 1/3 of the cervical wall, Grade 3 tumours and the presence of lympho-vascular space involvement. High-risk patients received whole pelvic radiotherapy between two and four weeks following surgery. Thirty-four patients (47.2%) were in the low-risk group and thirty-eight patients (52.8%) were in the high-risk group. Locoregional recurrences were diagnosed in three cases (8.8%) in the surgery group and in four patients (10.5 %) assigned to postoperative radiotherapy. The incidence of distant metastases was 2.9% in the group treated by surgery alone and 5.3% in the group treated by surgery and radiotherapy. Overall survival at five years was 91.2% in the low-risk group and 89.5% in the high-risk group of patients. Five-year overall survival, locoregional and distant metastases were similar in the low-risk and high-risk groups of patients, which emphasizes the value of whole pelvic radiation in patients with one or more unfavourable prognostic factors after radical surgery in Stage IB1 cervical cancer.

Key words: *cervical cancer, risk factors, adjuvant therapy*

Introduction

Uterine cervical cancer is the most common gynecological cancer worldwide with a yearly incidence of 500, 000 cases[1]. It is an important women's health problem in developing countries. Risk factors for cervical cancer include early onset of sexual activity, multiple sexual partners, lower socio-economic group and history of sexually transmitted disease. Human papilloma virus has been implicated as the major causative agent in this disease. Squamous cell carcinomas account for 80–85% of cases with adenocarcinoma and adenosquamous carcinomas responsible for 15% and 3–5%, respectively[2]. At the FIGO Congress in Montreal 1994, the Gynecologic Oncology Committee made some changes in the staging for cervical cancer. Stage IB comprises patients with microscopic stromal invasion more than 5.0 mm or with horizontal spread more than 7.0 mm and clinically visible lesion confirmed to the cervix. Stage IB1 presents clinical lesions no greater than 4.0 cm in size and Stage IB2 clinical lesions greater than 4.0 cm in size[3]. Signs range from abnormal cervical smear only to a cervix with exophytic or crater-like type lesions. Symptoms include abnormal vaginal bleeding, postcoital spotting and vaginal discharge.

The algorithm for the management of Stage IB cervical cancer includes radical hysterectomy and pelvic lymphadenectomy. Patients with lymph node metastases are treated by postoperative adjuvant pelvic radiation[4]. Positive surgical margins, parametrial involvement, tumor diameter, depth of stromal invasion, tumor grade and lympho-vascular space involvement are also risk factors for recurrences. Gynecology Oncology Group (GOG) study in 1990, reported clinical tumor size, depth of invasion of the cervix and lympho-vascular space involvement as

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independent prognostic factors. Patients with negative lymph nodes in Stage IB cervical cancer have 25% high risk factors[5]. Another GOG study in 1999, suggested that postoperative pelvic radiation reduced the risk of recurrences in patients with at least two risk factors: large tumor diameter, more than 1/3 stromal invasion and lympho-vascular space involvement[6]. Women with Stage IB or II cervical carcinoma with lymph node metastases post Wertheim hysterectomy and pelvic lymphadenectomy who were given adjuvant radiotherapy treatment had better survival than those undergoing surgery only in a multivariate analysis. Patients without pelvic lymph node metastases but with parametrial extension, tumour size greater than 4 cm, full thickness cervical stromal invasion and DNA index more than 1. 3 had significantly better five-year recurrence free survival rate if receiving postoperative radiotherapy[7].

In previously published studies patients with cervical cancer stage IB with unfavourable prognostic factors were classified in two groups, those who were and those who were not treated by radiotherapy after surgery. The five-year survival was significantly higher in those women who were treated compared to non-treated women by radiotherapy after surgery. Herein, we evaluate the whole pelvic radiation in women with vs. those without unfavourable prognostic factors after surgery of cervical cancer stage IB1. The recurrence of the disease was the endpoint of this evaluation.

Subjects, Material and Methods

Seventy-two patients were included into the study with a mean age 44.2±10.4 years. Initial evaluation included medical history, Pap smears and pelvic examination. When cytology has shown malignant cells in patients with no visible tumour we made diagnostic conization. In patients with clinically visible lesions confined to the cervix, diagnosis was confirmed by a directed punch biopsy. Preoperative evaluation included physical examination, complete blood count, blood chemistry tests, chest radiography, intravenous pyelogram, cystoscopy and sigmoidoscopy. All patients were treated by Wertheim hysterectomy and pelvic lymphadenectomy. When no residual tumour was found in the radical hysterectomy specimen, presurgical data from cone biopsies were used. The diagnoses of squamous cell carcinoma, adenocarcinoma or adenosquamous cervical cancer were made by a pathologist.

Low-risk group include the patients without unfavourable prognostic factors and they were treated only by surgery. High-risk group includes women with pelvic node metastases, clinical tumour size greater than 3.0 cm, depth of stromal invasion greater than 1/3 cervical wall, Grade 3 tumours and the presence of lympho-vascular space involvement. High-risk patients received radiotherapy between two and four weeks following surgery.

Postoperative radiotherapy was administered to the pelvic region according to a standardised protocol. The radiation was delivered by 4 fields' box technique or by *anteroposterior* and *posteroanterior* parallel-opposed pair of fields, using linear accelerator or cobalt-60 unit. The total tumour dose was 40–48 Gy in 20–24 fractions using 2 Gy daily fractions, five days a week.

Patients were evaluated by physical examination, ultrasound, Pap smear, blood counts, blood chemistries and chest radiography every three months for the first two years, every six months during the next three years and then annually. Computed tomography scan with contrast or magnetic resonance imaging of the abdomen and pelvis were done at six months and then yearly. Sites of recurrence were classified as local if detected in the pelvis or vagina, and distant if detected in extra-pelvic locations.

The differences were evaluated by Chi-square test with statistical significance set at $p<0.05$.

Results

From January 1995, to December 2001, seventy-two patients with FIGO Stage IB1 cervical cancer were primarily treated by radical surgery. The tumour cell type was squamous in sixty-one (84.7%) women, adenocarcinoma in nine (12.5%) and adenosquamous carcinoma in two (2.8%). Thirty-four patients out of 72 (47.2%) were low-risk and 38/72 (52.8%) were high-risk patients.

For the low-risk group of patients no further therapy was applied, while high-risk patients received postoperative whole pelvic radiation. The median interval between the operation and the first radiotherapy session was 24 days. During radiotherapy the patients were treated with medication or dietary measures, or both, for related symptoms.

Local recurrences in the pelvis or vagina were diagnosed in three patients (8.8%) in the group treated by surgery and in four patients (10.5%) treated by postoperative radiotherapy. Distant metastases involving the abdomen or lung, or both locations were analyzed. The incidence of distant metastases was 2.9% in the group treated by surgery alone and 5.3% in the group treated by surgery and radiotherapy (Table 1). No significant differences was observed (Chi-square=0,08, $p>0.05$).

In four patients there were grade 3 or 4 adverse effects, of which one out of 34 (2.9%) patient was treated by surgery alone and 3 out of 38 (7.9%) patients were treated by both surgery and radiotherapy.

TABLE 1
RECURRENCES OF IRRADIATED AND NONIRRADIATED GROUP OF PATIENTS

Site of recurrence	Surgery only (N=34)	Pelvic radiation (N=38)
Local	3 (8.8%)	4 (10.5%)
Distant	1 (2.9%)	2 (5.3%)
Total	4 (11.7%)	6 (15.8%)

TABLE 2
FIVE-YEAR SURVIVAL OF PATIENTS ACCORDING TO THE TREATMENT IN RELATION TO GOOD AND POOR PROGNOSTIC FACTORS

Prognostic factors	Number of cases	Pelvic radiation	5-year survival (%)
Low-risk group	34	No	91.2%
High-risk group	38	Yes	89.5%

Overall survival at five years was 91.2% in the low-risk group and 89.5% in the high-risk group of patients (Table 2); almost equal in both groups of patients.

Discussion

The role of postoperative irradiation was evaluated for patients with Stage IB cervical cancer with tumour-related risk factors. Three independent prognostic factors in the GOG study in 1990, clinical tumour size, depth of tumour invasion and lympho-vascular space involvement made GOG risk score. Score higher than 120 was correlated with 41% recurrence rate[5]. Sedlis et al. used a modification of the GOG scoring system and reported a 44% reduction of the risk of recurrences after adjuvant radiotherapy when a combination of three risk factors were present compared without postoperative irradiation[6]. Ayhan et al. reported that tumour size larger than 4 cm; lympho-vascular space involvement and vaginal involvement were independent prognostic factors in lymph node negative invasive cervical cancer. Depths of stromal invasion, parametrial, endometrial and myometrial involvement, however, were not independent prognostic factors. Women with one and two or more risk factors showed lower ratios of pelvic recurrences when receiving postoperative radiotherapy[8]. Pieterse et al. before 1996 received adjuvant radiotherapy for patients with pelvic node metastases, parametrial invasion or positive surgical margins. In 1997 they extended the indication for postoperative irradiation using a modification of the GOG scoring system. Patients with at least two of the three risk factors received total pelvic radiotherapy: pathologic tumour size greater than 40 mm, depth of invasion greater than 15 mm and presence of lympho-vascular space involvement. They found that a significantly larger percentage of the high-risk group of patients who did not receive radiotherapy had recurrence of disease (41% vs. 12%). Differences in five-year cancer specific survival and five-year disease free survival between the high-risk groups treated with radiotherapy and without radiotherapy after radical surgery were statistically significant[9]. In the recent study they found that adjuvant radiotherapy did not significantly increase the risk of bladder dysfunction, bowel symptoms, lymphedema and sexual function after 2-years follow-up[10]. Scharge et al. reported that lympho-vascular space involvement is an important prognostic variable and adjuvant pelvic radiation may decrease the risk of recurrence in this patients[11]. GOG study in 2005 suggests that pelvic radiotherapy after radical surgery significantly reduced the risk of recurrence and prolongs progression-free survival in women with Stage IB cervical cancer. Radiation appears to be particularly beneficial for patients with adenocarcinoma or adenosquamous carcinoma[12].

In another our study one hundred and forty eight patients with Stage IB squamous cell cervical cancer were primarily treated by radical surgery. Low-risk group included 70 patients without unfavourable prognostic factors that were treated by surgery alone. High-risk group included 78 women with one or more risk factors: pelvic node metastases, positive or close surgical margins, clinical tumour size greater than 4.0 cm, stromal invasion greater than 1/3 cervical wall, Grade 3 tumours and the presence of lympho-vascular space involvement. High-risk patients received postoperative whole pelvic radiation. Eleven (15.6%) women in the low-risk group developed cancer recurrences, nine (12.8%) local, and two (2.8%) distant. In high-risk patients there were sixteen (20.5%) cancer recurrences, eleven (14.1%) local and five (6.4%) distant. Overall survival at five years was 88.6% in the low-risk group and 84.7% in the high-risk group of patients[13]. More of recurrences in the high-risk group were seen in patients with IB bulky tumours and we reduced primarily radical surgery treatment for FIGO Stage IB1 cervical cancer.

In the recent study the low-risk group includes 34 patients without unfavourable prognostic factors who were treated by surgery alone. The high-risk group includes 38 women with one or more risk factors: pelvic node metastases, tumour size greater than 3.0 cm, stromal invasion greater than 1/3 cervical wall, Grade 3 tumours and presence of lympho-vascular space involvement. The high-risk patients received postoperative radiotherapy. Four (11.7%) women in the low-risk group developed cancer recurrences, three (8.8%) local, and one (2.9%) distant. In the high-risk group of patients there were six (15.8%) cancer recurrences, four (10.5%) local and two (5.3%) distant. Local relapses were treated by surgery and patients with distant metastases received chemotherapy. Women with squamous cell carcinomas received cisplatin-based regiments and patients with adenocarcinoma or adenosquamous type tumours received paclitaxel-based chemotherapy[14]. Five-year overall survival rate in the low-risk group treated by surgery and high-risk, surgery and radiotherapy treated group was similar, 91.2 and 89.5%, respectively. The adverse effects were not more expressed in the group of patients treated by adjuvant total pelvic irradiation after radical surgery. This emphasizes the value of whole pelvic radiation in patients with unfavourable prognostic factors in Stage IB1 cervical cancer.

Conclusion

In this study on a relatively small number of patients with cervical cancer stage IB1 with one or more unfavourable prognostic factors we intended to show the

value of postoperative adjuvant radiation. Our findings indicate that the number of local and distal metastasis were similar to those patients without unfavourable prognostic factors. The five year survival is also similar in both groups of patients. The adjuvant total pelvic irradiation after radical surgery might represent over treatment in some women but did not generate any side effects. From all these observations we consider that it is prove enough to practice whole pelvic radiation in patients with one or more unfavourable prognostic factors after radical surgery in Stage IB1 cervical cancer.

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ADJUVANTNA TERAPIJA NAKON RADIKALNE OPERACIJE RAKA VRATA MATERNICE

SAŽETAK

Prikazani su rezultati analize naše studije liječenja 72 bolesnice s rakom vrata maternice. Sedamdeset i dvije pacijentice s IB1 stadijem raka vrata maternice podvrgnute su radikalnoj histerektomiji i zdjeličnoj limfadenektomiji. Skupinu niskog rizika čine bolesnice bez nepovoljnih prognostičkih čimbenika i liječene su samo operacijom. Skupinu visokog rizika tvore žene s presadnicama u zdjeličnim limfnim čvorovima, veličinom tumora preko 3,0 cm, dubinom invazije strome većom od 1/3, nezrelim tumor ima i nazočnošću tumora u limfo-vaskularnim prostorima. Visoko rizične bolesnice primile su zračenje izvana na zdjelicu između dva i četiri tjedna nakon operacije. Trideset četiri bolesnice (47,2%) bile su niskog rizika, a trideset i osam (52,8%) ih je bilo visokog rizika. Lokalni recidiv bolesti je dijagnosticiran u tri slučaja (8,8%) u operiranoj skupini i u četiri bolesnice (10,5%) koje su određene za radioterapiju. Incidencija udaljenih metastaza bila je 2,9% u operiranoj skupini i 5,3% u operiranoj i zračenoj skupini. Peto-godišnje preživljenje je bilo 91,2% u skupini bolesnica s niskim rizikom, dok je u skupini bolesnica s visokim rizikom bilo 89,5%. Petogodišnje preživljenje, lokalne i udaljene metastaze bile su slične u bolesnica s niskim i visokim rizikom. Ovo ističe vrijednost zračenja zdjelice u bolesnica s jednim ili više nepovoljnih prognostičkih čimbenika nakon radikalne operacije IB1 stadija raka vrata maternice.

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