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High-risk HPV infection in Kosovar female population

DISSERTATION

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This dissertation was made at the Clinic of Obstetrics and Gynecology, University Clinical Center of Kosovo in Prishtina and at the University Hospital for Infectious Diseases “Fran Mihaljević” in Zagreb, Croatia.

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LIST OF ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
PV	papillomavirus
HPV	human papillomavirus
hr-HPV	high risk human papillomavirus
kb	kilo base
ORFs	open reading frames
ICTV	International Committee on Taxonomy of Viruses
L1	Late region 1
L2	Late region 2
VLPs	Virus like particles
E1-E7	early regions 1 to 7
p53	protein 53(tumor superior protein)
pRB	retinoblastoma protein
APC	antigen presenting cell
LC	Langerhans cell
KC	keratinocytes
DC	dendritic cell
IFN	interferon
IL	interleukin
TGF-b	transforming growth factor
MHC	major histocompatibility complex
NK	natural killer cell
CTL	cytotoxic T lymphocyte
CD4+	cluster of differentiations

Th1	T helper cell
CIN	cervical intraepithelial neoplasia
CC	cervical cancer
NILM	negative for intraepithelial lesions or malignancy
ASCUS	atypical squamous cells of undetermined significance
ASC-H	atypical squamous cells, cannot exclude high grade intraepithelial lesion
PAP	Papanicolaou smear
TZ	transformation zone
OC	oral contraceptives
IUD	intrauterine device
PCR	polymerase chain reaction
RT-PCR	real time- polymerase chain reaction
TMB	teramethylbenzidine
GP5+	general primer 5+
GP6+	general primer 6+
SD	standard deviation
CI	confidence interval

1. INTRODUCTION

Human papillomaviruses are small, double-strained DNA viruses that can infect cutaneous and mucosal epithelium in a large variety of vertebrates.

Up to date, more than 200 papillomavirus types were isolated, 120 types from humans, half of them can infect genital tract [1].

Human papillomaviruses cause warts and some type's cervical cancer and its precancerous lesions [2].

The onset of molecular cloning of HPV genome in 1980s made possible the study of an individual viral genes.

Worldwide population prevalence of HPV among women ranges from 2-44% [3]. These wide variations are explained by the divergence in the age range of the studied population and the sensitivity of the DNA assays used for HPV detection.

Age prevalence curve showed a clear peak in women under 25 years of age, with an subsequent decline, and increases again [4], with an second peak after, less prominent than first one and is observed in women older than 50 years old [5].

Cervical cancer is the second most common cancer in women, whereas in developing countries where 80% of them occur, it is even a principal cancer in women.

Nowadays, we have clear molecular and epidemiological data indicating that certain types of human papillomaviruses are the principal cause for cervical cancer [6,7]. This group is known as high risk HPV types (hr-HPVs).

1.1 Human papillomaviruses classification

Human papillomaviruses are small, non-enveloped, circular double strained DNA viruses, with an approximately 8 kb in size. Papillomaviruses can primarily infect cutaneous epithelia end mucosa of humans and other higher vertebrates.

Papillomaviruses (PV) were specified as a different family, Papillomaviridae, in the 7th Report of International Committee on Taxonomy of Viruses (ICTV). Human papillomaviruses are classified in five genera, named by Greek alphabet letters: alfa, beta, gamma, mu and nu [8] [Figure 1].

Human papillomaviruses DNA genome contain eight genes, encoding eight open reading frames (ORFs).

The onset of molecular cloning of HPV genome in 1980s made possible the study of an individual viral genes. Nevertheless, only in 1990s the cultivation and propagation of viruses in organotopic cultures made possible studying the viral genetics of HPV.

The availability of complete and partial genome sequences from a broad variety of PV types, as well as an increase in the number of PV isolates has emerged the need for taxonomic classification of Papillomaviruses [9].

Papillomaviruses have been traditionally referred as “types”. As the L1 ORF is best conserved gene in the structure of PVs, it has been used for the identification of new PV, as well as in classification and building up the polygenic tree.

A Papillomavirus type is recognized if the complete genome has been cloned and the DNA of L1 gene is at least 10% different from other known PV type.

Subtypes of papillomaviruses are defined if the differences in the genome of L1 are between 2-10%, whereas if there is less than 2% we are taking about the variant of PV [9].

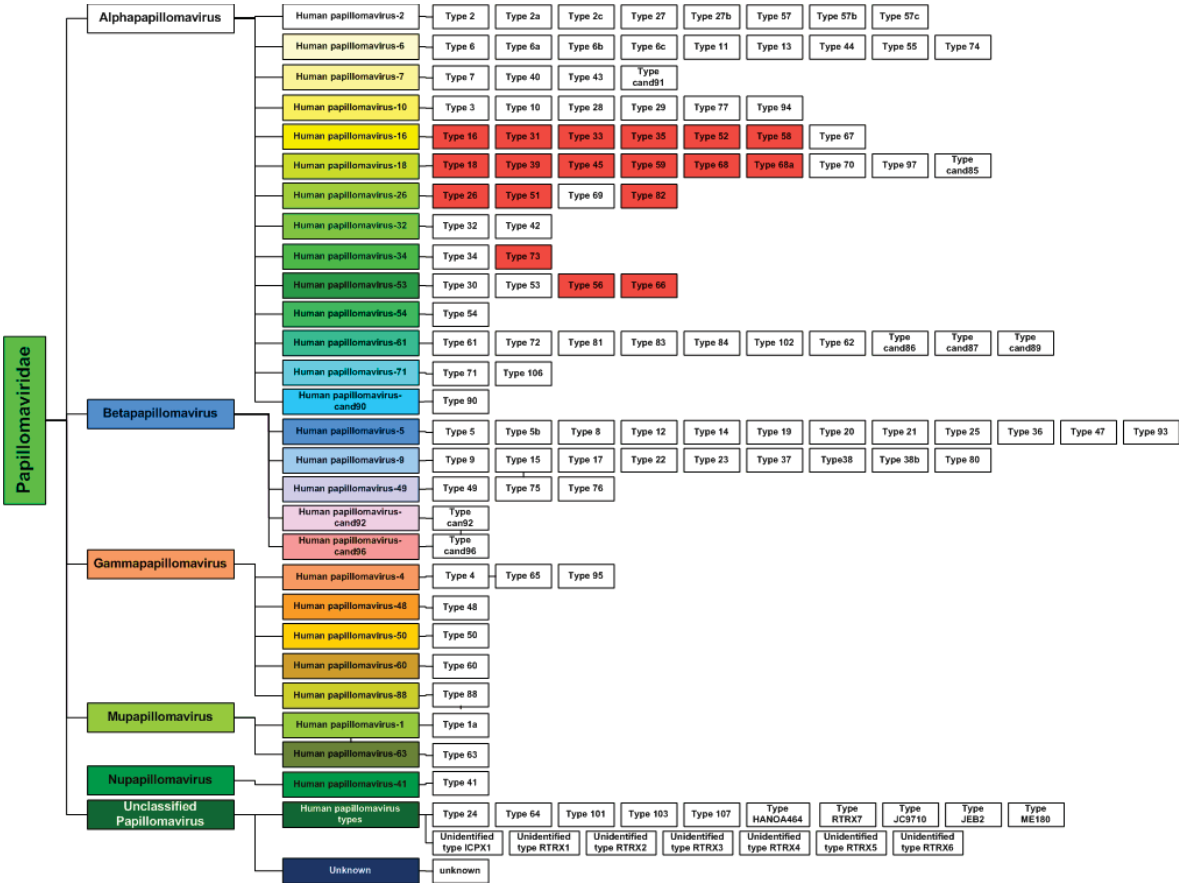

Figure 1. Classification of *Papillomaviridae*

1.2 Structure and the genome of the HPV

Papillomavirus as a small, non-enveloped DNA virus, containing the capsid that has two structural proteins, L1, 80% of total viral protein and L2. Viruses-like particles (VLPs) can be produced by an expression of L1, alone or in combination with L2 [10,11].

The genomes of all HPV types have around eight ORFs, each of them transcribed from the single DNA strain. The ORF has tree functional parts:

- Early (E) region that encodes proteins E1-E7, essential for viral replication
- Late (L) regions that encodes structural proteins L1 and L2, necessary for viral assembly

- Long control region (LCR), mostly as non-coding part, contains cis elements that are essential for the process of viral DNA replication and transcription [Figure2].

The E1 and E2 proteins directs the host cell factors to viral origin of replication [12,13] during the maintenance phase of the replication process, the E2 ensures that viral genomes are partioneted into the daughter cells (14) E2 is also the main regulator of viral gene transcription. E2 can activate or, more often, can repress viral transcription [14]. E4 plays role in a late stages of the virus life cycle, whereas E5 can play role in both early and late phases, by facilitating the immune evasion, by downregulation surface expression of proteins engaged in antigen exposure [15]. The proteins E6 and E7, plays a decisive role in carcinogenesis, by targeting different regulators of the cell cycle, like p53, pRb, Bak etc. [16,17]. These proteins are not well conserved, and both of them promotes cellular proliferation and inactivate cellular cycle checking points [18,19]. The In the HPV positive cancer-derived cells, the expression of oncoprotein E6 induces growth arrest followed by either apoptosis or senescence. [20-22]. Therefore, its an indication that HPV-positive cancers are “addicted” to E6 expression, in order to survive, so inhibiting the protein E6 key functions may lead to a promising strategy for counteracting the growth of HPV-positive tumors [Figure 2].

L1 and L2 proteins assemble in capsomers, and further forming icosahedral capsids around the HPV viral genome during the progeny virions [23].

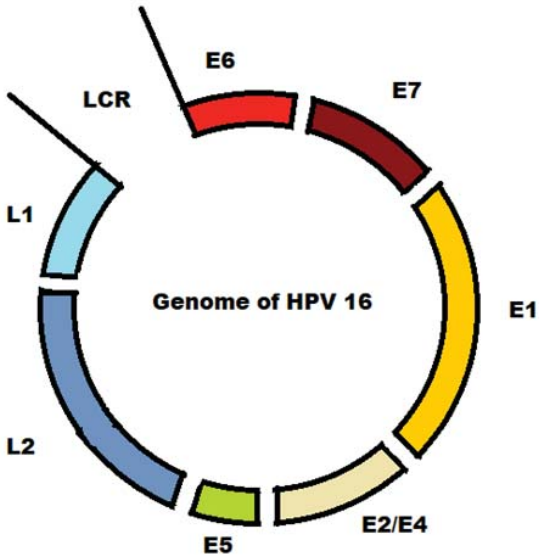

Figure 2. The genome of HPV

1.3. HPV life cycle

HPVs are highly epitheliotropic pathogens, and infection and viral cycle are completely reliant upon the expression of the complete process of keratinocyte differentiation. It is supposed that virus infects primitive basal keratinocytes, presumably at the site of micro injuries, which probably gain the stem cell phenotype during the wounding process [24,25]. All PVs induce proliferation of the infected cells. This proliferation mobilizes the cellular DNA replication and protein production machinery, which are hijacked by the virus to the benefit of its own replication [20,26-28].

Most HPV types that are designated as “low-risk” generate mild benign pathogenic effects, such as skin warts, mucosal lesions, or in the worst case, mucosal condylomas that require surgery [20,26,27]. However, for a subset of HPV types dubbed “high-risk”, viral genes sometimes do not get fully eradicated by the host. All or part of the viral genome remains maintained in at least one host cell, either in an episomal form or inserted in the host cell genome [29].

Human Papilloma Viruses are strongly associated with a group of malignancies, most notably cervical cancer (CC). The viral oncogenes have

the capability to promote, during a long period that may last up to three decades, further making changes to the infected cell, which may eventually lead to cancer [30]. Several studies have proposed the existence of multiple HPV target cells within the host epithelium.

Acknowledged the anatomical observations that a lot of cervical cancers are derived from the transformation zone (TZ), the association between infection of tissue stem cells and carcinogenesis has been proposed. There is an augmentative support for the hypothesis that stem cells of the TZ of the cervical epithelia are the primary site of persistent HPV infection [14,31].

The long after infection of injured basal cells, in HPV positive keratocytes and epithelial cells, suprabasal cells fail to back off from the cell cycle, and continue to support further the DNA synthesis and marker expression for cell proliferation [32], it is concept that there is a round of viral DNA replication, which is independent of the cell cycle and amplifies the viral copy number to about 50 to 100 copies per cell [33]. In the early phases of the high-risk HPVs life cycle, the expression of the potent E6 and E7 oncogenes is under control, and E6 and E7 transcripts of high-risk HPV types are barely detectable [34]. At the time when the infected keratinocyte move into the stratum spinosum initiating the cell cycle, then comes an prolonged upregulation of viral gene expression and the replication of viral DNA, amplifying the viral copy number to thousands of copies per cell, gross expression of the E6 and E7 early genes, and late genes expression of from the late promoter, leading finally to HPV formation [35].

Studies have demonstrated that E6 and E7 are responsible for most of the proliferative and transforming events that lead to carcinogenesis [9,36]. Altogether, E6 and E7 exhibit anti-apoptotic and pro-proliferative effects, HPV-induced cellular immortalization, similarly as cell adhesion and polarity, and cell differentiation-altering properties that facilitate a transient period of proliferation of the infected epithelial cells [37-40] [Figure 3].

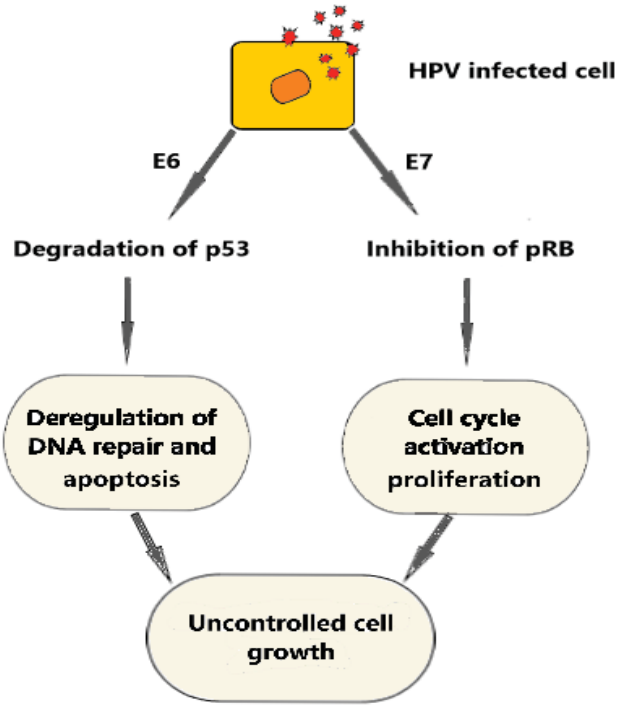

Figure 3. Role of E6 and E7 oncoproteins

1.4. Immunobiology of human papillomaviruses

Human papilloma viruses are highly infectious agents, by inducing chronic infection that have systemic sequelae and not necessarily kill the host. To achieve this form of persistency, HPV has the specific immune evasion mechanism that inhibits the host's detection of virus. Nevertheless, some epidemiological data suggest that the majority of ano-genital infections caused by oncogenic HPVs never become chronic [41]. In women, the incidence of new ano-genital infections by oncogenic HPVs decline with age, whereas persistence increases with age [42,43]. As HPVs are very diverse group in terms of genetics and its clinical presentation of the infection, it is very important reason why we cannot have a clear line between HPV acute and chronic infections.

Virus infects primitive basal keratocytes, probably targeting stem cells, but high level of viral expression occur only in upper layers of the stratum spinosum and granulomatosum of squamous epithelia [44].

For most the duration of the HPV infection cycle, there is little or no release into local milieu of proinflamatory cytokines which is important for antigen presenting cell (APC) activation and migration.

hr-HPV infection encourages the migration of immune cell to the dermis. Important roles during the immune response to infection in the epidermis layer have the macrophages, natural killer cells (NK), T lymphocytes, B lymphocytes, Langerhans cells (LC), KCs and dendritic cells (DC).Infection with hr-HPVs could effectuate the immune system to get more tolerant to the infection, thereby generating a environment vulnerable to further infection and facilitating progression into cervical intraepithelial lesions. There are some mechanisms that have been proposed and proved, like: It is proven that hr-HPV remains silent for a long period of time; and its duplication and assembly do not necessarily lead to cytolysis or the cytopathic death of the host cells [45]. Then, hr-HPV inhibits the synthesis of interferon (IFN) through E6 and E7 oncoproteins by interfering with IFN signaling pathways [46]. On the other hand, hr-HPV infection promotes the infiltration of regulatory T cell (Treg) and interleukin (IL)-10 or transforming growth factor β (TGF-β) production. Also, the hr-HPV infected cells express low levels of MHC class I, thereby resulting in impaired CTL function [47], and it compromises NK cell activation [48].

The ground for the progression from hr-HPV infection to cervical cancer is the impaired adaptive immunity. There are different immune cell profiles for the different stages of the disease progression in CIN and carcinogenesis. All the changes and alterations induced by hr-HPV infection are based in hr-HPV infection adapting the immune system to build a comfortable microenvironment for persistent infection and lesion progression [49].

The T cell activation is proven to be important in hr-HPV infection as shown in patients with HIV infection, compromised CD4+ T cells acts as the inducer for HPV-associated cancer appearance (45). In this case, the induction of incompetent CD4+ T cells infected by hr-HPV is the factor that promotes the progression of CIN lesion [50].

hr-HPV infection can also change the balance between type 1 T-helper cells (Th1) and Th2 cell is another property of cellular immunity [51].

One of the mechanisms by which hr-HPV regulates T cell activation is supported by E6 and E7 expression, the oncoproteins which would upregulate the expression of another molecules in the infected cells, such as vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and E-selectin (classical immunosuppressive molecules) [52].

Along with hr-HPV infection, immature DCs normally inhibit the activation of a proper immune response by CTLs and therefore making it easier lesion progression [53]. In patients with cervical cancer, the distribution of functional DCs are extremely low, or even completely absent.

The central signals to initiate the start of the immune reaction in squamous epithelia are nonexistent [54]. There is no blood born viremic phase of HPV life cycle, the virus is practically invisible to the host, who remains ignorant of the pathogen for the long period of time.

Viral capsides in epithelia should normally activate Langerhans cell (LC), but in the case of high risk HPVs, that doesn’t occur [55,56].

Despite the foremost attempts of the virus to putt-off the host defense system, most of HPV infection disappear with time. Infection with genital human papillomavirus (HPV), with both low- and high-risk types is a very common worldwide, but most infections resolve by the time, as a result of a cell-mediated immune response [57]. The majority of the low grade intraepithelial lesions are self-cleared as a result of a proper cell-mediated immune response headed against E2 and E6 oncoproteins [58-60]. Antibody concentrations achieved in animals and humans are low and many women do not show seroconversion [61,62].

There is also well known that antibody response to HPV infection is type specific [63,64].

Although 80 to 90% of the genital HPV infections resolve with time, about 10-20% of individuals do not become HPV DNA negative and can

develop cervical intraepithelial neoplasia (CIN2/3) and cervical cancer. This condition is characterized by the overexpression of HPV E6 and E7 proteins in the divided cells, and have ability to resist both the innate and the adaptive antiviral immune defense. Integration of HPV DNA into host chromosome is well known process that happen in high proportion in cervical cancers [65], are resistant to antiviral effect of IFN-b [66], and T-cell response to E2 and E6 are lost or reduced [67] [Figure 4].

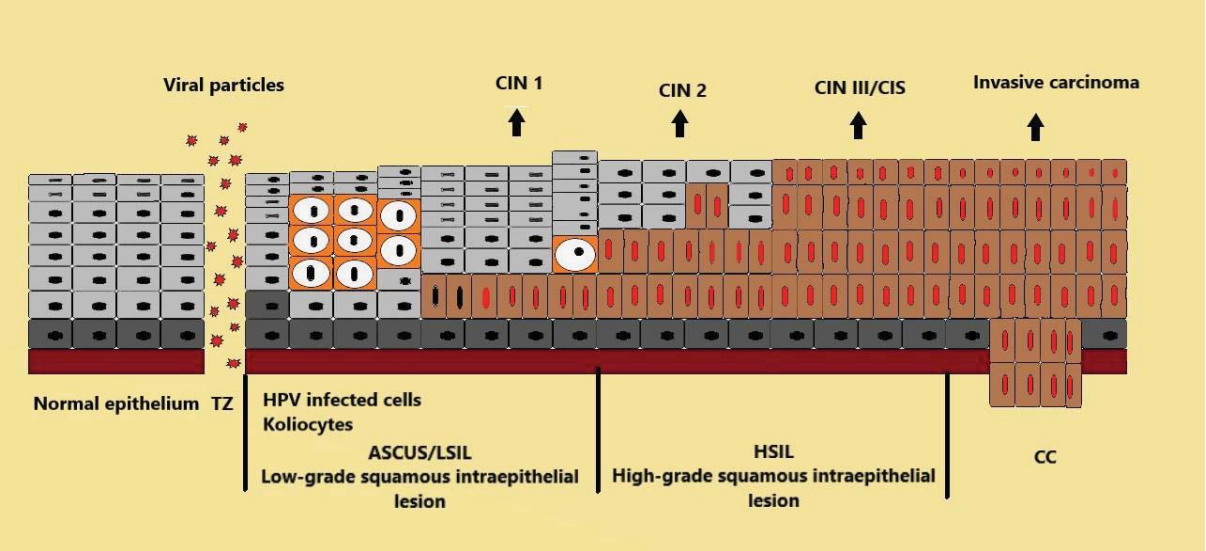

Figure 4. The impact of HPV infection on squamous epithelia

1.5. HPV transmission

The most common way of transmission is horizontal one, mostly by sexual activity through mucosa to mucosa and skin to skin contact [68,69]. Fair-chance of infection per intercourse is not clearly known but is evident that is high [68]. As the transmission route is the same, around 20-30 % of women sampled for testing has had concurrent infections with more than one HPV type [70,71]. Most women have a high chance to be infected with at least one or several types of HPV during they life [72].

Another form of HPV transmission is vertical, when a parent pas the infection to its newborn offspring, including a special form of vertical transmission – perinatal infection. Transmission of HPV from mother to child,

known as vertical transmission, was first suggested in the 1950s [73-76]. Some authors detected over 70% of HPV transmission from mother to neonate [77].

Perinatal transmission of HPV infection has been unequivocally demonstrated for the rare disease juvenile respiratory papillomatosis [78].

1.6. Clearance and persistence of HPV infection

Most women infected with a specific HPV type will not show evidence of that same type 6–12 months later [79-81].

In a prospective study conducted among female college students, around 70% of women, within 12 months of follow-up period after incident HPV infection, no longer had detectable levels of HPV DNA. After 18 months, over 80% appeared to have been cleared form the HPV infections [81]. Other cohort studies support this finding, several reporting a median duration of HPV detectability of approximately 1 year [80,82-84].

HPV 16 appears to have a particularly longer time of clearance relative [85] to other HPV types [86].

For papillomaviruses, most valuable evidence on latency come from animal models, where latency is considered a low-level viral genome maintenance in the basal layers of epithelia without a productive viral life cycle [87].

It is well known that on clearance time, other cofactors have an effect, like HPV genotype, host genetic background, age of sexual start-out, or other coinfections [88]. Nonetheless, our mechanistic knowledge is still far behind.
For HPVs, regarding the latency development, two ways of reaching it have been proposed: one that claims that infections may directly enter latency by escaping an acute phase, or it may also arise after a productive phase without successful clearance [87,89]. In both cases, latency is directly linked to acute infection dynamics.

In the wider meta-communities context, latency can additionally be a process that arises from diverse interactions between HPV and host cells. The molecular decision to start cell division in the basal layer is random and granted that the viral genome in order to replicate needs the cell division [90], latency-reactivation episodes could be mechanistically understood to show randomness in the time lapses of basal cell mitotic activity, without necessarily demanding viral act upon the host cell.

1.7. Prevalence of HPV infection

The estimated population prevalence of HPV infection among women worldwide ranges from 2% to 44% [91]. The very wide variation in estimates is mostly interpreted by differences in the age range of the populations studied and the distinctness in sensitivity of the DNA assay used for detection of HPV infection, and type-specific HPV DNA prevalence among HPV-infected women [92].

Data derived from multiple international studies, have shown that the median oncogenic HPV prevalence among all women was 15.1%, while the median oncogenic HPV prevalence among women older than 30 was 9.2% [91]. The prevalence of HPV infection is highest among women at younger age and evidently tends to drop off with increasing age [93]. Most studies conducted in several different regions in the world, have shown a decrease in HPV prevalence with age, with a peak prevalence of HPV infection in women under age 25, a decrease among women aged 35–54 and a second peak after age 55 [5,94,95].

1.8. HPV and cervical cancer

Experiments trying to establish a relationship between HPV infection and cervical cancer were initiated in 1970 [96]. In 1976 Meisels et al. [97] claimed that koilocytotic cells found in cervical smears of patients with flat dysplastic lesions corresponds to the cytopathogenic changes of a

papillomavirus infection. Early in 1985 the selective transcription of E5 and E6 genes in cervical cancer were established [98].

HPV is a prevalent pathogen, the epidemiology of which has mostly been studied in uterine cervix and the vagina. Major epidemiological studies identified the HPV16, HPV 18 and few others as a major risk factors for cervical Cancer [99,100].

The major steps known to be essential for cervical cancer carcinogenesis include: HPV infection, persistence of the infection, progression of precancerous lesions and eventually invasion.

Among over 200 HPV types, there are more than 40 mucoso-tropic viruses that infect the anogenital and upper aerodigestive tract [101]. Approximately 15 are considered to be high-risk types, including HPV 16,18,31,33,35,39,45,51, 52, 56, 58 and 68. Types 16 and 18 are most dangerous, since they cause more than 70% of cervical cancers.

The worldwide incidence of cervical carcinoma is more than 530000 cases per year, whereas mortality reaches 275000 deaths annually, of which approximately 85% occur in developing countries. Whereas, some authors have highlighted the elevated burden of cervical cancer in some South-Eastern European counties, like Romania, Bulgaria and is failing in Greece, Croatia and Slovenia [102].

1.9. Other risk factors for HPV infection

1.9.1. Number of sex partners

The most unvarying risk factor for HPV infection is increased number of sex partners. Several studies of women have demonstrated strong associations between lifetime number of sex partners and genital HPV acquisition [103-105] and in men [106]. Furthermore, it has been demonstrated that a woman's reported estimate of her lifetime number of sex partners is positively correlated with HPV infection in herself [105].

1.9.2. Smoking

HPV infection has been positively associated with the status of current smoking [107-109] and past smoking [110]. Many previous studies proved certain degree of association between cigarette smoking and prevalence, incidence, and persistence of HPV infections [111-116]. Another study investigating the relationship between smoking and oncogenic HPV infection could not found any correlation between number of cigarettes smoked per day and presence of HPV DNA [117]. Whereas, other studies investigating the relationship between smoking and HPV infection have failed to detect an association [118,119].

1.9.3. Oral contraceptive use

When taking about certain association between oral contraceptive (OC) use and HPV infection it is difficult to evaluate it correctly, due to the constant association between sexual activity and OC use. Many case-control studies has been shown that the use of oral contraceptives to be associated with cervical cancer and HPV positive prevalence [120,121]. Other study has found that after adjusting for some variables, like the age at first debut and lifetime number of sex partners, former OC users showed a borderline association with HPV 16, 18 and 31 seropositivity, while current OC users exhibited a borderline association with HPV 16 and HPV 18, but not with HPV 31 seropositivity [122].

Other studies have reported an association between cause and HPV DNA positivity only after adjusting for variables such as number of sex partners [123], but most studies have found no association [119,124].

1.9.4. Infection with multiple types

The infection with multiple HPV types in one individual is not uncommon and several studies that have tried to determinate the role of multiple HPV infections and HPV persistence [125,126] claim that infection with multiple HPV types was associated with persistence of HPV infection, a finding supported by Woodman et al. [127], who found that simultaneous infection

with HPV16 and another type resulted in longer duration of detectable HPV 16 than did infection with HPV 16 alone. Concurrent multiple HPV genotypes were detected in 36.93% (95%CI: 30.15-44.27) of the patients [128].

1.9.5. Development of lesions in cervix

Any of HPV types, oncogenic and non-oncogenic ones, can cause pathologies referred as low grade squamous intraepithelial lesions (LSIL) of the cervix, whereas most cervical lesions that are classified as high-grade SIL (HSIL), carcinoma in situ or invasive cancer results positive for oncogenic HPV types. HPV 16 or HPV 18 are found at about 70% of invasive cervical cancers [129] and about 90% of genital warts are caused by HPV 6 or HPV 11[130].

Persistent infection with HR HPV types is linked with an increasing probability of integration of the viral circular genome (episome) into the host chromosomes, leading to cancer development [131-133]. Integration of the viral genome coincide with the development of high-grade cervical intraepithelial neoplasia (CIN II/III) due to overexpression of the E6 and E7 oncogenes [26].

Some authors noticed a balanced rise in HPV 16 positivity and all the range thought the cervical diseases form NILM to cervical cancer in the all regions of the world [134].

The incidence of HSIL appears to be quite high among women infected with oncogenic HPV. In a cohort of women with no prior history of SIL, and tested positive on HPV test, the 2-year cumulative incidence of biopsy-confirmed HSIL was 28% and only 3% for women with no detectable HPV infection [135], others had an evidence of the predominance of HPV16 in HSIL lesions of the uterine cervix [128].

HPV 18, 45, and other types of alfa species have a potential for endocervical glandular lesions and cervical adenocarcinoma [136], pathologies that cannot be so precisely detected by cytological screening [137]. HPV 31, 33 and 58 have been reported to be related with the higher risk for CIN3 than other non HPV 16 HR types [108,138].

1.10. Prevention of HPV infection

Some authors claimed that by promoting absence and use of condoms could reduce the risk for cervical cancer [139], but others found out there is no complete protection against HPV transmission by condom use because the male anogenital skin area could not be completely covered [140].

Today the most effective primary prevention against the HR HPV induced cancers in women is the vaccination against the certain types of HPV.

The development of HPV L1 virus like particles (VLPs) vaccines is a tremendous advance in prevention against cervical cancer.

The VLP-s in vaccines are small, irregular shaped structures, composted of HPV major capsid L1 protein manufactured by DNA recombinant technology [141,142]. The capsid contain no genetic material [143].

Currently there are in use three vaccines, bivalent HPV vaccine Cervarix (Glaxo Smith Kline) covers HPV 16 and HPV 18, qudrivalent HPV vaccine Gardasil (Merck), covering HPV 6,11,16,18 and recently approved 9 valent HPV vaccine by Gardasil (Merck) is designed to protect against HPV 6 and 11, and 7 high risk HPV types (HPV 16/18/31/33/45/52/58) most common types found in cervical cancer worldwide. Approximately 90% of CC cases are attributed to infection with 7 HR HPV types targeted by (vHPV vaccine) [144].

All vaccines are Vaccines are injected intramuscularly, hence HPV VLPs induces the production of neutralizing antibodies against the HPV [145,146].

Although the vaccination against HR HPV types is a promising feature in a fight against the cervical cancer, there is still a long way until it can cover a considerable number of women worldwide.

Hence, the screening against the cervical cancer should be the primary focus in developing countries. The implementation of a proper and organized cytology-based cancer screening program, which enables to detect precancerous lesions in early stages has helped to decrease the incidence and mortality of cervical cancer.

HPV testing is also approved in primary screening in women older than 30 years [147].

Within 2 years of a newly acquired infection, 60% of young women developed an antibody response to HPV-16, compared with only 4%–13% of men [148].

Compared with men, women tend to exhibit more-robust cell-mediated and humoral immune responses to infectious agents in general [149]. The most assertable explanation is that high levels of estrogen promote antibody production, thereby increases immunocompetence, whereas androgens such as testosterone, suppress immune function [150].

The main conclusion from these sero-epidemiological studies is that the antibody response to HPV16 proteins does not invariably occur during a natural HPV infection. For example, only in about half of the women with normal cytology and HPV DNA presence in their cervical epithelium, a humoral immune response to VLPs is induced. The VLP-specific antibodies are neutralizing and genotype-specific and have been crucial for the development of preventive HPV vaccines [151,152].

2. HYPOTHESIS

To prove if HPV 16 and 18 are more prevalent high-risk HPV subtypes in Kosovar female population.

3. AIMS AND THE PURPOSE OF THE RESEARCH

General aim:

- To determine the prevalence of high risk HPV infection in Kosovar women

Specific aims:

- To determine the prevalence of specific high risk genotypes isolated in our samples
- To identify the relationship between high risk HPV positive women and histopathological findings in the women included in our study
- To determine the relationship between the high risk HPV positive women and other cofactors that may influence the on it, like:
  - parity,
  - pregnancy termination,
  - women's sexual activity
  - use of contraceptives
  - tobacco use
  - their profession and family incomes,
  - existence of previous sexually transmitted diseases
  - other systemic or gynecological disorders
  - their partner age and profession

4. MATERIAL AND METHODS

4.1. Materials

Our study has included 260 women, aged 18 to 65 years old, from the different regions of Kosovo that has been attending outpatient unit of the Department of Genecology and Obstetrics at the University Clinical Center of Kosovo, from August 2015 to February 2016.

Prior to enrolment to the study, all of the women were provided written inform consent and completed a questionnaire containing general information about them and their partners, their parity, sexual behavior, use of contraceptives, tobacco and drug use, previous history of sexually transmitted diseases and their social and income.

During the gynecological examination, to each women enrolled in the study, one cervical smear was obtained for cytological examination, and another cervical smear was stored in 1ml of Digene Specimen Transport Medium (STM) (Qiagen, Hilden, Germany) at −20 °C for subsequent HPV typing.

Cytology: Cytological examination was performed by an experienced and licensed pathologist at the Institute of Pathology, University Clinical Center of Kosovo, University of Prishtina, who was unaware of the HPV status of the patients.

From the group of women enrolled in the study, only 199 specimens where in the condition to be tested for HPV genotyping, the rest of tubes has been destroyed or not saved in the proper manner so we decided to exclude them from the study.

HPV typing were performed in the University Hospital for Infectious Diseases, Zagreb, Croatia and the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia.

4.2. Methods

4.2.1. Cytology

Each women enrolled in the study conventional PAP smear was performed. The results are classified regarding the Bethesda system. The group of women that showed no pathologies in the cytological results were grouped as NILM. The others were listed as ASCUS, ASC-H and 2 cases of cervical carcinoma were additionally confirmed by the histopathology.

4.2.2. Detection of HPV DNA and HPV typing

Detection of hr-HPV DNA was performed using a clinically validated RealTi*m*e High Risk HPV test (RealTi*m*e) (Abbott, Wiesbaden, Germany), which enables concurrent individual typing for HPV16 and HPV18, as well as pooled detection of 12 other carcinogenic HPV types (HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68), and also includes an internal process control for sample adequacy, DNA extraction, and amplification [153,154]. To amplify HPV targets a primer mix was used, consisting of 3 forward primers and 2 reverse primers targeting a conserved L1 region. Signal for 14 HR HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52,56, 58, 59, 66, and 68) is generated with the use of fluorescent labelled probes. Internal Control (IC) amplicons are generated with a primer set targeting an endogenous human beta globin sequence. The Abbott RealTi*m*e hr-HPV assay detects the endogenous human beta globin sequence as sample validity control for cell adequacy, sample extraction and amplification efficiency.

During the amplification reaction on the Abbott, the target DNA is amplified by DNA Polymerase in the presence of dNTPs and magnesium.
In the Abbott RealTi*m*e hr-HPV assay, the DNA Polymerase is first activated at 92°C for 10 minutes. During each subsequent round of thermal cycling, a high temperature is used to melt double-stranded DNA strands apart, followed by a low temperature where primers anneal to their respective targets and are extended to generate double-stranded DNA products.

Amplification of both targets (HPV and IC) takes place simultaneously in the same reaction.

The target sequence for the Abbott RealTi*m*e hr-HPV assay is in the conserved L1 region of the HPV genomes.

In addition to several other transport media [153], the assay has also been clinically validated for use with cervical specimens collected with Digene STM (Qiagen) [155,156].

In specimens positive for other carcinogenic HPVs, HPV typing was subsequently performed using the Linear Array HPV Genotyping Test (Roche LA) (Roche Molecular Diagnostics, Branchburg, NJ) and, if necessary, also with an HPV52 type-specific real-time PCR assay and an in-house GP5+/GP6+ PCR assay, as described previously [154, 157].

The Linear Array HPV Genotyping Test (Linear Array) (Roche Molecular Diagnostics, Pleasanton, CA) is a standardized HPV genotyping test used widely. Linear Array combines PCR-amplification and reverse-line blot hybridization for simultaneous detection of 36 individual HPV genotypes and one subtype, including all high risk HPV genotypes (HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59, HPV 68, HPV 73, and HPV 82) [158]. A pool of HPV primers present in the Master Mix is designed to amplify HPV DNA from 37 HPV genotypes14 including 13 high risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Capture probe sequences are located in polymorphic regions of L1 bound by these primers. An additional primer pair targets the human s-globin gene to provide a control for cell adequacy, extraction and amplification.

AmpliTaq Gold DNA Polymerase is utilized for “hot start” amplification of the HPV target DNA and the s-globin control. First, the PCR reaction mixture is heated to activate AmpliTaq Gold DNA polymerase, to denature the viral DNA and human genomic DNA and to expose the primer target sequences. As the mixture cools, the primers (both upstream and downstream) anneal to the target DNA. The AmpliTaq Gold DNA polymerase, in the presence of Mg2+ and excess dNTPs, extends the annealed primers along the target templates to produce an approximately 450 base pair double-stranded HPV target DNA molecule or a 268 base pair s-globin DNA molecule termed an amplicon. This process is repeated for a number of cycles, each cycle effectively doubling the

amount of amplicon DNA. Amplification occurs only in the region of the HPV genome or s-globin gene between the appropriate primer pair.
The entire genome is not amplified.

Following PCR amplification, the HPV and the s-globin amplicon are chemically denatured to form single-stranded DNA by the addition of Denaturation Solution. Aliquots of denatured amplicon are then transferred to the appropriate well of the typing tray that contains hybridization buffer and a single LINEAR ARRAY HPV Genotyping Strip that is coated with HPV and s-globin probe lines. The biotin-labeled amplicon will hybridize to the oligonucleotide probes only if the amplicon contains the matching sequence of the complementary probe.

In addition, the LINEAR ARRAY HPV Genotyping Strip is coated with one cross-reactive oligonucleotide probe that hybridizes with HPV genotypes 33, 35, 52 and 58.

Following the hybridization reaction, the LINEAR ARRAY HPV Genotyping Strip is stringently washed to remove any unbound material. Streptavidin-Horseradish Peroxidase Conjugate is then added to the strip. The strip is than washed in order to remove any unbound Streptavidin-Horseradish Peroxidase Conjugate and a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) is added to each strip. In the presence of hydrogen peroxide, the bound streptavidin-horseradish peroxidase catalyses the oxidation of TMB to form a blue coloured complex, which precipitates at the probe positions where hybridization occurs.

The LINEAR ARRAY HPV Genotyping Strip is then read visually by comparing the pattern of blue lines to the LINEAR ARRAY HPV Genotyping Test Reference Guide [Figure 5a and Figure 5b].

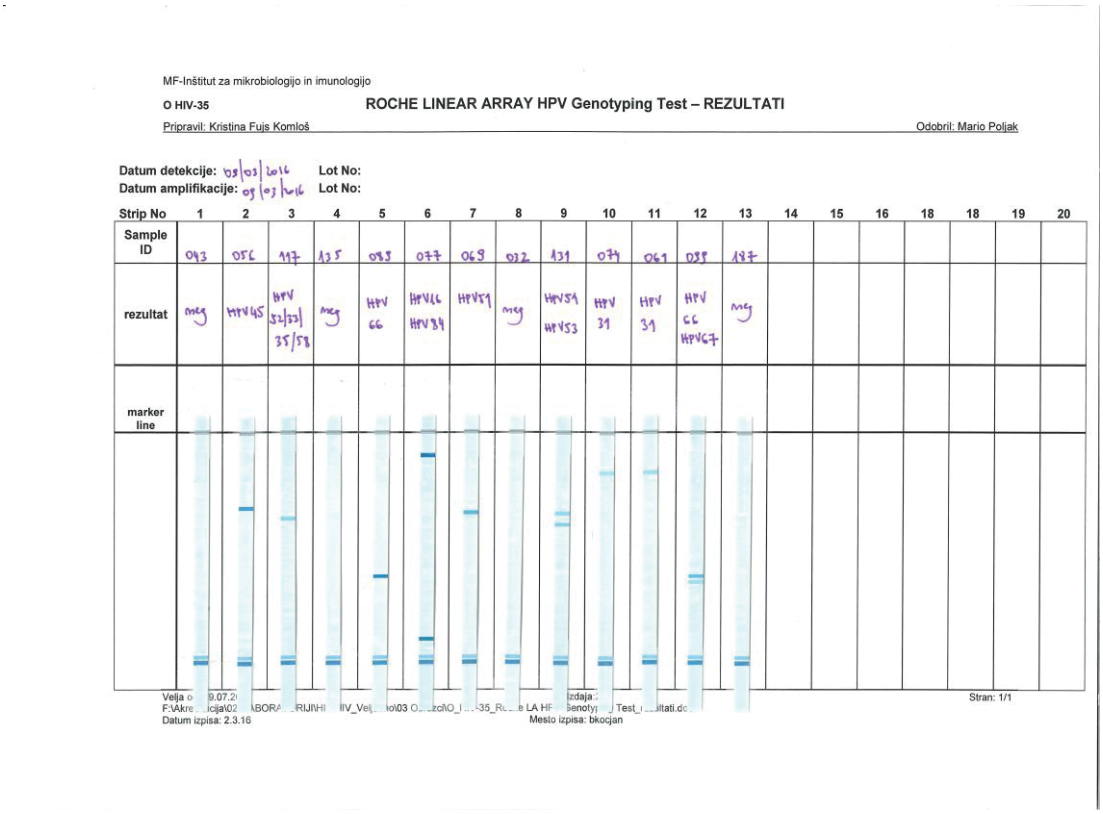

Figure 5a. Genotyping strips produced after LINEAR ARRAY HPV testing

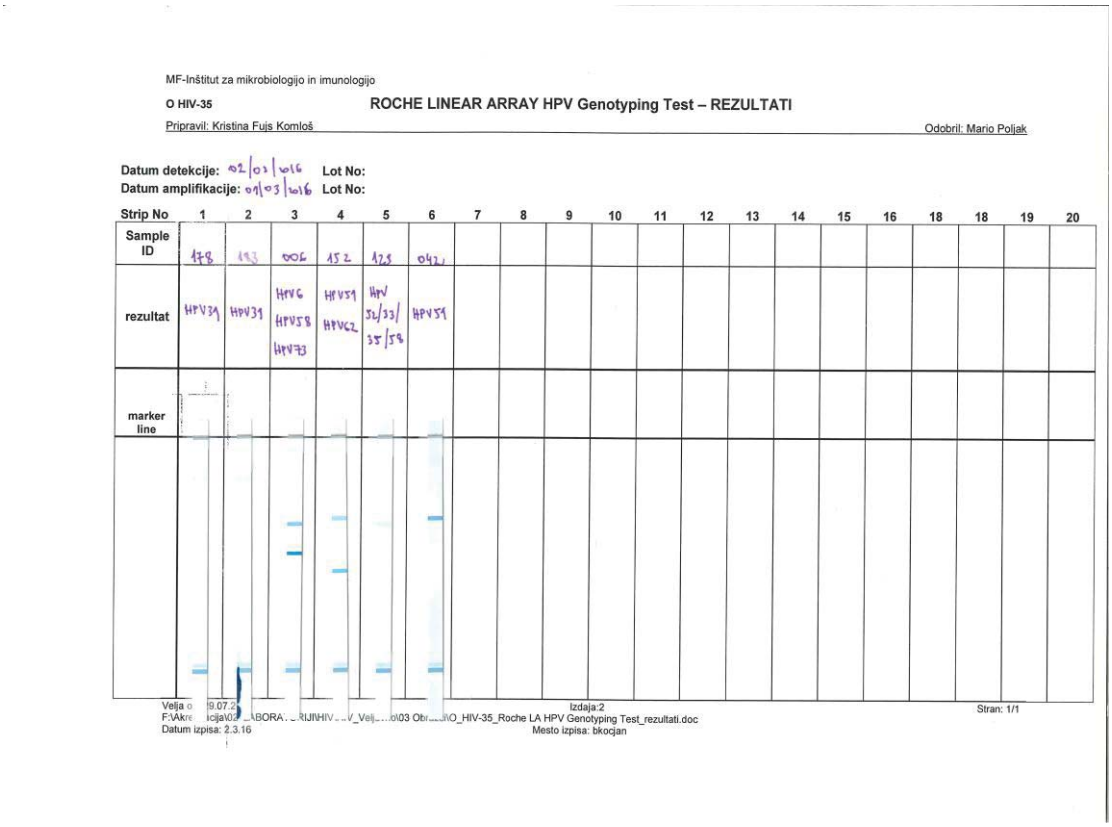

Figure 5b. Genotyping strips produced after LINEAR ARRAY HPV testing

Because of intellectual property rights for HPV 52 detection, this genotype is identified using a cross-reactive HPV 52 probe that hybridizes also with HPV 33, HPV 35 and HPV 58 [159]. A sample is defined as HPV 52 positive if it reacts with the cross-reactive probe, but not with the HPV 33, HPV 35 or/and HPV 58 genotype-specific individual probes [160].

The assay was performed on a LightCycler 1.5 Real-time PCR instrument (Roche Diagnostics) under the following conditions: initial DNA polymerase activation at 95 ◦C for 10 min, followed by 45 cycles of 95 ◦C for 10 s (denaturation), 60 ◦C for 30 s (annealing) and 72 ◦C for 1 s (extension). The temperature transition rate was set to 20 ◦C/s for each of the steps. Acquisition of the fluorescence signal was performed on the F1 (530 nm) channel at the end of the elongation step of each cycle. The final step consisted of cooling at 20 ◦C/s to 40 ◦C with a 30 s hold. The analytical sensitivity of the RT-PCR assay was evaluated using triplicates of serial 10-fold plasmid dilutions spanning from 1×107 to 1×101 DNA copies per reaction and the detection limit was established to be at least 10 viral copies. The 95% detection limit was calculated by probit analysis The HPV DNA status was determined by sequencing GP5+/GP6+ or PGMY09/PGMY11 PCR products, as described previously [157] for PCR amplifications, up to 5_l (up to 100 ng) of each of the samples was used per 25_l reaction [160].

4.3. Statistical analyses

To assess the associations between sociodemographic characteristics and overall HPV infection the univariate and multiple logistic regression analyses were used. A likelihood ratio test was used to investigate the associations between sociodemographic characteristics and hr-HPV types. Associations with $p < 0.05$ were treated as statistically significant. Analyses were performed with SPSS, version 23.

5. RESULTS

5.1. General characteristics of the women included in the study

After we have excluded the samples that were damaged, a total of 199 women, 20 to 63 years old, with a mean age of 41.8 years (*SD* ± 9.3 years) were included in the study [Table 1]. The majority of women has been declared as Albanian (198/199, 99.5%), most of them have graduated from a secondary school (107/199; 53.8%), 20,6% (44/199) has finished the higher education and the rest were with elementary school or less (51.199, 25.6%).

The vast majority of them were married (176.199, 88.4%), 6.5% (13/199) divorced or widow and only 5% (10/199) single, 71.9% (143/199) had no job, declared as housewives or farmers, 25.6% (51/199) were employed and 5 out of 199(2.5%) were students.

70.9% (141/199) of women included in the study lives in a small families (only parents and their children), 27.1% in a large families, whereas only 2% (4/199) lives alone.

Most of the women has declared they have sufficient income (104/199, 52.3%), 27.1% (54/199) have insufficient income and with good family incomes were 20.6% (41/199) [Table 1].

Only 25.1% (50/199) were smokers, with a mean (SD) number of cigarettes 11.3. No women has been declared as alcoholic or drug user.

Mean (SD) number of partner is 1.1. Partners mean age was 45.5 years, 99% of them were of Albanian nationality, and the majority of them have at least finished the secondary school [Table 1].

Table 1. Socio-demographic characteristics of women included in the study

	N = 199 (%)
(Mean) age (SD)	41.8 (9.3)
≤ 40 years old	79 (39.7)
> 40 years old	120 (60.3)
Education	
finished elementary school or less	51 (25.6)
finished secondary school	107 (53.8)
more than secondary school	41 (20.6)
Employment status	
Employed	51 (25.6)
housewife or farmer	143 (71.9)
Student	5 (2.5)
Marital status	
Married	176 (88.4)
divorced/widow	13 (6.5)
Single	10 (5.0)
Family size	
Single	4 (2.0)
small family (parents & children)	141 (70.9)
large family (parents, children and other family members)	54 (27.1)
Family income*	
Insufficient	54 (27.1)
Sufficient	104 (52.3)
Good	41 (20.6)
Smoking	
Yes	50 (25.1)
No	149 (74.9)
Mean (SD) number of cigarettes (n = 50)	11.3 (6.3)
Alcohol	0 (0)
Drugs	0 (0)
Partner's mean age (SD) (n = 183)	45.5 (10.3)
Partner's nationality	
Albanian	197 (99)
Turk	1 (0.5)
Swiss	1 (0.5)
Partner's education	
elementary or less	51 (25.6)
Secondary	107 (53.8)
high education	41 (20.6)

Categorical and continuous variables are shown as frequencies (percentages) and as mean (standard deviation), respectively.*The family's financial situation was evaluated by patients themselves.

The mean age of menarche was 13.7 years, and average age at first intercourse was 21.8 years, were 21.1%(42/199) have had they sexual debut before 19 years of age, 89.4% had given birth to one or more children and 50.3% (100/199) had at least one abortion (pregnancy termination) [Table 2]. Only one third of the women (59/199. 29.9%), had previous regular gynecological examination, were 15.6% (31/199) declared other concomitant gynecological disease, 25.1% (50/199) with concomitant chronic diseases, and prior STD, 16.1% (32/199).

The majority of the women (116/199. 58.3%) previously underwent PAP smear testing, were 33.7% of them had one year testing interval, whereas 13.6% of them were testing every 3 years, had a regular menstrual cycle (126/199; 63.3%), were not using contraception (166/199; 83.4%) [Table 2].

Table 2. Characteristics of women included in the study – health issues

	N = 199 (%)
Mean age (SD) of menarche	13.7 (1.48)
Age at first sexual intercourse	
< 18 years old	31 (15.6)
≥ 18 years old	168 (84.4)
Mean (SD) number of partners	1.1 (0.27)
Delivery	
Yes	178 (89.4)
No	21 (10.6)
Mean (SD) number of deliveries (n = 178)	3.2 (1.6)
Pregnancy termination	
Yes	100 (50.3)
No	99 (49.7)
Number of abortions	
0	99 (49.7)
1	57 (28.6)
2	26 (13.1)
3 or more	17 (8.5)
Menstrual cycle	
Regular	126 (63.3)
Irregular	40 (20.1)
Postmenopausal	33 (16.6)

Contraception use	
Yes	33 (16.5)
no	166 (83.4)
Regular gynecological examination	59 (29.9)
Concomitant gynecological disease	31 (15.6)
Prior STD	32 (16.1)
Concomitant chronic disease	50 (25.1)
Previous PAP smear	116 (58.3)
Time of previous PAP smear (n =116)	
1 year or less	67 (33.7)
2 years	2 (1)
3 years	27 (13.6)
4 years or more	20 (10.1)

* Data is shown as frequency (percentage) for categorical and as mean (SD) for continuous variables.

5.2. Cytology results

The vast majority of women (165/199; 82.9%) had a normal cytological result (negative for intra-epithelial lesion or malignancy, NILM). Atypical squamous cells of undetermined significance (ASCUS) were detected in cervical specimens of 30/199 (15.1%) women and atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H), and cervical cancer were detected in two women, respectively [Table 3].

Table 3. Cytology results of the women involved in the research

	N = 199 (%)
Negative for intra-epithelial lesion or malignancy (NILM)	165 (82.9)
Atypical squamous cells of undetermined significance (ASCUS)	30 (15.1)
Atypical squamous cells, cannot exclude high-grade squamous Intraepithelial lesion (ASC-H)	2 (1.0)
Cervical cancer	2 (1.0)

5.3. Detection and typing of HPV infection

The crude overall prevalence of hr-HPV infection with any of the targeted 14 HPV types was estimated at **13.1%** (26/199; 95% confidence interval (95% CI: 9.1–18.5%) [Table 4]. As shown in Table 4 and illustrated in Figure 6, infections with HPV16 were overall the most common, followed by HPV31 and HPV51, and HPV18.

Among 26 hr-HPV positive women, 21 were as a single infection and 5 of them as a multiple hr-HPV infections.

Table 4. Overall distribution of hr-HPV types among 199 women from Kosovo

			Total	
HPV Type	**Single**	**Multiple**	**N**	**%**
Negative			173	86.9
Positive	21	5	26	**13.1**
16	6	1	7	3.5
18	3	-	3	1.5
31	4	-	4	2.0
33	1	-	1	0.5
45	1	-	1	0.5
51	2	2	4	2.0
52	2	-	2	1.0
53	1	1	2	1.0
56	1	-	1	0.5
58	-	1	1	0.5
62	1	1	2	1.0
66	1	1	2	1.0
67	-	1	1	0.5
73	-	1	1	0.5
84	-	1	1	0.5

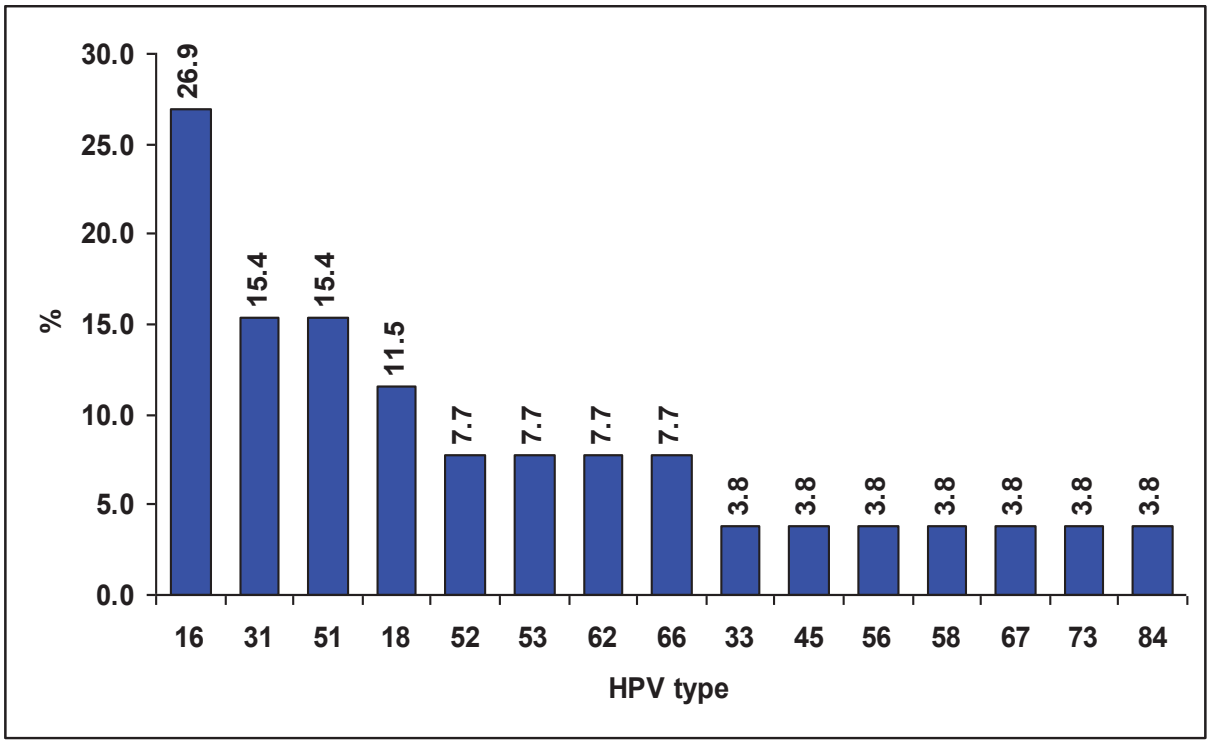

Figure 6. Percentages of identified hr-HPV types

Similarly, HPV16 was also the most frequently detected HPV type in patients with NILM, followed by HPV31 and HPV51. Patients with ASCUS were most frequently infected with HPV18 [Table 5].

Both cervical cancer cases determined by the cytological examination were additionally confirmed using histopathology. Using Roche LA and an in-house GP5+/GP6+ PCR assay, single HPV16 and HPV66-positive cervical specimens were additionally positive for HPV84 and HPV67, respectively, two HPV51-positive cervical specimens were additionally positive for HPV53 and HPV62, respectively, and the HPV58-positive cervical specimen was additionally positive for HPV6 and HPV73 [Table 6].

Table 5. Overall prevalence of hr-HPVs among 199 women from Kosovo according to the cytology results

HPV types	All women (N = 199)		Cytology results							
	N	Prevalence (%) (95% CI)	NILM (N = 165)		ASCUS (N = 30)		ASC-H (N = 2)		Cervical cancer (N = 2)	
			N	Prevalence (%) (95% CI)	N	Prevalence (%) (95% CI)	N	Prevalence (%) (95% CI)	N	Prevalence (%) (95% CI)
Any HR-HPV	26	13.1 (9.1-18.5)	1 6	9.7 (6.1-15.2)	7	23.3 (11.8-40.9)	1	50.0 (9.5-90.6)	2	100 (34.2-100)
HPV16/HPV18	10	5.0 (2.8-9.0)	5	3.0 (1.3-6.9)	2	6.7 (1.9-21.3)	1	50.0 (9.5-90.6)	2	100 (34.2-100)
HPV16	7	3.5 (1.7-7.1)	5	3.0 (1.3-6.9)	0	0 (–)	1	50.0 (9.5-90.6)	1	50.0 (9.5-90.5)
HPV18	3	1.5 (0.5-4.3)	0	0 (–)	2	6.7 (1.9-21.3)	0	0 (–)	1	50.0 (9.5-90.5)
HPV31	4	2.0 (0.8-5.1)	3	1.8 (0.6-5.2)	1	3.3 (0.6-16.7)	0	0 (–)	0	0 (–)
HPV33	1	0.5 (0.1-2.8)	1	0.6 (0.1-3.4)	0	0 (–)	0	0 (–)	0	0 (–)
HPV45	1	0.5 (0.1-2.8)	1	0.6 (0.1-3.4)	0	0 (–)	0	0 (–)	0	0 (–)
HPV51	4	2.0 (0.8-5.1)	3	1.8 (0.6-5.2)	1	3.3 (0.6-16.7)	0	0 (–)	0	0 (–)
HPV52	2	1.0 (0.3-3.6)	1	0.6 (0.1-3.4)	1	3.3 (0.6-16.7)	0	0 (–)	0	0 (–)
HPV56	1	0.5 (0.1-2.8)	1	0.6 (0.1-3.4)	0	0 (–)	0	0 (–)	0	0 (–)
HPV58	1	0.5 (0.1-2.8)	0	0 (–)	1	3.3 (0.6-16.7)	0	0 (–)	0	0 (–)
HPV66	2	1.0 (0.3-3.6)	1	0.6 (0.1-3-4)	1	3.3 (0.6-16.7)	0	0 (–)	0	0 (–)

Abbreviations: NILM (negative for intra-epithelial lesion or malignancy), ASCUS (atypical squamous cells of undetermined significance), ASC-H (atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion), 95% CI (95% confidence interval).

Table 6. Distribution of hr-HPV types among 26 women infected with any of the 14 hr-HPVs according to the cytology results

HPV types	N	%	Cytology results							
			NILM		ASCUS		ASC-H		Cervical cancer	
			N	%	N	%	N	%	N	%
Any HR-HPV	26	100	16	100	7	100	1	100	2	100
HPV16/HPV18	10	38.5	5	31.3	2	28.6	1	100	2	100
HPV16	7	26.9	5	31.3	0	0	1	100	1	50.0
HPV18	3	11.5	0	0	2	28.6	0	0	1	50.0
HPV31	4	15.4	3	18.8	1	14.3	0	0	0	0
HPV33	1	3.9	1	6.3	0	0	0	0	0	0
HPV45	1	3.9	1	6.3	0	0	0	0	0	0
HPV51	4	15.9	3	18.8	1	14.3	0	0	0	0
HPV52	2	7.7	1	6.3	1	14.3	0	0	0	0
HPV56	1	3.9	1	6.3	0	0	0	0	0	0
HPV58	1	3.9	0	0	1	14.3	0	0	0	0
HPV66	2	7.7	1	6.3	1	14.3	0	0	0	0

Abbreviations: NILM (negative for intra-epithelial lesion or malignancy), ASCUS (atypical squamous cells of undetermined significance), ASC-H (atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion).

5.4. Association between sociodemographic characteristics and HPV infection

As shown in Table 7, using univariate logistic regression analysis to investigate potential differences in the sociodemographic characteristics of HPV-negative vs. HPV-positive patients, statistically significant associations with HPV infection were found only for women's age (p = 0.015), age at the time of first sexual intercourse (p = 0.006), delivery (p = 0.026), and history of pregnancy termination (p = 0.033). Despite the fact that the prevalence of HPV infection was the highest in women 30 to 39 years old (21.2% (11/52); mean: 20.8%; standard deviation (SD): ±6.4), followed by women 20 to 29 years old (19.1% (4/21); mean: 19.0%; SD: ±10.5), 40 to 49 years old (9.6% (8/83); mean: 9.6%; SD: ±4.2), and over 50 (7.0% (3/43); mean: 7.0%; SD: ±5.8) (Figure 7&8), the differences were not statistically significant (p = 0.127). However, women over 40 had lower odds for HPV infection (odds ratio (OR) = 0.36; 95% CI: 0.15–0.84) [Table 7 and Figure 2].

Moreover, lower odds for HPV infection were also observed among women that were at least 18 years old at sexual debut (OR = 0.28; 95% CI: 0.11–0.69), had given birth to at least one child (OR = 0.32; 95% CI: 0.11–0.91), or had a history of pregnancy termination (OR = 0.39; 95% CI: 0.16–0.95) [Table 7]. Due to the small number of units in the category of the dependent variable (HPV infection), only three independent variables (age at inclusion in the study: up to 40 years old and over 40, age at first sexual intercourse: below 18 and at least 18 years old or older, and history of delivering at least one child), were included in the multiple logistic regression analysis. When controlling for other variables in the model, all three independent variables were statistically significantly associated with HPV infection, with OR = 0.37 (95% CI: 0.15–0.94) and p = 0.036 for overall older age (over 40 years old), OR = 0.18 (95% CI: 0.06–0.50) and $p < 0.001$ for older age at sexual debut (at least 18 years old), and OR = 0.28 (95% CI: 0.09–0.92) and p = 0.035 for history of delivering at least one child.

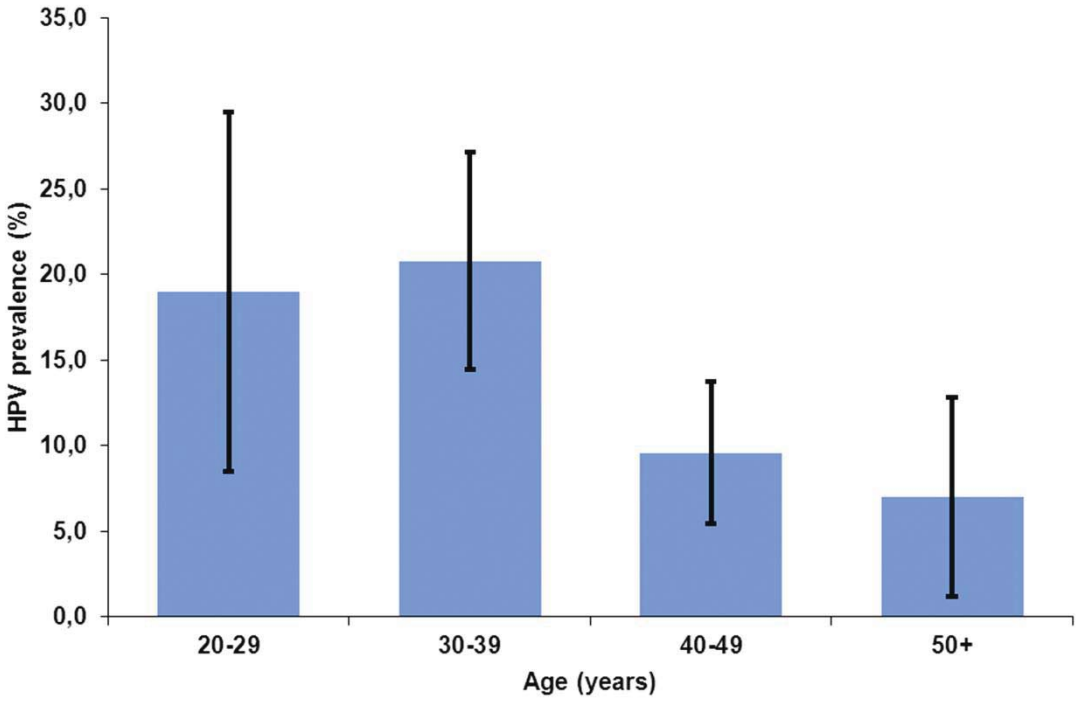

Figure 7. Age-specific HPV prevalence with standard deviation among 199 women from Kosovo included in the study

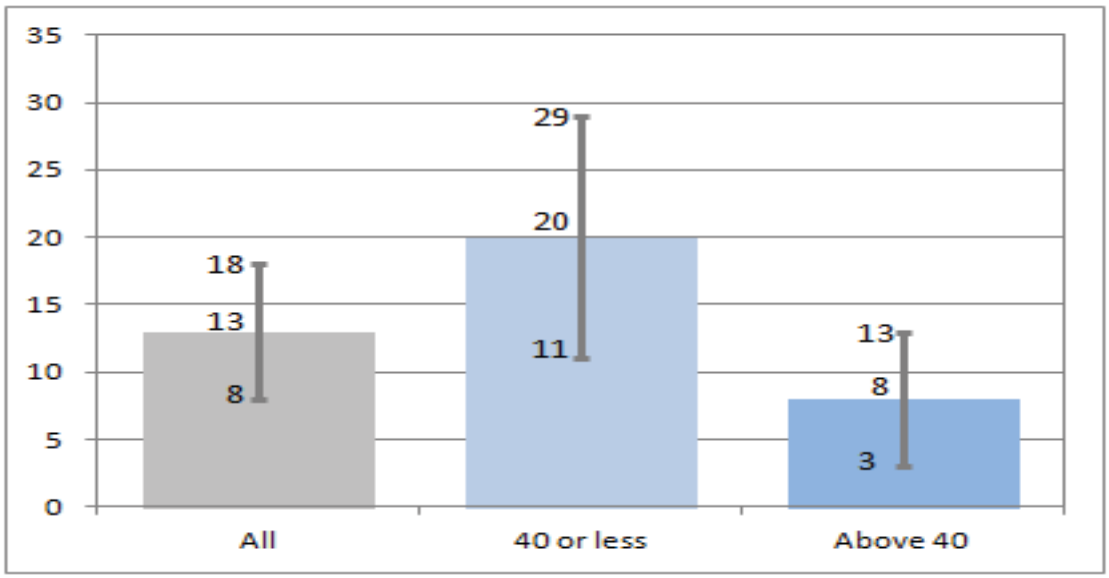

Figure 8. Among women younger than 40 the prevalence is 20% (95% CI: 11 – 29%) and among older 8% (95%CI: 3 – 13%).

Table 7. Association between socio-demographic characteristics and HPV infection (results of univariate logistic regression analysis)

	HPV-negative (n = 173)	HPV-positive (n = 26)	OR (95% CI)	*P*-value
(Mean) age (SD)	42.4 (9.2)	38.4 (9.9)	0.96 (0.91; 1)	0.044
≤ 40 years old	63 (36.4)	16 (61.5)	1	
> 40 years old	110 (63.6)	10 (38.5)	0.36 (0.15; 0.84)	0.015
Education				
finished elementary school or less	45 (26)	6 (23.1)	1	
finished secondary school	92 (53.2)	15 (57.7)	1.22 (0.44; 3.36)	0.697
more than secondary school	36 (20.8)	5 (19.2)	1.04 (0.29; 3.69)	0.950
Employment status				
Employed	44 (25.4)	7 (26.9)	1	
housewife or farmer	125 (72.3)	18 (69.2)	0.91 (0.35; 2.31)	0.835
Student	4 (2.3)	1 (3.8)	1.57 (0.15; 6.18)	0.702
Marital status				
divorced/widowed	10 (5.8)	3 (11.5)	1	
Married	155 (89.6)	21 (80.8)	0.45 (0.11; 1.77)	0.244
Single	8 (4.6)	2 (7.7)	0.83 (0.11; 6.26)	0.859
Family size				
Single	3 (1.7)	1 (3.8)	1	
small family (parents&children)	124 (71.7)	17 (65.4)	0.41 (0.04; 4.18)	0.453
large family (parents, children and other family members)	46 (26.6)	8 (30.8)	0.52 (0.05; 5.66)	0.593
Family income*				
Insufficient	47 (27.2)	7 (26.9)	1	
Sufficient	88 (50.9)	16 (61.5)	1.22 (0.47; 3.18)	0.683
Good	38 (22)	3 (11.5)	0.53 (0.13; 2.19)	0.380
(Mean) age (SD) at first sexual intercourse	21.9 (4.2)	20.8 (5.4)	0.94 (0.84; 1.04)	0.230
< 18 years old	22 (12.7)	9 (34.6)	1	
≥ 18 years old	151 (87.3)	17 (65.4)	0.28 (0.11; 0.69)	0.006
Delivery				
No	15 (8.7)	6 (23.1)	1	
Yes	158 (91.3)	20 (76.9)	0.32 (0.11; 0.91)	0.026
Pregnancy termination				
No	81 (46.8)	18 (69.2)	1	
Yes	92 (53.2)	8 (30.8)	0.39 (0.16; 0.95)	0.033
Menstrual cycle				
Irregular	33 (19.1)	7 (26.9)	1	
Regular	108 (62.4)	18 (69.2)	0.79 (0.30; 2.04)	0.620
Postmenopausal	32 (18.5)	1 (3.8)	0.15 (0.02; 1.27)	0.081

Contraception				
No	145 (83.8)	21 (80.8)	1	
Yes	28 (16.2)	5 (19.2)	1.23 (0.43; 3.54)	0.697
Smoking				
No	131 (75.7)	18 (69.2)	1	
Yes	42 (24.3)	8 (30.8)	1.39 (0.56; 3.42)	0.477

Categorical and continuous variables are shown as frequencies (percentages) and as mean (standard deviation), respectively. *The family's financial situation was evaluated by patients themselves. Abbreviations: OR (odds ratio), 95% CI (95% confidence interval).

The results of the multiple logistic regression analysis therefore suggest that women younger than 40, who were younger than 18 at the time of first sexual intercourse and that have not yet given birth, are at greater risk for HPV infection [Figure 9&10].

The share of women according to important factors associated with HPV infection are summarized in Figure 9. Among women that were HPV negative there is higher share of women with delivery or abortion in comparison to women that were HPV positive. In this group there is a higher share of women above 40 years old and women that were of age when having first sexual intercourse. There is also lower percentage of women that had more than one partner.

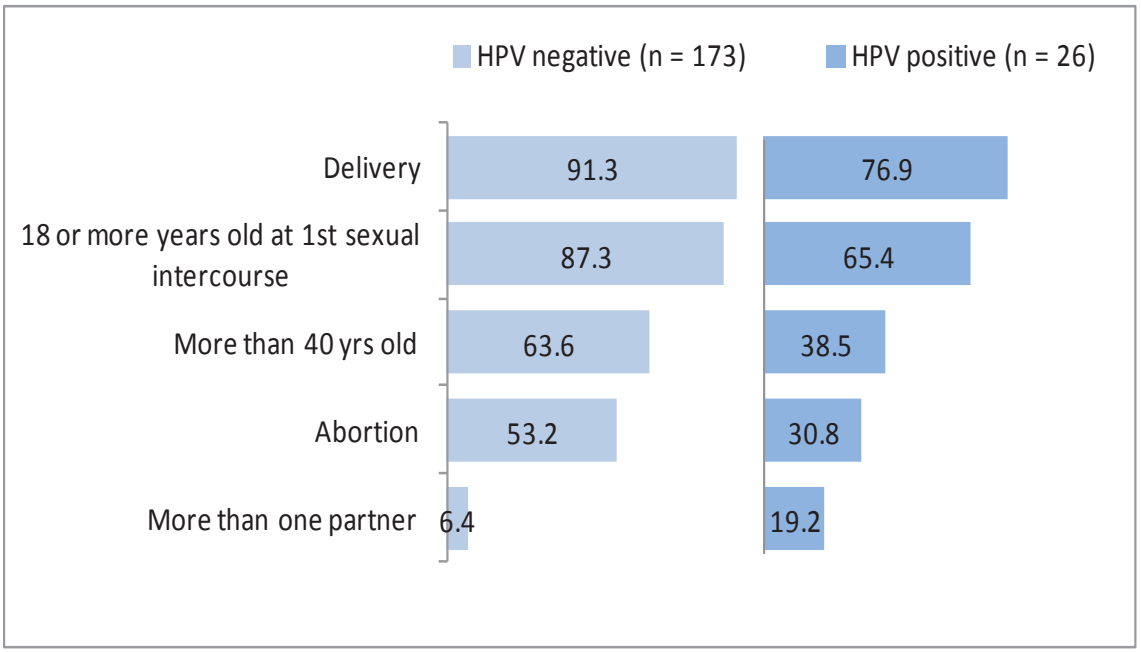

Figure 9: Percentage of women by important factors associated with HPV infection.

Odds ratios for HPV infections with 95% confidence intervals for important factors are summarized in Figure 10.

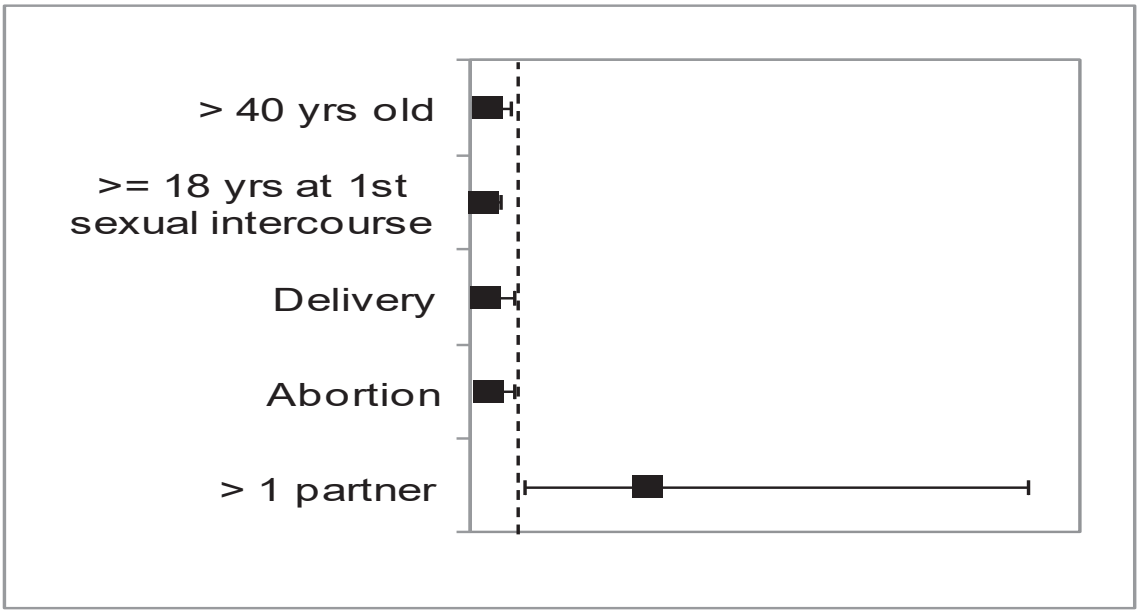

Figure 10: Odds ratios with 95 % confidence intervals for all relevant parameters.

For the multiple logistic regression the minimum sample size requirements according to Agresti's (2007) rule of thumb is 10 cases in the smaller category of dependent variable per independent variable. We have 26 HPV positive cases therefore 2 to 3 independent variables can be included in the multiple logistic regression model.

Further analysis showed that there is a statistically significant association between delivery and abortion ($p = 0.002$) and between age and delivery ($p < 0.001$). To avoid multicollinearity and comply with 1:10 rule, the final model will include three predictors that is current age (above 40 or at most 40), age at the first sexual intercourse (bellow 18 or 18 or more) and number of partners.

Results are shown in Table 8. All three predictors are statistically significantly associated with HPV infection. Risk factors associated with HPV are therefore first sexual intercourse when being younger than 18, being 40 or younger and having more than one partner.

Table 8. Association between current age, age at 1st sexual intercourse, number of partners and HPV infection (results of multiple logistic regression model)

	OR (95% CI)	P-value
> 40 year old	0.29 (0.11; 0.70)	0.007
≥ 18 years at the 1st sexual intercourse	0.22 (0.08; 0.59)	0.003
More than one partner	4.05 (1.20; 13.61)	0.024

5.5. Association between sociodemographic characteristics and hr-HPV types

As shown in Table 9, the results of the likelihood ratio test suggested that women infected with HPV16 were older in comparison to women infected with other hr-HPV types (p = 0.037). In addition, all women infected with HPV16 have given birth to at least one child, whereas only 68.4% of women infected with other hr-HPVs had biological children (p = 0.036). Other characteristics were not statistically significantly associated with specific hr-HPV types.

Table 9. Association between socio-demographic characteristics and hr-HPV types (results of likelihood ratio test).

	Other HPV type (n = 19)	HPV16 (n = 7)	*P*-value
Age			
≤ 40 years old	14 (73.7)	2 (28.6)	0.037
> 40 years old	5 (26.3)	5 (71.4)	
Education			
finished elementary school or less	6 (31.6)	0 (0)	0.107
finished secondary school	10 (52.6)	5 (71.4)	
more than secondary school	3 (15.8)	2 (28.6)	
Employment status			
Employed	5 (26.3)	2 (28.6)	0.725
housewife or farmer	13 (68.4)	5 (71.4)	
Student	1 (5.3)	0 (0)	
Marital status			
Married	15 (78.9)	6 (85.7)	0.303
divorced/widowed	3 (15.8)	0 (0)	
Single	1 (5.3)	1 (14.3)	
Family size			
Single	1 (5.3)	0 (0)	0.706
Small (parents&children)	12 (63.2)	5 (71.4)	
Large (parents, children and other family members)	6 (31.6)	2 (28.6)	
Family income*			
Insufficient	6 (31.6)	1 (14.3)	0.652
Sufficient	11 (57.9)	5 (71.4)	
Good	2 (10.5)	1 (14.3)	
Age at first sexual intercourse			
< 18	7 (36.8)	2 (28.6)	0.691

≥ 18	12 (63.2)	5 (71.4)	
Delivery			
Yes	13 (68.4)	7 (100)	0.036
No	6 (31.6)	0 (0)	
Pregnancy termination			
Yes	6 (31.6)	2 (28.6)	0.882
No	13 (68.4)	5 (71.4)	
Contraception			
Yes	4 (21.1)	1 (14.3)	0.691
No	15 (78.9)	6 (85.7)	
Smoking			
Yes	6 (31.6)	2 (28.6)	0.882
No	13 (68.4)	5 (71.4)	

*The family's financial situation was evaluated by patients themselves.

Comparing the results of high risk HPV positive women and their respective professions, we found out that the most affected group are women that works as a cleaner (37.5% of them are high risk HPV positive), followed by the hairdressers (25%), and students (20%) [Table 10].

Table 10. Correlation between the profession and HPV positivity

	HPV Negative		HPV Positive		Total	
	N	**%**	**N**	**%**	**N**	**%**
Total	**173**	86.9	**26**	**13.1**	**199**	**100.0**
Profession						
Cleaner	5	62.5	3	37.5	8	100.0
Clerk	2	100.0	-	-	2	100.0
Commercial	2	100.0	-	-	2	100.0
Cooker	1	100.0	-	-	1	100.0
Economist	7	87.5	1	12.5	8	100.0
Farmer	1	100.0	-	-	1	100.0
Hairdresser	3	75.0	1	25.0	4	100.0
Housewife	123	87.2	18	12.8	141	100.0
Journalist	1	100.0	-	-	1	100.0
Chemist	1	100.0	-	-	1	100.0
Lawyer	5	83.3	1	16.7	6	100.0
Linguist	1	100.0	-	-	1	100.0
Nurse	11	91.7	1	8.3	12	100.0
Sociologist	1	100.0	-	-	1	100.0
Student	4	80.0	1	20.0	5	100.0
Tailor	2	100.0	-	-	2	100.0
Teacher	3	100.0	-	-	3	100.0

We have analyzed the high risk HPV samples and their relationship with the menarche and menstrual cycle regularity, and our results show no statistical significance regarding these variables [Table 11].

Table 11. Menarche and Menstrual cycle regularity and HPV positivity

	HPV Negative		HPV Positive		Total		
	N	%	N	%	N	%	
Total	**173**	**86.9**	**26**	**13.1**	**199**	**100.0**	***P-value***
Menarche							
10-11	9	90.0	1	10.0	10	100.0	P=0.954
12-13	73	85.9	12	14.1	85	100.0	
14-15	73	86.9	11	13.1	84	100.0	
16+	18	90.0	2	10.0	20	100.0	
Menstrual cycle							
Irregular	33	82.5	7	17.5	40	100.0	P=0.158
Postmenopausal	32	97.0	1	3.0	33	100.0	
Regular	108	85.7	18	14.3	126	100.0	

After analyzing the use of contraception and the results of HPV positive women we did found statistical significance (p=0.045), and it is worthy to mention that the most affected women were those who use oral contraception pills with 26,7% resulting HPV positive, and none of them who uses condoms appeared to have high risk HPV infection [Table 12].

Table 12. Contraception and HPV infection

	HPV Negative		HPV Positive		Total		
	N	%	N	%	N	%	
Total	**173**	**86.9**	**26**	**13.1**	**199**	**100.0**	***P-value***
Contraception							
Condom	10	100.0	-	-	10	100.0	P=0.045
IUD	7	87.5	1	12.5	8	100.0	
no contraception	145	87.3	21	12.7	166	100.0	
oral contraceptives	11	73.3	4	26.7	15	100.0	

We have also analyzed the correlation between HPV infection and the previous history of cervical screening. We could not find any statistical significance at this point. Also, there was no statistical significance after analyzing the time interval between the cervical screening (PAP smear) and HPV infection [Table 13].

Most of the women included in our study, have declared that they had no regular gynecological examinations (140/199), but there was no statistical significance found regarding this variable and HPV infection [Table 13].

Table 13. Correlation between HPV infection and the previous history of cervical screening

	HPV Negative		HPV Positive		Total		
	N	**%**	**N**	**%**	**N**	**%**	
Total	**173**	86.9	**26**	13.1	**199**	**100.0**	**P-value**
Have you performed PAP smear before?							
no	71	85.5	12	14.5	83	100.0	P=0.805
yes	102	87.9	14	12.1	116	100.0	
When you have performed PAP smear?							
>3 yr	17	85.0	3	15.0	20	100.0	P=0.821
1 yr	58	86.6	9	13.4	67	100.0	
2 yr	2	100.0	-	-	2	100.0	
3 yr	25	92.6	2	7.4	27	100.0	
no	71	85.5	12	14.5	83	100.0	
Do you have regular gynecological examinations?							
no	124	88.6	16	11.4	140	100.0	P=0.389
Yes	49	83.1	10	16.9	59	100.0	

6. DISCUSSION

Cervical cancer (CC), as a preventable disease, still continuous to be the fourth most common cancer in women worldwide [161].

In developed countries, good preventive programs are established which enables women to get screened, making most pre-cancerous lesions of the cervix diagnosed at early stages when they can easily be treated. Early treatment prevents up to 80% of cervical cancers in developed countries [162]. Whereas, in developing countries, leak of an effective screening means that the disease in these women is often not identified in time, leading to an advance stages, including the cancer itself. In addition, possibilities for treatment of such late-stage disease may be insufficient, resulting in a higher rate of death from cervical cancer in these countries.

Unfortunately, in Kosovo, up to date we have no exact data for incidence, morbidity and mortality from cervical cancer.

It is well known that HPV is a major cause for the development of Cervical Cancer [4,7,163]. Infections with over 40 HPV types from the clinically most important *Alpha*-PV genus are considered to be the most frequently sexually transmitted infections in both genders [164]. In addition, worldwide, up to 4.5% of incident cancer cases, including cervical, anogenital, and head and neck cancers, are etiologically associated with HPV infection [165].

The data for the specific high risk HPV types and its distribution are the most necessary tool for developing new tests in HPV screening and producing vaccines that will cover most of HPV high risk types [166,167].
Moreover, cervical cancer accounts for 83% of all HPV-related cancers, with the majority occurring in women originating from less-developed countries [168].

In female population from Kosovo, the crude overall prevalence of infection with any of the 14 HR-HPVs was estimated at **13.1%.**

But, our results, shows lower prevalence comparing the studies conducted in other neighbor countries, as FYROM with 35.7% [169] and Serbia (Vojvodina region) with 50.5% [170].

From our results, it turned out that HPV 16 is the most prevalent high risk genotype with 26.9% of all hr-HPV positive tested women. This result corresponds with most studies conducted worldwide, in Europe and the region [4,92,171]. The second most prevalent hr-HPV types were HPV 31 and HPV 51 with 15.4%, each. HPV 18 comes as a fourth most prevalent type with 11.5% of HPV positive cases tasted in our study.
The rest of hr-HPV types found in our study were HPV 52,53,62,66 with 7.7 % each, and less frequent types were HPV 33,45,56,58,67,73 and 84.

Our results corresponds with the slightly difference with other results reported earlier from our neighbors, as in FYROM most prevalent hr-HPV were HPV 16, 31, 53 and 18, in Albania is HPV 16, 53, 31, 18, Vojvodina region in Serbia HPV 16,31,51 and 52 [169,170,172].

The HPV 31 and 51 were also reported in other studies in Europe, 31 as a second most prevalent in Croatia [173], also Germany[174] and Netherland [175] reported HPV 51 as the second most prevalent high risk genotype.

Also, one of our aims in the study was to analyze the hr-HPV positivity in correlation with cytological findings of the women included in the study.

From our results we found out that from the overall prevalence of hr-HPVs is 9.7 % among women with NILM (negative for intraepithelial lesion) in the cytology screening, 23.3 % were diagnosed with ASCUS; The two cases diagnosed with ASC-H and two other with SSC (confirmed also by the histopathology), were all positive on hr-HPV (HPV 16 and HPV 18, respectively).

Interestingly, in our study population the overall hr-HPV prevalence in NILM (9.7% (6.1-15.2) was lower than in studies on a total of 10,744 eligible women from other countries in Central and Eastern Europe (9.7%; 95% CI: 6.1 – 15.2% vs. 18.0%; 95% CI: 17.0 – 19.0%, respectively) [171,176-183], with the reported high incidence rates of cervical cancer and related mortality rates

[184]. Our results were concordant with those obtained in the largest study in the region to date, performed on 4,199 Slovenian women (hr-HPV prevalence of 10.7%; cervical cancer incidence rate per 100,000: 13.4; mortality rate due to cervical cancer per 100,000: 6.1) [184,185] as well as with global data (hr-HPV prevalence of 10.4%; cervical cancer incidence rate per 100,000: 15.1; mortality rate due to cervical cancer per 100,000: 7.6) [4,184].

Because persistent infection with hr-HPVs is a necessary, but non-sufficient, etiological factor of cervical cancer [186-188], several contributory factors have been described previously [189]. In this study, the results of the univariate and multiple logistic regression analyses suggested that women over 40 (univariate logistic regression: $p = 0.015$, OR = 0.36; multiple logistic regression: $p = 0.036$, OR = 0.37) were at lower risk for HPV infection.
Our results are concordant with global data [189,190], reporting that the incidence/prevalence of HPV infection peaks at a younger age (below 25–35 years), soon after the start of sexual activity, and declines with age due to the spontaneous clearance of HPV infections [4,190].

In our study, statistically significant associations with HPV infection were found for age at the time of first sexual intercourse, the results of the univariate and multiple logistic regression analyses suggested that women who were older than 18 at sexual debut (univariate logistic regression: $p = 0.006$, OR = 0.28; multiple logistic regression: $p < 0.001$, OR = 0.18) are at lower risk acquiring HPV infection. Our findings are in concordance with those of other authors that claim that the beginning of sexual activity in early age, enlarges the risk for HPV infection(190,191), but there are some authors that claimed the opposite [192,193].

Other findings that shows statistical significance in our study is the history of deliveries. Our data tells that women with a history of delivering at least one child (univariate logistic regression: $p = 0.026$, OR = 0.32; multiple logistic regression: $p = 0.035$, OR = 0.28) and the women with a history of pregnancy termination also have lower odds ($p = 0.033$, OR = 0.39) for HPV infection. Similarly as in our study, there are authors that has published before,

claiming that having more deliveries, increases the risk for HPV infection and cervical cancer.

In other case control studies, a very strong link was found between cervical cancer, cervical intraepithelial lesions and an increasing number of pregnancies [108,194-196]. But also, some authors did not observe any association between parity and CIN [197,198], another author found out that in the hr-HPV positive women, childbirth but not pregnancy was a predictive for CIN3+ [199]. There are suggestions that high parity might increase the risk for CC because it maintains the transformation zone on exocervix for many years, being more exposed to HPV and other cofactors [200].

Interestingly, according to the likelihood ratio test, in comparison to women infected with other hr-HPV types, women infected with HPV16 were older (p = 0.037) and have given birth to at least one child (p = 0.036).
Regarding the other factors analyzed in our study, like number of partners in life, very large number of women has declared that they had only one partner in a lifetime, which may be insincere answering, due to cultural background, were having more partners is still a taboo among middle age women in Kosovo.

From the results of multiple logistic regression, we found the statistical significance (p=0.024), were women having more than one partner are at higher risk for HPV infection. Our results are similar to the one published earlier, confirming the importance of the risk that brings the greater number of partners on inquiring the HPV infection [201], thus leading to the risk for even multiple infections with hr-HPV-s. Some authors have observed a significant increased risk of cervical diseases in individuals with multiple sexual partners compared to individuals with few partners, both in non-malignant cervical disease and in ICC [202].

We did found statistical significance in contraceptive use among hr-HPV infected women (p=0.045). Majority of the women included in the study declared that they do not use any contraceptive method. Probably, this group of women uses coitus interruptus as a method of contraception, which is widely used by the couples in Kosovo. Among the participants that uses other

methods, women that uses oral contraceptives are the most affected by hr-HPV infection. Whereas, the women that uses condom were all hr-HPV negative. Many case-control studies has been shown that the use of oral contraceptives to be associated with cervical cancer and HPV positive prevalence [203,204]. Sex hormones can alter the immune response to HPV 16 virus particles [205]. Hormonal contraception has been hypothesized to facilitate carcinogenesis mediated by HPV through increased expression of viral oncogenes [206,207], and tumor persistence and promotion [208,209].

The association between condom use and decreased risk for HPV infection has been described [210-212], whereas some authors claims that condom use may not be very effective in preventing HPV infection [213], because HPV lives in the skin of pubic area [214].

All the women included in the study declared they are not alcoholic nor drug users. One quarter of them are active smokers. Our results do not show any statistical significance regarding tobacco use and HPV infection. Most of the recent epidemiological studies confirmed the positive association between smoking and the risk for cervical cancer development [107,109]. IARC classified tobacco as a cause of CC. Whereas, Syrjanen K at all [112], concludes that cigarette smoking is not an independent risk factor of CIN2+. One explanation of an increased risk of HR-HPV infection among smokers is the mitogeneic effect of nicotine and cotinine, by causing DNA damage and also impair the host local immune defense mechanisms [215,216].

We also couldn’t find any statistical significance when analyzing HR-HPV positive women and their marital status, their level of education, family size, employment, economical background, by analyzing their income, declared by themselves. Some authors have noticed that low income environment and impossibility to use proper hygienic resources for personal hygiene are at higher risk for developing cervical cancer [217].

Our study, also has failed to show any relationship between the menarche, menstrual cycle regularity and the risk for HPV infection.

More studies, with larger number of participants, should be conducted to show any possible correlation of HPV infection and these variables.

We have analyzed the relationship between previous PAP test screenings, the intervals between screening and the HPV infection.

In our findings we could not find any statistically significance on it.

7. CONCLUSIONS

To the best of my knowledge, this study is the first study reporting the hr-HPV prevalence and distribution of HPV types in Kosovo, as well as the relationship between the hr-HPV infection and other related socio-demographic factors.

Through the development of this project a series of results was obtained:

a) The crude overall prevalence of hr-HPV infection with any of the targeted 14 HPV types was estimated at **13.1%** (26/199; 95% confidence interval (95% CI): 9.1–18.5%).
b) Infections with HPV16 were overall the most common, with 26.9% of all hr-HPV types identified, followed by HPV31 and HPV51, with 15.4% each, and HPV18 with 11.5%.
c) In our study, multiple HPV types were detected in five cervical smears (HPV16+HPV84, HPV66+HPV67, HPV51+53, HPV51+62, and HPV6+58+73)
d) Similarly, HPV16 was also the most frequently detected HPV type in patients with NILM, followed by HPV31 and HPV51. Patients with ASCUS were most frequently infected with HPV18.
e) After analyzing the potential differences in the sociodemographic characteristics of HPV-negative vs. HPV-positive patients, statistically significant associations with HPV infection were found for women's age (univariate logistic regression: $p = 0.015$, OR = 0.36; multiple logistic regression: $p = 0.036$, OR = 0.37). Women over 40 had lower odds for HPV infection.
f) Statistically significant associations with HPV infection were found for age at the time of first sexual intercourse ($p = 0.006$), where lower odds for HPV infection were also observed among women who were older than 18 at sexual debut (univariate logistic regression: $p = 0.006$, OR = 0.28; multiple logistic regression: $p < 0.001$, OR = 0.18).
g) Also, statistically significant associations with HPV infection were found at women with a history of delivering at least one child (univariate

logistic regression: p = 0.026, OR = 0.32; multiple logistic regression: p = 0.035, OR = 0.28), they were at lower risk for HPV infection.

h) The results of the univariate logistic regression analysis additionally suggested that women with a history of pregnancy termination also have lower odds (p = 0.033, OR = 0.39) for HPV infection.

i) Interestingly, according to the likelihood ratio test, in comparison to women infected with other hr-HPV types, women infected with HPV16 were older (p = 0.037).

j) In addition, all women infected with HPV16 have given birth to at least one child, whereas only 68.4% of women infected with other HR-HPVs had biological children (p = 0.036).

k) After analyzing the use of contraception and the results of HPV positive women we did found statistical significance (p=0.045), and it is worthy to mention that the most affected women were those who use oral contraception pills with 26,7% resulting HPV positive, and none of them who uses condoms appeared to have high risk HPV infection.

l) Regarding the other factors analyzed in our study, like number of partners in life, very large number of women has declared that they had only one partner in a lifetime, which may be insincere answering, due to cultural background, were having more partners is still a taboo among middle age women in Kosovo. From the results of multiple logistic regression, we found the statistical significance (p=0.024), were women having more than one partner are at higher risk for HPV infection.

After the first 10 years of routine use of HPV vaccines, comprehensive clinical trials and real-life data have confirmed the vaccines' safety and effectiveness.

Even though the introduction of HPV vaccination has the potential to substantially reduce the incidence of cervical cancer cases, the full effect on women of all ages will be detectable no sooner than after 30 years. Thus well-organized cervical cancer screening programs will still play a crucial role in the

prevention of cervical cancer and bridging the gap until the full effect of HPV vaccines can be observed [219].

In comparison to cytology-based screening, HPV-based screening is more effective and efficient for the prevention of invasive cervical cancer. Therefore, HPV testing is an invaluable part of guidelines for cervical cancer screening, triage, and follow-up after treatment. Even though more than 190 distinct HPV tests and at least 127 test variants were commercially available in only a fraction of HPV tests fulfill the criteria for use in primary cervical cancer screening, with RealTi*m*e, used in this study, being one of them [220].

The World Health Organization suggests that, in countries without established cytology-based cervical cancer screening, HPV-based screening programs, in combination with the implementation of HPV vaccination, could further accelerate screening benefits and reduce the burden of cervical cancer.

Despite the fact that a comprehensive strategy, including HPV vaccination and HPV-based cervical cancer screening, has been demonstrated to be cost-effective in nearly all countries, progress toward prevention is often hindered due to relatively low access to vaccines and limited use of cervical cancer screening, especially in low-income countries [221].

We hope that the results of this study will also persuade the authorities to implement nationwide cervical cancer screening and HPV vaccination programs in Kosovo.

Our results are concordant with global data, reporting that the incidence/prevalence of HPV infection peaks at a younger age (below 25–35 years), soon after the start of sexual activity, and declines with age due to the spontaneous clearance of HPV infections.

In conclusion, this study is the first study reporting the hr-HPV prevalence and distribution of HPV types in Kosovo.

Because more than 70% of cervical precancerous lesions identified in the study could have been prevented using nationwide HPV-based cervical cancer screening and HPV vaccination programs, it is of outmost importance to implement both programs in the national health care system in Kosovo as soon as possible.

8. ABSTRACT IN CROATIAN

Ciljevi: Trenutačni sustav zdravstvene zaštite Kosova ne podržava organizirani nacionalni probir karcinoma vrata maternice i program cijepljenja protiv human papiloma virusa (HPV). Do sada, nisu dostupni pouzdani podaci o incidenciji i mortalitetu raka vrata maternice na Kosovu, takoder ni o prevalencije visokorizičnih HPV-a i raspodjela HPV tipova. Cilj nam je utvrditi prevalenciju i raspodjelu visokorizičnih HPV tipova prije cijepljenja i procijeniti povezanost sociodemografskih obilježja i povećanog rizika od HPV infekcije u žena s Kosova.

Metode: Detekcija DNK visokorizičnih HPV tipova u cervikalnim obriscima žena koje su bile na rutinskoj ginekološkoj i citološkoj obradi provedena je primjenom klinički validiranog HPV testa, Abbott RealTime HPV testa, Roche Linear Array HPV genotipizacijskog testa, HPV52 tip-specifičnog realnog PCR-a i GP5 + / GP6 + / 68 PCR.

Rezultati: Ukupna prevalencija svih HPV-a visokog rizika procijenjena je na 13,1% (26/199, 95% intervala pouzdanosti (CI): 9,1-18,5%), pri čemu je HPV16 najčešći tip (7/26; 26,9 %), zatim HPV31 i HPV51, od kojih je svaki detektiran u 4/26 (15,4%) cervikalnih uzoraka, HPV18, detektiran u 3/26 (11,5%) uzoraka, HPV52 i HPV66, svaki detektiran u 2/26 (7,7%) uzoraka , i HPV33, HPV45, HPV56 i HPV58, svaki detektiran u jednom (3,9%) uzorku. Žene iznad 40 godina (OR = 0,36), starije od 18 godina pri prvom spolnom odnosu (omjer vjerojatnosti (OR) = 0,28), one koji su rodile barem jedno dijete (OR = 0,32), i one koje su imale prestanak trudnoće (OR= 0,39) imale su niži rizik za HPV infekciju.

Zaključak: Obzirom na to da bi se na Kosovu moglo spriječiti više od 70% prekanceroznih lezija vrata maternice pomoću HPV cjepiva protiv raka vrata maternice i cjepiva protiv HPV-a na nacionalnoj razini, od najveće je važnosti što prije implementirati preventivne programe u nacionalnom zdravstvenom sustavu.

9. ABSTRACT IN ENGLISH

PhD thesis: High risk HPV infection in Kosovar female population

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Objectives: Kosovo's current health care system does not support organized nationwide cervical cancer screening and human papillomavirus (HPV) vaccination programs. To date, no reliable data are available on cervical cancer incidence and mortality in Kosovo, or on high-risk HPV (hr-HPV) prevalence and HPV type distribution. Our aim is to determinate the pre-vaccination prevalence and distribution of hr-HPVs and to assesses the associations between sociodemographic characteristics and increased risk of HPV infection in women from Kosovo.

Methods: Detection of hr-HPV DNA was performed in cervical swabs of women that underwent routine gynecological and cytological evaluation by using a clinically validated Abbott RealTi*m*e High Risk HPV test, Roche Linear Array HPV Genotyping Test, HPV52 type-specific real-time PCR and an in-house GP5+/GP6+/68 PCR.

Results: The crude overall prevalence of any of the hr-HPVs was estimated at 13.1% (26/199; 95% confidence interval (CI): 9.1–18.5%), with HPV16 being the most common type (7/26; 26.9%), followed by HPV31 and HPV51, each detected in 4/26 (15.4%) cervical specimens, HPV18, detected in 3/26 (11.5%)

specimens, HPV52 and HPV66, each detected in 2/26 (7.7%) specimens, and HPV33, HPV45, HPV56, and HPV58, each detected in a single (3.9%) specimen. Women over 40 (OR = 0.36), older than 18 at sexual debut (odds ratio (OR) = 0.28), those that had delivered at least one child (OR = 0.32), and those that had a history of pregnancy termination (OR = 0.39) were at lower risk for HPV infection.

Conclusion: Because more than 70% of cervical precancerous lesions could have been prevented in Kosovo using nationwide HPV-based cervical cancer screening and HPV vaccination, it is of outmost importance to implement both programs in the national health care system as soon as possible.

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11. LIST OF TABLES AND FIGURES

TABLES

FIGURES

12. CANDIDATE'S CURRICULUM VITAE

Pranvera Zejnullahu Raçi was born on 26th of March, 1976 in Prishtina, Republic of Kosovo.

She finished the School of Medicine in the University of Prishtina, and later on she continued her further professional education, specializing in the field of Gynaecology and Obstetrics in the University Clinical Centre of Kosovo. While working as a gynaecologist, she gained interest in a field of Gynaecological Oncology and cervical diseases prevention.

Due to her interests in this area she continued with PhD studies, in a field of Biomedicine and Health Sciences in the School of Medicine, University of Zagreb.

Pranvera is a co-author in few publications in international journals and participant at different medical congresses and conferences, in country and abroad.

In her research, she has tried to reveal a real situation with HPV infection, necessity of implementing a proper cervical cancer screening program in Kosovo, as well as possibility for starting the vaccination against the HPV.

This research has a great contribution for Public Health in Kosovo, in sense of having a clear picture on hr-HPV infection, and the distribution of certain HPV types among the female population of Kosovo. This study may also serve as a starting point for having into consideration vaccination against HPV in near future.