The role of HPV in diagnosis and management of cervical premalignancies

Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

Proefschrift

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Reviews in Medical Virology 2007, in press (combined with chapter 8)

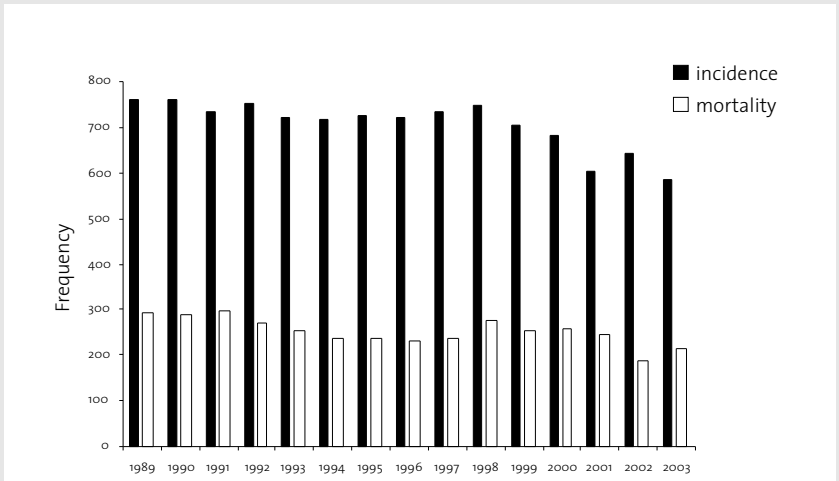
General Introdution

Cervical cancer

Cancer of the uterine cervix is a major cause of death worldwide. In 2000 cervical cancer was the second most common cancer in women accounting for more than 11% of all feminine cancers. Annually, an estimated 490,000 women are diagnosed with invasive cervical cancer globally.[1] The majority of the cases occur in developing countries, where it is frequently second in magnitude, with a lifetime risk of 1.5%.[2] In developed countries it accounts for 3.6% of the new cancers, with a lifetime risk of 0.8%. In the developed parts of the world cervix cancer is only seventh in magnitude with fewer cases than cancer of the corpus uteri and ovary.[1] Fortunately, mortality rates are substantially lower than the incidence rates. Globally the mortality to incidence ratio is 0.47, still accounting for more than 230,000 deaths yearly. In the Netherlands, incidence declined from 762 in 1989 to 584 in 2003, see Figure 1. The age-standardized mortality rate decreased as well from 3.3 deaths per 100,000 women in 1989 to 2.0 deaths per 100,000 women in 2003.[3] Most probably the influence of the population-based screening programme contributes to these declines.[1] The reductions are solely observed in squamous cell carcinomas, the incidence of adenocarcinomas appears to increase especially in young women aged 15-29.[4] Apart from non-specific symptoms like intermenstrual and post-coital bleeding the development of cervical cancer usually occurs without symptoms. Cervical cytological changes can be detected in an early and pre-invasive stage of the disease. Detection and treatment of these precursor lesions can effectively prevent cervical cancer.[5]

Cervical cancer is believed to have a co-factorial aetiology in which human papillomavirus (HPV) infections are considered the most important factor.[6,7] The risk of acquiring an HPV infection is highly associated with early sexarche, promiscuous precarious sexual behaviour, and sexual contact with promiscuous partners.[7] The cofactors, i.e. long-term oral contraceptive use, high parity, other sexually transmitted infections, cigarette smoking, and viral cofactors as viral load, integration, genotype and variants influence the likelihood of HPV persistence and progression towards a (pre-)malignant cervical lesion.[8]

Figure 1 Incidence and mortality of cervical cancer in The Netherlands



Anatomy of the uterine cervix

Non-keratinising squamous epithelium covers the ecto-cervical area (i.e. the external part of the cervix), whereas the endo-cervix (i.e. the internal part of the cervix) is covered with glandular columnar epithelium. The border between these two epithelia is called the squamo-columnar junction (SCJ). From puberty onwards columnar epithelium is replaced by squamous epithelium in a physiological process of metaplastic transformation. The SCJ is shifted more towards the endo-cervix thus forming a new SCJ. The transformation zone (TZ) is the area between the original and new SCJ. Due to the high cell-turnover rate, this zone is presumably more susceptible to oncogenic influences.[9] The majority of all (pre) malignant lesions develop in this particular area.[10] Generally, cervical lesions are detected using cervical cytological, i.e. cervical scrapes or smears. Colposcopy and histological examination are performed respectively to identify and finally diagnose the lesion.

Detecting, identifying, and diagnosing cervical lesions

Carcinoma *in situ* of the uterine cervix had already been acknowledged a precursor of invasive cervical cancer in 1932 by Broders.[11] Subsequently, Pap Anicolaou and Traut demonstrated that exfoliative cytology could be used to detect both *in situ* and invasive carcinomas of the uterine cervix.[12] Cervical lesions potentially progressing towards invasive carcinoma were initially diagnosed as dysplasia characterized by a disturbed epithelial architecture and cellular atypia of the epithelial cells. In the late 60's Richart hypothesized that cervical cancer develops from non-invasive stages, thereby introducing the terminology of cervical intraepithelial neoplasia (CIN).[13] Decades later it is generally accepted that CIN precedes squamous cell carcinoma of the uterine cervix. The pre-malignant CIN lesions are classified solely by histomorphological criteria, i.e. nuclear atypia, presence, frequency and localization of mitotic figures and the loss of polarity of the nuclei. According to the thickness of the epithelium involved in dysplastic alterations, CIN is hereby subdivided in low-grade lesions CIN 1 (involvement of less than one third of the epithelial layer), and high-grade lesions CIN 2 (one to two thirds involved) and CIN 3 (up to the full thickness is involved). CIN 1 is considered equivalent to mild dysplasia, CIN 2 to moderate dysplasia, and both severe dysplasia and carcinoma *in situ* are equal to CIN 3.

Twenty years after the introduction of Richart's CIN nomenclature the Bethesda System for reporting results of cervical cytology was developed.[14] The objective of this uniform system of terminology was to provide clear guidance for the improvement of patient management through the establishment of an optimal differentiation between lesions with a low- and high-risk of progression to carcinoma. The classification's final revision in 2001 intended an even more standardized approach in women with equivocal cervical smears.[15] Although, the Bethesda system and its local versions are used frequently, various other nomenclatures for the cytological and histological description of cervical abnormalities circulate and are sometimes used interchangeably. In the Netherlands for instance a modified Papanicolaou system, the CISOE (in Dutch KOPAC) classification was used since the introduction of the nationwide cytological screening in 1988. In this system five items are scored according to the acronym **C**omposition, **I**nflammation, **S**quamous epithelium, **O**ther abnormalities and endometrium and **E**ndo-cervical columnar epithelium. The S, O, and E are the only parameters specifying the smear classification as used in the other nomenclatures concerning cytological pathology. In 1996 this classification system was revised, resulting in CISOE-A (in Dutch KOPAC-B), where the **A** stands for the adequacy of the cervical smear.[16] Due to the revisions, cytology and histology are more concordant. Borderline nuclear changes resulting from inflammatory epithelial changes or from atrophic cells for instance are now classified as Pap 1 (i.e. normal pap-smear) instead of Pap 2. This has led to a significant decrease in Pap 2 smears diagnosed in the Dutch population-based screening programme from 9.8% in 1990 to 1.9% in 2000 (P<0.001), without any changes in the detection rate of high-grade cervical lesions (stable at 0.9%).[17] Simultaneous to the revision concerning the Pap 2 smears, screening-age limits, interval-time between recommended smears, and repeat and referral recommendations were changed. This caused a decrease in total

Table I Overview of the most frequently used cytological and histological classification systems, modified from Bulk, et al. (with permission).[17]

Bethesda 2001		WHO	CIN	BSCC	CISOE-A			Pap
Squamous	**Glandular**				**S**	**O**	**E**	
Unsatisfactory				Inadequate	0	0	0	Pap 0
NILM BCC/NILM/Atrophy		Normal	Normal	Normal	1 1	1 2	1-2 1-2	Pap 1
ASC-US/ASC-H	AGC			BNC	2-3	3	3	Pap 2
LSIL		Mild dysplasia	CIN 1	Mild dyskaryosis	4	4	4	Pap 3a
HSIL	AGC favour neoplastic	Moderate dysplasia	CIN 2	Moderate dyskaryosis	5	5	5	Pap 3a
		Severe dysplasia		Severe dyskaryosis	6	6	6	Pap 3b
	AIS	Carcinoma *in situ*	CIN 3		7	-	7	Pap 4
Invasive carcinoma	Adeno-carcinoma	(Micro-) invasive carcinoma	Carcinoma	Invasive carcinoma	8-9	7-8	9	Pap 5

WHO: World Health Organization; CIN: cervical intraepithelial neoplasia; BSCC: British Society Clinical Cytology; CISOE-A: the Dutch cervical cytology classification; S: Squamous epithelium; O: Other abnormalities and endometrium; E: Endocervical columnar epithelium; Pap: Papanicolaou classification; BCC: benign cellular changes; NILM: negative for intraepithelial lesion or malignancy; ASC-US: atypical squamous cells of undetermined significance; ASC-H: atypical squamous cells cannot exclude HSIL; HSIL: high-grade squamous intraepithelial lesion; AGC: atypical glandular cells; BNC: borderline nuclear changes; LSIL: low-grade squamous intraepithelial lesion; AIS: Endocervical adenocarcinoma *in situ*

number of smears (approximately 27%), although the number of regular screening smears increased (approximately 10%). In Table I the most frequently used cytological and histological classification systems are listed and related next to one another.

The current population-based screening programme

Non-invasive abnormalities of the uterine cervix are generally detected through cervical smears. Opportunistic or non-organised screening can reduce cervical cancer rates, but it may not adequately cover the targeted population, thereby specifically missing those at highest risk.[18] Large-scaled organised detection of cervical pathology i.e. population-based screening programmes (PSP), perform substantially better than non-organised programmes.[19] Depending on the screening interval and the age range of target group, PSP could establish a decline in mortality rate of 80-95%, providing that the participation rate is maximal.[20] Screening interval and accordingly lifetime number of recommend smears varies significantly throughout Europe, from 5-yearly interval and 7 smears in Lithuania, Finland, and parts of France, to yearly interval and more than 60 smears in Germany and Luxembourg.[21] Since, liquid based cytology is at least as good as conventional cytology[22], the present Dutch cervical cancer screening is based on either method supported by screening laboratory. In general, family doctors perform the cervical smears. It consists of a 5-yearly screening of women aged 30-60 years, leading to a recommended lifetime number of 7 smears. Approximately 70% of the invited women actually participate in the Dutch screening programme, this leads to a mortality reduction of 56%.[23] Optimising the sensitivity of the screening method and the screening interval, and increasing the participation rate of the target population to 100%, the mortality reduction rate will increase to 70%. This could for instance be achieved by adding a high-risk human papillomavirus test with a sensitivity of 100%.[23]

According to the consensus guidelines from 1996, cytology indicating either atypical squamous cells of undetermined significance (ASC-US) or a low-grade squamous intraepithelial lesion (LSIL) (also BMD; borderline and mild dysplasia) need to be repeated after 6 months. In case of persistent cytological abnormalities the patient is referred to the gynaecologist for colposcopic examination, whereas normalised cervical smears are repeated 12 months later. Patients with smears more severe than LSIL are immediately referred for a similar assessment. Based on the latest guidelines of the Dutch Pathology Association approved and published in July 2006, all BMD smears are repeated after 6 months and tested for presence of Human papillomavirus (HPV)[24], see “HPV assessment in screening” below.

Management and natural behaviour of abnormal cytology

Colposcopy guided biopsy samples are obtained to histologically diagnose a cervical abnormality; this is considered the gold standard. Adequate colposcopic images visualize the entire transformation zone, the impressions are enhanced by application of a 3% acetic acid solution. The classification of colposcopic impressions is a prediction of the expected histopathological findings. The quality of estimating the extent and severity of the lesion is related to experience and skills of the colposcopist and can thus be highly biased. Average inter-observer agreement for instance appeared to be moderate (52.4%) in experienced colposcopists, the level of agreement interpreting CIN 1 and CIN 2 lesions was lower than the agreement in no-CIN and CIN 3 lesions.[25] Ideally, a biopsy ought to be taken from all colposcopically suspected areas. However, generally a biopsy is only taken from the most severe or susceptible lesion visible. This could lead to misclassification of the underlying CIN lesion due to sampling error of multifocal lesions or histologically heterogeneous lesions.[26]

Subsequent to colposcopy and biopsy, various treatment options for high-grade CIN are available depending on the preference and experience of the gynaecologist. Regardless of the ablative therapy method used, the risk of an invasive cancer of the uterine cervix is reduced by 95% during the first 8 years following a successful treatment of pre-malignancies. Nonetheless, the chance of developing invasive cancer of the cervix for a treated woman is still increased five times compared to healthy individual.[27] To date, there is no convincing evidence suggesting a superior surgical treatment technique for high-grade CIN.[28,29] However, for several decades the large loop excision of the transformation zone (LLETZ) has been the most widely used method treating cervical pre-malignancies.[30-33] Using the LLETZ, CIN lesions are in general adequately eradicated, while anatomy and function of the cervix are maintained.[31,34]

Since, this multiple step strategy has increased the number of colposcopy appointments, time between referral and definite diagnosis, and subsequently expenses, Bigrigg and colleagues introduced a one-step protocol.[35] In case colposcopic assessment revealed a high-grade CIN lesion LLETZ was immediately performed. In a patient-control study, the histological diagnosis of the "see-and-treat" procedure has recently shown to be highly comparable with the diagnostic findings of the multi-step procedure.[36] Using the one or three step large loop excision treatment success rates varying from 81% to 98% can be achieved.[28,31,33,34,37,38]

Unfortunately, over-treatment of low-grade CIN is the penance for the accessibility and the low specificity of the LLETZ procedure. For achieving adequate and well-deliberate treatment, knowledge on the pro- and regressive behaviour of squamous intraepithelial cervical lesions is compulsory. In a review Östör showed that CIN 1 will progress to CIN 3 in 10% of the cases and to invasive cancer in only 1%.[39] CIN 3 lesions on the other hand will advance to invasive carcinomas in 12%, whereas spontaneous regression will occur in 33%.[40] Projections by Peto and colleagues, however implied that without a screening programme around 40% of the CIN 3 cases would eventually progress to invasive cancer if left untreated.[5] Of the women diagnosed with a single ASC-US smear only 7% have shown progression to a high-grade CIN lesion within 24 months, while 68% normalised.[41] Uncertainty however endures about the exact timescale of pro- and regression of "any-grade" CIN. If in defensive medicine all women with abnormal or persistently abnormal smears would be referred for colposcopy, this would irrevocably lead to a substantial overkill. Especially, since a large proportion of the women with ambiguous cervical cytology (i.e. Borderline and mild dysplasia (BMD) smears) will never develop cervical cancer.[42-44] Nevertheless, the risk of developing cervical cancer in BMD smears should not be underestimated, but should be carefully deliberated in the light of unfavourable health effects as unnecessary referrals, high anxiety levels[45,46], and cost effectiveness.[47] A not insignificant factor in the decision-making is that neither cyto- nor histomorphological markers of cervical intraepithelial abnormalities can predict the biological behaviour of CIN. Recently however, several studies found that abnormal cytology in women who tested negative for high-risk human papillomavirus will definitely not progress.[42,44]

Human papillomavirus (HPV)

A large body of clinical, molecular and epidemiologic studies have shown that a persistent infection with high-risk human papillomavirus (hr-HPV) is needed for the development of both cervical cancer and its high-grade non-invasive precursors.[43,44,48-53] This association is unique in cancer epidemiology, since it is the largest association ever identified.[43,52]

Papillomaviruses (PVs) are small, nonenveloped viruses with 55 nm diameter icosahedral capsids that contain double-stranded DNA genomes of approximately 8,000 base pairs. Based on their capsid structure and biochemical composition these microorganisms were originally lumped with the polyomaviruses in one family, the Papoviridiae. Since taxonomic classifications should reflect natural relationships, it was concluded that these viruses form two separate families. The International Committee on the Taxonomy of Viruses (ICTV) has recognised this and separated the families accordingly, Papillomaviridae and Polyomaviridae.[54]

Papillomaviruses are widely spread throughout the animal kingdom, each of those viruses being highly specific for their respective hosts. They specifically infect squamous epithelia and cause a generation of warts.[55] The infectious aetiology of warts was first described by Chandler in 1845. While removing a large circular condyloma, he accidentally injured his assistant beneath the thumbnail. "There appeared after a short time a wart, which was repeatedly destroyed, but reappeared, until the nail of the injured thumb was removed" (cited in Ullmann).[56] Two hundred years ago genital warts and even cervical carcinoma were regarded manifestations of venereal diseases not uncommon for the era.[57] In 1917, this assumption was abhorrently disputed by exposing extracts of a penile condyloma on sites of the forearm of a researcher and the genital mucosa of a "virgo intacta". The unfortunate subjects respectively developed flat warts and genital condylomata.[58] In the 30s of the last century several experiments had shown that the warts transmitted from cottontail rabbits to domestic rabbits were susceptible to malignant progression in the domestic animals.[59,60]

Regardless of their small size, the molecular biology of PVs is very complex. It consists of a 'late region', a non-coding long control region, and an 'early region'. The two late

region open reading frames (ORFs) L1 and L2 encode two structural proteins composing the major and minor capsid proteins, respectively. The non-coding region contains regulatory elements. The early region ORFs encode proteins that are supposed to be expressed before the onset of viral DNA replication. Three of the early ORFs are the oncogenes E5, E6, and E7, modulating the transformation process. Moreover, there are two regulatory proteins, E1 and E2, modulating replication and transcription. Moreover, E2 negatively influences the expression of E6 and E7.[55]

Although human papillomavirus was already identified as crystalline virus-like particles in 1949[61], it took approximately 30 years before HPV DNA from condylomata acuminata was characterised and cloned.[62-64] Eventually, this has led to the discovery of associated HPV sequences in cervical cancer.[65] In the following years the full genomes of various papillomviruses became available. Presently, the complete genomes of almost all isolated papillomavirus types have been fully sequenced.[54] The rapid increase in number of PVs identified, uttered the need for a taxonomic classification. Using phylogenetic criteria the taxonomic levels 'family', 'genus', 'species', 'types', 'subtypes', and 'variants' evolved. Since the L1 ORF is the most conserved gene within the genome, it has together with the E6 and E7 ORFs been used for identification of new papillomavirus types, subtypes and variants for the last two decades.[54] In order to identify a new type, the L1, E6, and E7 ORFs should differ by more than 10% from the closest type known. Differences of 2-10% lead to detection of a new subtype, whereas differences of less than 2% need to be observed in case of intratype variants.

The papillomavirus genotypes infecting humans can be divided in cutaneous and mucosotropic types, based on their tissue preferences. This variability in local tissue tropism is probably explained by the presence of type-unique binding sites for cellular factors on the non-coding region of the viral genome.[66] The cutaneous types are found in the skin and cause common warts. The mucosotropic HPV types infect the mucous epithelium of the oropharynx and anogenital tract. Approximately 40 different genotypes exhibit a tropism for the mucosa of the anogenital tract. Considering their carcinogenicity these anogenital HPV types have been subdivided into low-risk types (lr-HPV), probable high-risk types and high-risk types (hr-HPV)[67], although some controversy remains regarding the probable high-risk genotypes.[68] Almost all squamous cell cervical cancers harbour hr-HPV types.[52] High-risk HPV 16 alone accounts for 50% of the cervical cancer cases worldwide.[69] Other predominating types are HPV 18, 31, and 45. Together with HPV 16 they account for 80% of the cases.[6] In a pooled analysis of multiple case-control studies the IARC estimated an odds ratio of 158 for the association between cervical cancer and the presence of HPV.[67] Although the evidence of HPV induced carcinogenesis is strongest for cervical cancer, HPV infections have, with lesser evidence, also been associated with carcinoma of de vagina, vulva, penis, anus, skin, and oropharynx.[70]

HPV mediated carcinogenesis

Persistently hr-HPV positive women with abnormal cytology are reported to have a 327 times increased risk of developing high-grade lesions.[43] And although a genital HPV infection can occur in an early stage, persistence of a type-specific hr-HPV infection is considered a necessary requirement in the carcinogenesis of cervical cancer.[71] The established key-event of HPV-induced oncogenesis is the integration of viral-DNA in the human genome.[55]

The expression of the viral proteins is very tightly regulated and dependent on cell differentiation. Once the integrity of the basal layer is compromised due to micro-traumata or environmental changes HPV can infect the epithelium. Recently it was suggested that following absorption to the cell surface, capsomeric structure remains largely unchanged for many hours.[72] Uncoating may be facilitated by the disassembly of intracapsomeric interpentamer disulphide bonds in the reducing environment of the cell's cytoplasm hereby allowing transportation of viral DNA into the nucleus.[73] The viral genome is maintained as low copy number episomes in the basal layer of the epithelium. Vegetative DNA amplification occurs only in squamous epithelial undergoing terminal differentiation. Viral mRNA can be detected in low levels in the infected basal cells, but transcription of the viral genome is predominantly increased in the differentiated layers.[74] Papillomaviruses completely depend on the host cell's replication mechanism for DNA synthesis. Preservation of viral DNA as an episome is however most likely done by expression of E1 and E2.[75]

Frequently, hr-HPV DNA integrates near common fragile sites of the host genome[76], and this is believed to occur somewhere in the gradual process of progression of a

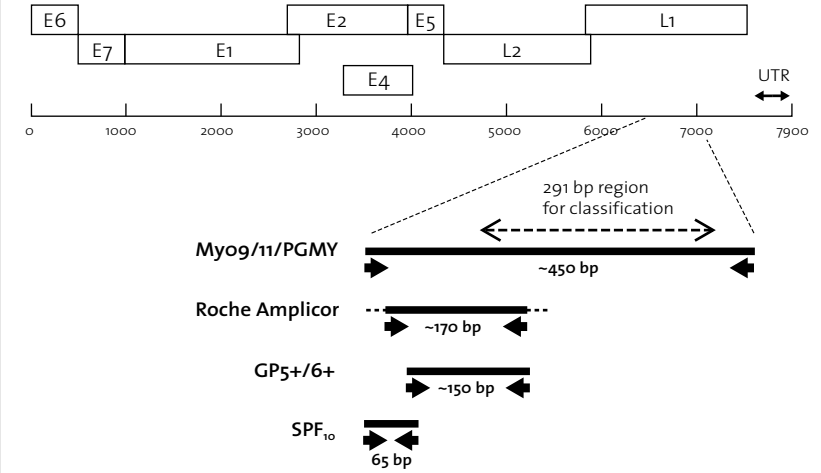
CIN lesion. Integration not infrequently results in the disruption of the viral E2 region, inducing an over-expression of viral E6 and E7 oncoproteins.[77,78] Whereas in low-grade CIN E6 and E7 are expressed in low levels in the basal layer, the expression increases throughout all epithelial layers in high-grade CIN lesions and cervical cancer. The proteins encoded by E6 and E7 show high resemblance with oncoproteins of other DNA tumour viruses. Successively, the oncoproteins interfere with two crucial mitosis-regulating pathways of the host cell, the p53-pathway and pRb-pathway, respectively. The E6 protein targets the p53 protein, which normally induces growth arrest or apoptosis. The binding of E7 to the active form of the retinoblastoma protein (pRb) causes inactivation of pRb and subsequent release of the host transcriptional factor E2F. This factor will eventually disrupt the cell cycle regulation. Accordingly, E6/E7-induced inactivation of these pathways results in hyperproliferation (E7 related) and genetic instability, numerical and structural chromosome aberrations and immortalization (all E6 related) (reviewed by Zur Hausen).[79]

In cervical carcinomas E6-induced chromosomal aberrations as deletions, translocations and inversions have been reported in chromosomes 1, 3, 6, 9, 11, and 17.[80] Structural rearrangements of chromosome 1 for instance have been described in 90% of the cervical cancers.[81] Also, the mean number of chromosome copies per nucleus, i.e. chromosome index (CI), for chromosome 1 shows a significant positive correlation with CIN grading.[82,83] E7-induced hyperproliferation expresses the proliferation-associated Ki-67 antigen that is recognized by the monoclonal antibody MIB1.[84] The Ki-67 labelling index (percentage of MIB1 positive cells) has been propound a promising alternative method for classification of CIN lesions.[85-87] As opposed to cyto- or histomorphological markers of a CIN lesion, the parameters related to aberrations of chromosome 1 and those related to cell proliferation are suggested to have predictive value regarding the natural behaviour of CIN.

HPV detection and genotyping

Since HPV cannot be cultured in conventional cell cultures and serological assays are of limited value as they are not able to distinguish between present and past infection, accurate diagnosis of HPV infections relies on the detection of viral nucleic

Figure 2 The outline of the HPV-DNA genome, presented in linear form with the positions of the early (E) and late (L) genes, and untranslated regions (UTR) indicated, as well as the positions and size of the four most widely used primer-sets for HPV detection/genotyping. Adapted form Molijn, et al. (with permission).[88]



acid (NA).[88] HPV DNA can be detected in cytological smears and histological samples by a number of methods that have been developed over the past 25 years. Amongst these techniques are i) non-amplified NA hybridisation methods as Southern Blot, ii) signal-amplified, immunoassay-based NA hybridisation methods as the Hybrid Capture assay, and iii) a variety of target-amplification systems like type-specific polymerase chain reaction (PCR) and consensus-primer PCR techniques. The non-amplified NA hybridisation methods are insensitive[89] and very laborious. To overcome these problems and to increase both sensitivity and specificity various amplification methods were developed (see for review Molijn, et al.).[88] These NA amplification techniques could also enlarge the appropriateness for mass screening. Snijders, et al. recently stated that the efficacy of population based screening programmes solely using cervical cytology could benefit from adding hr-HPV testing.[90] Accordingly, many ongoing international research projects have been assessing the feasibility of introducing hr-HPV tests in the available routine screening.

Apart from categorising the tests based on the technique used, contemporary tests can be divided according to their actual purpose, i.e. hr-HPV detection or HPV genotyping. Tests distinguishing high-risk HPV infections from no HPV infection for instance could be specifically valuable for mass-screening purposes. Especially, since merely hr-HPV genotypes are essential in the carcinogenesis of the uterine cervix. The non-radioactive signal-amplification method Hybrid Capture II (hc2, Digene Corp., Gaithsburg, Maryland, USA) and the recently developed target-amplification method Roche Amplicor® HPV Test (Roche Molecular Systems, Inc., Branchburg, NJ, USA) are two of these alleged "hr-HPV plus/minus" screening methods which are currently commercially available, CE marked and frequently used. The hc2 test is however the only FDA registered HPV screening assay.[91] Since, both tests only differentiate between an infection with one (or more) of 13 hr-HPV genotypes and no hr-HPV infection, neither one of the assays allow individual identification of specific genotypes, nor do they have the ability to identify infections harbouring multiple genotypes. This is regrettable as recent studies have provided evidence for a difference in oncogenic potential between the different hr-HPVs[92] arguing for the importance of HPV genotyping in a clinical setting. Outside of the clinical setting, HPV genotyping is a key-characteristic of studies evaluating i) the epidemiology of HPV infections worldwide[93-95], ii) HPV vaccination/surgical treatment trials [88,96-100], and iii) cervical cancer screening and triage.[43,90,101,102]

In order to detect and genotype HPV DNA in a single sample using type-specific (TS) primers, multiple polymerase chain reactions ought to be used separately. However, particularly if multiple genotypes are present this method is too laborious. Additionally, the type-specificity of each PCR TS primer set should be validated. As an alternative, a broad spectrum of HPV types can be amplified using consensus or general primers. Predominantly these primers target a section within the most conserved region amongst HPVs, the L1 ORF.[88] Various primer designs are available for HPV assessment. The most commonly used are the GP5+/6+ PCR system, the Roche Amplicor®, the PGMY primer set, and the SPF_{10} primer set. These PCR primer sets vary substantially in their design and the size of the PCR product they amplify, see Figure 2. Generally, the PCR efficiency is dependent on the amplimer size; the larger the product the less efficient the reaction. Formalin-fixed and paraffin-embedded samples frequently show DNA degradation, therefore HPV assessments using primer sets generating large PCR products are inappropriate and their efficiency is considerably lower than primer sets generating smaller PCR products.[103] In contrast the various consensus primer assays show comparable results in detecting HPV in liquid-based cytology samples. Subsequent to the amplification of HPV DNA, reverse hybridisation of the amplicon to multiple oligonucleotides provides the possibility to simultaneously type up to 37 different low-risk, possible high-risk and high-risk HPV genotypes. The oligonucleotide probes which recognize the different genotypes are frequently tailed with poly(dT) and immobilised as parallel lines to membrane strips. The assays called line blot assay (LBA), line probe assay (LiPA) or LINEAR ARRAY (LA) require only little amount of PCR product. A range of scientific papers have assessed and, on occasion, compared the various tests. Generally these tests are judged advantageous in the ability to rapidly genotype HPVs present in samples with high sensitivity and specificity.[104-108] Thereby, minimizing the possibility of misclassification.[104]

Besides the importance of specific HPV genotypes in the risk of (pre-)malignant deterioration of cervical lesions[92], other viral cofactors as viral load and viral integration appear to influence the progression likelihood.[8] Real-time PCR techniques have been developed to quantify HPV in clinical samples. However, due to low multiplicity for different hr-HPV types, real-time PCR methods are not (yet) suitable as a high-throughput screening tool.[109] Moreover, the Hybrid Capture II provides a semi-quantitative measurement of HPV-DNA. The estimated hc2 load correlates well with the precise load generated by real-time PCR.[110] Abnormal cervical smears with high viral loads have been suggested to have an increased risk to progress.[111-115] These conclusions however could not be verified by other studies.[116-118] The physical status of HPV DNA, i.e. episomal, mixed, or integrated, measured through the real-time PCR evaluation of the HPV 16 E2/E6 ratio is suggested a potential diagnostic marker for progressive cervical lesions.[119,120] In general, the frequency of viral integration increased in parallel with the severity of the lesion. However, several studies have already reported a lack of sensitivity of this specific assay.[121,122] Moreover, a study by Arias-Pulido and co-workers showed that exclusive viral integration occurs in 8.7% of the carcinomas *in situ* and only in 15.2% of the cervical cancers, whereas mixed infections were detected in 29.4% and 45.7% of the cases, respectively.[121] This suggests the existence of other potential sites for HPV integration.[76] Whether the E2/E6 ratio real-time PCR for HPV integration is already usable in routine detection of HPV integration remains questionable.

HPV assessment in screening

As mentioned previously hr-HPV infection plays the key role in the multi-step carcinogenesis of cervical cancer. The sensitivity of hr-HPV testing for the detection of high-grade lesions is approximately 95%.[123] The negative predictive value (NPV) of hr-HPV testing for the detection of high-grade CIN, i.e. the proportion of patients with negative hr-HPV test results who are correctly diagnosed not having nor developing high-grade CIN, is almost as high as 100%.[124,125] This by far exceeds the sensitivity of conventional smears and liquid based cytology, respectively 58% and 84%.[124] HPV assessment could therefore play a significant role in population based screening programmes for cervical cancer, i.e. detecting of abnormal cervical cytology[126], management of equivocal smears[42,127], and follow-up management of women treated for high-grade CIN.[128,129]

However, the specificity of a positive high-risk HPV DNA test for high-grade CIN is lower than that of both conventional and liquid based cytology. Also, the positive predictive value (PPV) of hr-HPV assessment for the detection of high-grade cervical lesions -i.e. the proportion of patients with a positive hr-HPV test who are correctly diagnosed with a high-grade CIN- is low compared to the PPV of cytology.[124,125] Insignificant positive HPV test results are especially inconvenient for sexually active young women who experience the highest HPV prevalence. Providing an adequate immune response, the vast majority of these women will clear the HPV infection within 12-24 months.[130,131] Moreover, 70% of the HPV induced low-grade CIN will regress spontaneously, whereas the regression rate is 100% in hr-HPV negative women.[102] Viral clearance often precedes the cytological normalisation. In countries provided with a well-organised screening programme, this natural behaviour of HPV infections and the absence of a proper test discriminating persistent and transient infection, are responsible for the scepticism replacing cytology with HPV testing.[132] Introducing such an all-exclusive HPV detection strategy could however be a promising alternative to a cytological assessment in countries lacking an organised population based programme.

Adding high-risk HPV detection to the conventional cytological screening could safely lengthen the interval period by 2-5 years in the Netherlands.[133] Which would lead to a substantial decrease in the recommended lifetime number of smears.

Particularly for countries with an opportunistic screening policy and therefore high numbers of recommended smears (e.g. Germany and Luxembourg) this could reduce costs considerably. The NPV of double negative tests, i.e. normal cytology and no presence of high-risk HPV, is close to 100%.[134] Moreover, in several studies progression towards high-grade CIN lesions was not detected in women with an HPV-negative borderline and mild dysplasia (BMD) smear.[127,135] Providing that all hr-HPV negative BMD women (65% of all BMD women) have their subsequent smear taken at the next screening round (after 5 years) and all hr-HPV positive women are reassessed after 6 months, referral to the gynaecologist for colposcopic assessment of this group could be markedly reduced. Berkhof and colleagues have recently shown that this approach is cost-effective.[136]

Not to trivialize is the suggestion that incorporating HPV assessment would have a positive impact on the detection of glandular lesions,[137] these rare but aggressive lesions are easily missed through regular cytological screening.[132] High-risk HPV assessment might be helpful in detection of these lesions.[138,139]

Since primary and exclusive HPV testing will lead to too many false positive referrals and HPV assessment combined with cytology will increase screening cost (especially if a liquid based approach has to be introduced simultaneously), triage management could be highly valuable. Considering triage, either abnormal cytological smears could be triaged using HPV assessment or persistently hr-HPV positive women could be triaged using cytology. The prospective, randomised clinical ALTS (ASCUS-LSIL Triage Study) trial concluded that HPV triage in ASC-US women is at least as sensitive as immediate colposcopy-guided biopsy for detecting CIN 3.[140] For the LSIL smears the ALTS-trial was not able to determine a useful triage strategy, these smears were best management by direct colposcopy.[141] More recently however Bias et al. suggested that hr-HPV negative persistent borderline and mild dysplasia smears will not develop cervical cancer, do therefore not need to be referred and can thus be followed in the regular population based screening programme.[42] Based on repetitive positive high-risk HPV test colposcopy referrals could be reduced by approximately 50% in BMD smears.[42,44,127,140,142] Too high levels of HPV positivity however will negatively affect a triage management, in a UK study for instance 80% of the BMD smears appeared to be HPV positive.[143] Since HPV predominantly affects young women, age could be a determining factor in triage. Cuzick and colleagues suggested

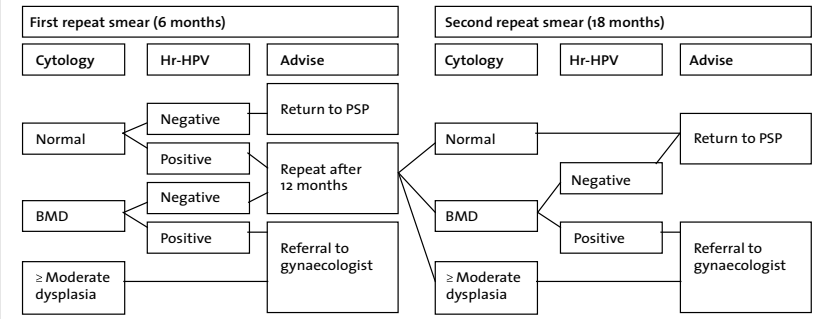
using HPV testing for primary screening in women older than 30 years, thereby using cytology to triage HPV-positive women. This approach could potentially improve detection rates of high-grade CIN without increasing the colposcopy referral rate.[127] How to manage the triage of younger hr-HPV positive women remains however equivocal.

The previously described HPV assays generally detect 13 of the 15 most common hr-HPVs. Adding more high-risk and probable high-risk genotypes will probably be of too little relevance for cervical cancer. Moreover, the possible increased sensitivity will be at the costs of a marked specificity loss.[68] Recently, Castle, et al. discovered that hr-HPV 16 positive women had a significant five fold increased risk for developing high-grade CIN compared to women infected with any other high-risk HPV genotype.[92] This suggests that, the clinical implications of HPV genotyping can be even more significant than HPV detection. However, HPV genotyping is too expensive to introduce as a routine screening tool.

Following the introduction of high-risk HPV assessment in the USA, other countries will eventually implement hr-HPV testing. Only recently (July 2006) the Dutch Pathology association (NVVP) for instance has approved the new guidelines considering the triage of persistent BMD smears.[24] In case of an initial BMD smear, cytology is repeated after 6 months. If the repeated smears indicated moderate dysplasia or worse, the women is immediately referred for colposcopic examination, whereas in all other cytological diagnoses hr-HPV is tested and recommendations are as indicated in Figure 3. For the countries still awaiting implementation of HPV testing, the most obvious questions rising are; which strategy and which assay to use and what are the costs? Regarding cost-effectiveness, both HPV DNA testing strategies, HPV triage and combination testing, appeared to be more effective than status quo screening policy in the UK, France, Italy and the Netherlands.[144] The cost-effectiveness of HPV genotyping as risk assessor has not been studied yet.

In addition to the discussion on which HPV assessment strategy to use, the issue of the most appropriate detection method has risen. Particularly the dramatic variation in HPV prevalence in healthy women (normal cytology) probably depending on the assessment moment and on the HPV assay used has contributed to this debate. Herein, difficulties concerning analytical versus clinical sensitivity and specificity

Figure 3 Flow chart of the new policy of the Dutch pathology association (NVVP) regarding the follow-up of abnormal cytology and the HPV triage, modified from "de praktijkrichtlijn versie 2.0".[24]

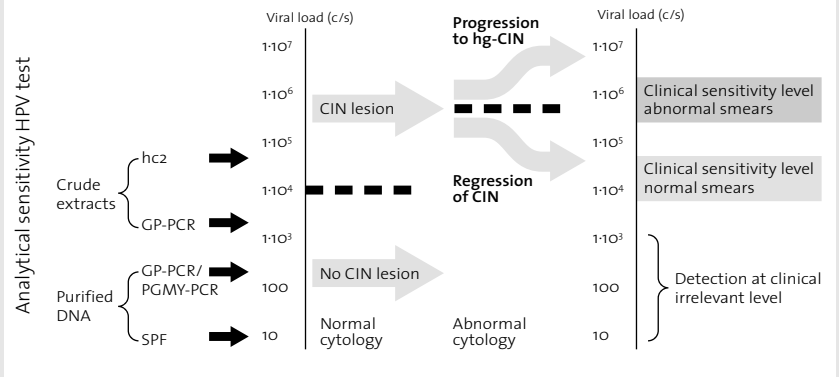


Hr-HPV: high-risk Human papillomavirus; PSP: population-based screening programme; BMD: borderline and mild dysplasia

need to be overcome. The analytical performance measures refer to the proportion of HPV positive and negative women who are correctly classified by a given assay. The clinical sensitivity refers to the proportion of affected women (≥ CIN 3) who are correctly identified by a positive hr-HPV test, whereas the clinical specificity signifies the women without a lesion who are correctly classified negative by a given test.[90] In case a specific test is able to distinguish as little as 10 HPV DNA copies per sample, this could be very relevant from an analytical perspective. Both the virologist and the epidemiologist would welcome an assay with such a high analytical sensitivity, i.e. always detecting the virus if present. Thereby not taking the clinical implication of the test result in to account; in a sample that is barely hr-HPV positive, it is highly unlikely a high-grade CIN is detected. Recent studies have for instance shown that HPV 16 viral load measured using quantitative real-time PCR methods is proportional related to the severity of CIN lesions.[104,115] These data support the concept that an increased HPV viral load in normal cytology increases the risk of developing pre-malignant cervical lesions. Persistently high viral loads are subsequently associated with progression of the lesion, whereas clearance of a lesion is preceded by a decrease in viral load (Figure 4).[90]

As previously mentioned, the observed variations in HPV prevalence and the following risk estimates for high-grade lesions may be partly attributed to the HPV assay used. Based on existing experiments, Snijders and colleagues concluded that of the most widely used consensus HPV test, the Hybrid Capture II assay has the lowest analytical sensitivity and the SPF_{10} the highest. Both the GP5+/6+ and the PGMY primer sets have intermediate analytical sensitivities.[90] The relation between analytical and clinical sensitivity of the four different assays is schematically represented in Figure 4.

Figure 4 The concept of HPV load in cervical smears in relation to biological behaviour and the schematic representation of the relationship between the analytical sensitivity and the potential clinical sensitivity levels (for lesions ≥CIN 3) for normal and abnormal cervical smears. Modified from Snijders, et al. (with permission).[90]



Physicians ought to be aware of the stated differences and should be able to use and translate this information to the management of the individual patient. Accurately studying both the epidemiology of HPV and the global impact of HPV infections, and monitoring the HPV vaccination trials however, require a test with the highest analytical sensitivity possible.[90]

Self-sampling

The participation rate of the Dutch population-based screening programme (PSP) is approximately 70%. Tragically, half of the cervical cancers are diagnosed in the remaining non-participating 30%. The reason why women do not participate varies from being afraid of the procedure or the possible diagnosis to having a too busy schedule. Although hr-HPV testing might have a beneficial effect in general patient management, the viral infection is often presented as or associated with sexually transmitted diseases. Implementing or adding an HPV test might affect a woman's sexual relationship in terms of trust, fidelity and blame if a positive result is found, this could in turn negatively influence the compliance rate.[23] Especially, if the general practitioner performing the test is acquainted with the partner involved. Genital self-sampling is a more anonymous and user-friendly alternative method for the collection of vaginal material. Since it is virtually impossible for a woman to obtain a desired cervical scrape of the transformation zone, self-sampled material is not suitable for cytological diagnoses. Material from vaginal lavages or self-sampling brushes is however highly representative for the cervical HPV status.[145-147] Recently, Brink and colleagues have described a paper in preparation in which 1.6% of the PSP non-responders with a valid self-sample test appeared to have a high-grade cervical intraepithelial lesion.[109] This is significantly higher than the high-grade CIN prevalence in the POBASCAM trial.[148] These results not only show that women who do not participate in a well-organised screening programme indeed have a higher risk developing cervical cancer[109,145,146], but also that hr-HPV testing on self-sampled materials might be a promising opportunity increasing the efficiency of population-based screening programmes worldwide.[109] Moreover, it illustrates the need for HPV tests with both high enough sensitivity and specificity and subsequent adequate patient management, in order to detect and subsequently treat high-grade cellular abnormalities of the uterine cervix in an early stage thereby reducing the risk of cervical cancer development.

Aim and outline of this thesis

The management of abnormal cervical cytology and pre-malignant cervical lesions has been debated for many decades. Traditionally, cytological and histological examinations were used for the screening, diagnosis, and follow-up of cervical abnormalities. However, since it is now widely accepted that high-risk human papillomavirus (hr-HPV) infections play a significant, but not sufficient, role in the development of virtually all cervical carcinomas worldwide, HPV assessment has become increasingly more issued. This sexually transmittable infection (STI) is highly prevalent in the general population, especially in sexually active young adults. Previous investigations have shown STI are more prevalent in subfertile patients. Chapter 2 describes the distribution of normal and abnormal cervical cytology in women eligible for *In vitro* fertilisation (IVF) and compares these smears with the smears of women in the population-based screening programme for cervical cancer. In chapter 3, cervical cytology is studied with regard to the follow-up of women who underwent a LLETZ procedure for a high-grade cervical lesion in a retrospective study. Chapter 4 reports the presence and persistence of (high-risk) HPV genotypes in histological samples, and cytological follow-up samples of women who were treated for a high-grade CIN. Accurate genotyping of HPV is essential when studying i) the epidemiology of HPV, ii) evaluating the efficiency of HPV vaccines or surgical procedures of the cervix, and iii) cervical screening and triage. A recently developed HPV genotyping assay is compared with a more established assay in chapter 5. Not every HPV positive women will eventually develop a (pre-) malignancy of the uterine cervix. Assessment of various proliferation markers in cervical smears could triage progressive and regressive CIN lesions in case of HPV positivity. This is studied in chapter 6. The worldwide advance and influence of HPV assessment assays is irreversible and also self-sampling will become customarily. Chapter 7 reports the efficiency of HPV detection using a new method of sample storage and transportation. And finally in chapter 8, future directions are discussed in general, based on the results of the studies presented in this thesis.

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Abnormal cervical cytology in women eligible for IVF

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Abstract

Chlamydia trachomatis is more prevalent in subfertile women than in the general population and is the leading cause of tubal factor subfertility. As *C. trachomatis* infections are sexually transmitted, it can be expected that infections with human papillomavirus (HPV) are also more prevalent in this group of women. HPV is a necessary cause for the development of cervical (pre-)malignancies. We therefore hypothesized that subfertile women are more likely to have HPV-induced cervical abnormalities compared to the general population. In this retrospective case-control study, all cervical smears of women visiting the fertility clinic for IVF (cases) and of women attending the population-based screening programme for cervical cancer (controls) were retrieved from an electronic database and assessed. The cases (n=669) showed significantly more abnormal cervical smears compared to the controls (n=77 055) (6.1 and 3.9%, respectively, P < 0.02). The probability that subfertile women eligible for IVF are diagnosed with a high-grade cervical lesion is almost twice as high compared to women in the general population. We therefore suggest to take a cervical smear from all women referred for fertility problems.

Introduction

Sexually transmitted infections (STIs) in adolescents and young adults (i.e. 10-19 and 20-24 years of age, respectively) are a significant burden for health care worldwide. In the United States this age group represents approximately 25% of the sexually active population; however, they account for more than 65% of all registered STIs.[1,2] *Chlamydia trachomatis* and human papillomavirus (HPV) infections are STIs of specific interest because of the high prevalence rates and the association with tubal factor subfertility and cervical cancer, respectively. Both can be considered a marker reflecting promiscuous, precarious sexual behaviour noticeably increasing in the general population.[3]

Prevalence of *C. trachomatis* infections varies from 5% in asymptomatic women in the general population[4] to 24% in women assessed for fertility problems.[5] Adolescence, nulligravidity, promiscuity and unmarried state are independently associated with an increased risk for *Chlamydia* infection.[4] In a small percentage of infected women, an ascending infection will lead to pelvic inflammatory disease and ultimately to tubal factor subfertility, the third cause of subfertility in industrialized countries.[6]

The estimated lifetime risk of contracting a genital HPV infection is 80%, whereas 50% of the sexually active women will be infected within 2 years following the sexarche.[7] Molecular and epidemiologic studies have shown that a persistent infection with high-risk HPV is the most important risk factor for the development of both cervical cancer and its precursors.[8-10] Close to all of the cervical cancers[11], 84% of high-grade cervical intraepithelial neoplastic lesions (CIN) and 74% of low-grade CIN lesions harboured the high-risk virus.[12] Recent studies have shown that HPV persistence is associated with concurrent *Chlamydia* infection[13] and that *C. trachomatis* antibodies were associated with a significant increase of squamous cell cervical cancer.[14]

The presumed increase in promiscuous, precarious sexual behaviour results in sexually active adolescents who are at risk of acquiring HPV and *C. trachomatis* infections unaware of the fact that (tubal factor) subfertility and (the treatment of) severe cervical pathology could negatively affect family planning even many years following an infection. Because women with fertility problems have the highest

Chlamydia prevalence, suggesting promiscuous, precarious sexual behaviour and thus possible HPV infections, we hypothesize that women visiting fertility clinics are more likely to harbour high-grade cervical lesions than women in the general population. This study was undertaken to study this hypothesis.

Materials and methods

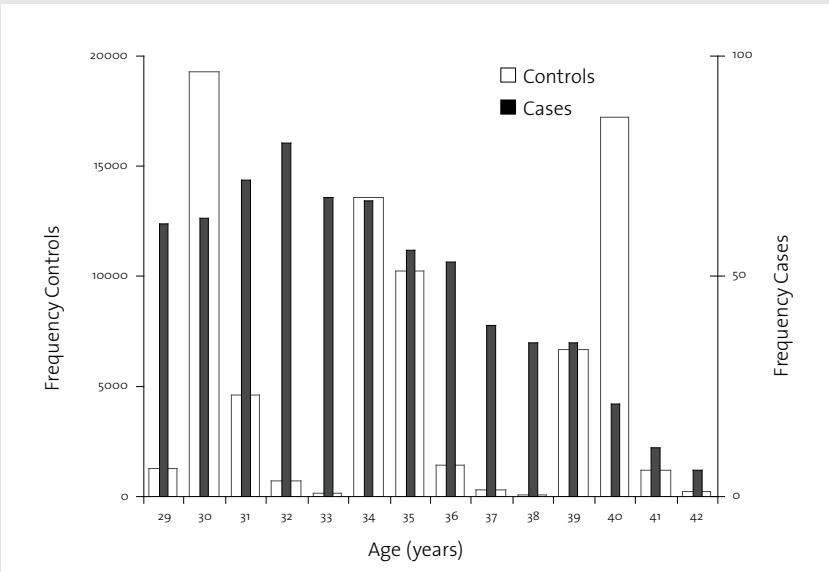
In this retrospective case-control study, the cases were women who consulted the fertility clinic of the Department of Obstetrics and Gynaecology of the Radboud University Nijmegen Medical Centre in the period from 1 January 2000 to 31 December 2003 and who had had either *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) treatment. According to the policy of the department, cervical smears were taken before starting IVF or ICSI. The controls comprises women who had had a cervical smear taken within the population-based screening programme for cervical cancer in the same period. These smears were taken by a general practitioner. Because, both fertility clinic and screening programme operated in the same region, demographic features of both cases and controls were considered comparable.

Women aged 29-42 years were included. The upper limit was applied since IVF is not performed in women older than 42 years, whereas the lower limit is based on the age of the first screening within the population-based screening programme. Although this programme starts at age 30, some women have their first smear taken at 29 years of age, explained by the fact that women are generally invited in the year they are turning 30. Applying these criteria led to the inclusion of 669 cases and 77 055 controls.

The cytological results were retrieved from the local pathology database and were classified using the Dutch CISOE-A classification.[15] This classification system discriminates between normal cytology, borderline nuclear changes (BNC), mild dyskaryosis, moderate dyskaryosis, severe dyskaryosis, carcinoma *in situ*, and carcinoma. Smears diagnosed equal to or more severe than BNC were considered abnormal. Furthermore, it is hospitals policy to perform colposcopy guided histology in all smears indicating ≥ moderate dyskaryosis. Smears showing BNC or mild dyskaryosis are repeated after 6 months and are referred for colposcopic evaluation in case of persistence. On the basis of classification system guidelines, all cervical smears classified as 'insufficient quality for reliable diagnosis' were repeated within 6 weeks. Presuming that all 'insufficient' smears were reiterated, all cervical smears classified accordingly were excluded from the controls. With regard to the cases, 'insufficient' smears were identified and replaced by the first consecutive cervical smear of sufficient quality. In case the only available smear was 'insufficient', the woman was excluded from the study.

To study the relation between the incidence of abnormal smears and the primary cause of subfertility, additional data were collected from the electronic database of the fertility clinic. Only the most important subfertility diagnosis was listed in the database and thus taken into consideration. All women of subfertile couples, irrespective of age and fertility treatment modality, who had had a cervical smear and who were diagnosed with subfertility were taken into account. For this part of the study 1629 women were included.

Figure 1 Age distribution of 669 cases and 77 055 controls.



Statistics

The chi-square test was used to test for significant differences between cases and controls in categorical nominal variables. Fischer's exact test was used in case of two by two tables. Univariate logistic regression analysis was used to test differences in the incidence of severe abnormalities between groups. The (crude) odds ratio (OR) with 95% confidence interval (95% CI) is presented. Multivariate logistic regression was used to test differences in the incidence of severe abnormalities between groups adjusted for age. The adjusted odds ratio (adj OR) with 95% CI is presented. All test results with a probability (P) of <0.05 were considered to be statistically significant. The statistical analyses were performed with SAS version 8.2 software.

Results

Meeting the inclusion criteria, that is age between 29 and 42 years and cervical smear taken of sufficient quality, the cases consisted of 669 women aged 34±3 years, whereas the mean age of the 77 055 controls was 35±4 years. Age distribution for cases and controls is shown in Figure 1.

The distribution of cervical cytology in the two groups is summarized in Table I. In comparison to the cases, the control group showed more normal smears. Smears indicating BNC and the smears showing moderate dyskaryosis or more severe were more frequently observed in the cases. The percentage of abnormal cytology (BNC or more severe) as observed in the cases was significantly different from the abnormal cytology as expected based on the findings in the control group (P=0.014; chi-square test).

Table I Percentage of the distribution of cervical cytology in the cases (*n=669*) and the controls (*n=77055*)

Cytology	Cases	Controls
Normal	93.87	96.06
BNC	3.74	2.36
Mild dyskaryosis	0.45	0.56
Moderate dyskaryosis	0.60	0.34
Severe dyskaryosis	0.75	0.38
Carcinoma *in situ*	0.45	0.27
Carcinoma	0.15	0.02

BNC: borderline nuclear changes

From moderate dyskaryosis onwards, it is compulsory to evaluate and verify the cytological abnormalities using (colposcopy-guided) histology. Table I summarizes that significantly more cases (1.95%) than controls (1.01%) had a cytological diagnose equal to or more severe than moderate dyskaryosis (P=0.017; OR 1.94 [95% CI = 1.12-3.37]). Although the age distribution in the cases differed from the distribution in the controls (Figure 1), this did not affect the significant difference (adj OR 1.77 [95% CI = 1.19-2.65]).

To study a possible association between cytological abnormalities and the various diagnoses of subfertility, the cervical smears of all women at the fertility department who had been diagnosed concerning their subfertility were assessed (disregarding age or IVF-status), as is summarized in Table II. In 38.6% of the women having an abnormal smear, subfertility was explained by female factors being menstrual cycle problems, cervical factor, tubal/uterine factor, and endometriosis (see Table II). However, the vast majority (61.4%) of cervical abnormalities equal to or more severe than BNC were found in women of couples diagnosed with male factor subfertility, couples with sexual problems and unexplained subfertile couples.

The results of the diagnoses of the smears of women with tubal factor subfertility were compared with those of women with subfertility of other origin (excluded is male subfertility) (Table III). Although, smears indicating BNC, mild and severe dyskaryosis, and carcinoma *in situ* appeared more frequently in the women suffering tubal factor subfertility, the differences were not statistically significant (P=0.356; Fischer's exact test).

Discussion

In this study, abnormal cytology (i.e. ≥ BNC) was observed significantly more often in women eligible for IVF treatment (i.e. the cases) as compared with the women in the population-based screening programme for cervical cancer (i.e. the controls) (6.1% and 3.9%, respectively [chi-square test, P<0.02]). These results are in contrast to the observations by Lundqvist and colleagues. In a similar study, they found abnormal cytology in 2.3% of women admitted for IVF and in a corresponding 4.1% of the healthy control women from a screening programme.[16] However, in the latter study, both case and control group were substantially smaller, encompassing just 214 and 197 women, respectively. In contrast to this study, Lundqvist, et al. did not apply age limits -the cases were aged 20-40 years whereas the controls were aged 25-59 years. Moreover, the results could neither be interpreted scientifically nor compared to our results since statistical analyses were not performed.

The most important factor known in the development of CIN and cervical cancer is a persistent type-specific high-risk HPV infection.[8-10] Because this study is entirely based on electronic data, HPV status was not and could not be included. Hormonal stimulation promotes HPV replication *in vitro*[17] and in vivo.[18] Long term exogenous oestrogen exposure in humans has shown an increased detection rate of HPV[19] and appeared to be a risk factor for high-grade cervical lesions.[20]

However, because the cases in our study consisted of nonpregnant women eligible for IVF, who neither used oral contraceptives nor received hormonal treatment at intake, higher hormone levels were considered unlikely to explain the observed difference. Moreover, Strehler and colleagues did not find a significant difference in cervical HPV DNA prevalence between infertile women undergoing ovarian hormonal stimulation and healthy control women.[21] In addition, the study of Lundqvist and co-workers did not report significant differences in hr-HPV genotypes prevalence in IVF women compared to healthy controls (7% and 9.1%, respectively).[16] Even cumulative HPV prevalence rates in infertile and healthy women do not seem to be significantly different. Van Ham and colleagues found a cumulative HPV prevalence of 75% in infertile women with normal cytology[22], whereas Brown and colleagues found 82% in healthy adolescents.[1]

Table II The number (percentages) and totals of the distribution of cervical cytology in all subfertile women for the various subfertility diagnoses

Subfertility diagnosis	Normal [*n* (%)]	BNC [*n* (%)]	Mild dyskaryosis [*n* (%)]	Moderate dyskaryosis [*n* (%)]	Severe dyskaryosis [*n* (%)]	Carcinoma *in situ* [*n* (%)]	Carcinoma [*n* (%)]	Total [*n* (%)]
Male subfertility	644 (93.6)	25 (3.6)	5 (0.7)	4 (0.6)	5 (0.7)	5 (0.7)	0 (0)	688 (100)
Menstrual cycle disorder	239 (93.5)	11 (4.3)	1 (0.4)	2 (0.8)	0 (0)	0 (0)	0 (0)	253 (100)
Cervical factor	44 (93.6)	2 (4.3)	0 (0)	1 (2.1)	0 (0)	0 (0)	0 (0)	47 (100)
Tubal/Uterine factor	167 (88.8)	14 (7.4)	2 (1.1)	1 (0.5)	3 (1.6)	1 (0.5)	0 (0)	188 (100)
Endometriosis	67 (91.8)	6 (8.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	73 (100)
Sexuological problems	12 (92.3)	0 (0)	0 (0)	1 (7.7)	0 (0)	0 (0)	0 (0)	13 (100)
Unexplained	342 (93.2)	15 (4.1)	2 (0.5)	1 (0.3)	5 (1.4)	1 (0.3)	1 (0.3)	367 (100)
Total	**1515 (93.0)**	**73 (4.5)**	**10 (0.6)**	**10 (0.6)**	**13 (0.8)**	**7 (0.4)**	**1 (0.06)**	**1629 (100)**

BNC: borderline nuclear changes

The pathogenesis of cervical intraepithelial neoplasia is however not solely dependent on HPV. Smoking, promiscuity, early sexarche, long-term contraceptive use and immunosuppres-sion have been proposed as co-factors. Moreover, an independent role for the most prevalent STI, i.e. *C. trachomatis* was suggested by several epidemiologic and case control studies.[14,23-25] Although endocervical glandular cells are the targets for *C. trachomatis*, the association between *Chlamydia* and cervical carcinoma was only applicable to squamous cell carcinomas.[14] However, the association is modest in comparison with the strong effect of HPV infections. Possibly, a *C. trachomatis* infection might increase the host susceptibility to HPV or enhance the effects of HPV.[26,27] Because, *C. trachomatis*-seropositive women appear to elicit a humoral-mediated rather than a cell-mediated immune response to particular antigens they may have an impaired ability to clear HPV.[28] This hypothesis is supported by the finding that a self-reported history of previous *Chlamydia* infection was the most significant risk factor for the persistence of a HPV infection.[29] Therefore, it remains unlikely that *C. trachomatis* individually acts as a carcinogen of the cervix, but it may act as a trigger or an enhancer for HPV-mediated carcinogenesis of the uterine cervix. Both microorganisms are however sexually transmittable and evidently related to promiscuous, precarious sexual behaviour.

Another consequence of persistent *C. trachomatis* infections is a significant increase in the likelihood of developing tubal pathology and, subsequently, tubal factor subfertility.[30] In addition to the association between *Chlamydia* infection and cervical pre-malignancies, we expect a higher frequency of abnormal smears in subfertile women suffering from tubal pathology. However, despite one case of moderate dyskaryosis, three cases of severe dyskaryosis and one case of carcinoma *in situ* in the 'tubal pathology' group, the data of this study do not support this hypothesis, because the majority of the abnormal cytological results were found in the 'unexplained subfertility' and the 'male subfertility' groups. This latter group is of specific interest since HPV has been found in substantial proportions of the sperm cells of patients who attended fertility clinics[31,32] and in the vas deferens of vasectomized middle-aged men.[33] Moreover, HPV negatively affects sperm cell motility, and the incidence of asthenozoospermia appears associated with sperm harbouring HPV.[34] Although insufficiently studied, this work emphasizes the interesting possibility of sperm cells as a carrier for HPV transmission in unprotected sexual intercourse, eventually leading to cervical (pre-) malignancies.

Table III The number (percentages) and totals of the distribution of cervical cytology in women diagnosed with tubal/uterine factor subfertility (*n*=188) and women diagnosed otherwise considering subfertility (*n*=753)

Cytology	Tubal/uterine factor [*n* (%)]	Other factors [*n* (%)]	Total [*n* (%)]
Normal	167 (88.8)	704 (93.5)	871 (92.6)
BNC	14 (7.4)	34 (4.5)	48 (5.1)
Mild dyskaryosis	2 (1.1)	3 (0.4)	5 (0.5)
Moderate dyskaryosis	1 (0.5)	5 (0.7)	6 (0.6)
Severe dyskaryosis	3 (1.6)	5 (0.7)	8 (0.9)
Carcinoma *in situ*	1 (0.5)	1 (0.1)	2 (0.2)
Carcinoma	0 (0.0)	1 (0.1)	1 (0.1)
Total	**188 (100)**	**753 (100)**	**941 (100)**

Male factor subfertility was not included in the groups. BNC: borderline nuclear changes

Two other or alternative confounding factors could however explain the observed differences in the present study: i) an urbanization trend and ii) intra- and interobserver variability. Squamous and glandular cell abnormalities were observed more frequently in cities exceeding 250 000 inhabitants in comparison to towns of 20 000 - 250 000 inhabitants and villages of less than 20 000 inhabitants.[35] This positive urbanization trend has also been described in association with HPV, bacterial vaginosis and Gardnerella infection.[35,36] However, both the case and the control groups of women assessed in this study populate the same geographical area that encompasses only towns and villages. The town/village ratio was assumed to be equally distributed over the cases and controls. Several studies have described a significant intra- and interobserver variability in subclassification of squamous and glandular pre-malignancies based on cytology[37-40] and histology.[41] Although all patients in this study lived in the same area, the control group smears were taken by a different group of healthcare workers (i.e. general practitioners) and the smears were assessed by various laboratories in the district, whereas all smears from the cases were taken by gynaecologists and subsequently evaluated by the pathology department of the Radboud University Nijmegen Medical Centre.

The higher prevalence of severe abnormal cytology in subfertile women visiting the outpatient clinic because of IVF treatment compared to the general population in the same geographical area suggests that sexually transmitted diseases are more present in subfertile women. Because, pathologists might be influenced by the clinical origin of a cervical smear, thereby judging smears from cases in a more enquired perspective, a possible bias could be considered. Provided that the results of this study are validated in a prospective randomized investigation with standardized sampling and assessment methods, we suggest to structurally perform cytological examination of the uterine cervix in all women assessed for fertility problems. Especially since cytological abnormalities are induced or enhanced by highly prevalent STIs that are related to promiscuous, precarious sexual behaviour and appear to be more common in patients visiting subfertility clinics.

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Long-term follow-up after large-loop excision of the transformation zone: evaluation of 22 years treatment of high-grade cervical intraepithelial neoplasia

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Abstract

Early treatment of cervical intraepithelial neoplasia (CIN) significantly reduces the risk of invasive cancerous progression. Residual and recurrent high-grade CIN should be detected and retreated in an early phase. Therefore, a post-surgery cytological follow-up protocol was introduced at 3, 6, 9, and 12 months and yearly thereafter for 5 years. The aim of this study is to evaluate the long-term experience in treating high-grade CIN using large-loop excision of the transformation zone (LLETZ). Additionally, the long-term follow-up in this study gains the opportunity to document the pattern of disease recurrence beyond 5 years. The average follow-up of the 1696 women included in this study was 6.5 years. Overall, 8.5% of the patients who underwent LLETZ showed a high-grade repetitive CIN and three patients had invasive carcinoma. Eighty percent of those lesions were probably residual, whereas 20% of all high-grade repetitive lesions appeared more than two years after initial surgery and were considered recurrent lesions. Half of the recurrent lesions occurred more than 5 years after LLETZ.

Introduction

Cancer of the uterine cervix is a major cause of death. In developed countries population-based screening programmes for cervical cancer to identify precursor lesions have significantly contributed to a reduction in mortality and morbidity of this disease. However, worldwide annual incidence and mortality rates are still high, respectively 500 000 and 230 000.[1]

Using any form of cervical ablative therapy, the risk of an invasive cancer of the cervix is reduced by 95% during the first 8 years subsequent to successful treatment of high-grade cervical intraepithelial neoplasia (CIN 2/3). Nonetheless, the chance of developing invasive cancer of the cervix for women conservatively treated for CIN is still increased five times compared to the general population.[2] To date, there is no convincing evidence suggesting a superior surgical technique for treating CIN.[3,4] However, for decades, one of the most widely used conservative methods is the large-loop excision of the transformation zone (LLETZ).[5,6] Using the LLETZ, CIN lesions are in general adequately eradicated, while anatomy and function of the cervix are maintained.[7-9]

Treatment success of LLETZ -defined as not having recurrence in the follow-up period of 24 months- varies from 81% to 98%.[3,7,8,10-12] The vast majority of treatment failures transpires during the first two years following the procedure.[13] Repetition of CIN may be due to inadequate treatment of the initial lesion (residual) or development of a new lesion (recurrence).[10] In order to verify the adequacy of the treatment cytological follow-up is performed. Dutch guidelines from 1998 recommend cytological follow-up at 6, 12 and 24 months after a LLETZ procedure with histologically proven high-grade CIN lesion, after which the patient is referred back to the population-based screening programme for cervical cancer.[14]

Prior to its introduction as treatment modality by Prendiville in 1989[12], we had already adopted the technique of the large loop excision. Preponderantly for scientific purposes, the Radboud University Nijmegen Medical Centre had formulated guidelines, posing cytological follow-up was to be executed at 3, 6, 9, 12 and 24 months after LLETZ. Subsequently, the patients' general practitioners were advised to perform a cervical smear annually during 5 years. Thereafter, the patients were

sent back to the cervical cancer-screening programme (cervical smear every 5 years to age 60). The purpose of this early and frequent collection of cervical cytology is particularly important in order to obtain sufficient data to document the effectiveness of the treatment[15] and to evaluate our policy regarding 'LLETZ and follow-up' for the future. Therefore, the objective of this study was to present and evaluate our 22 years of experience in treating high-grade cervical intraepithelial lesions using diathermy loop excision. Additionally, the long-term follow-up in this study gains the opportunity to document the pattern of disease recurrence beyond 5 years as suggested by Flannelly and colleagues.[15]

Materials and methods

Retrospectively, we have assessed all 2579 cases who underwent LLETZ at the outpatient clinic of the Department of Obstetrics and Gynaecology of the Radboud University Nijmegen Medical Centre from January 1981 to December 2001. Background information, data, and diagnoses of all patients were retrieved from an electronic database. From this group we have included only the patients (n=1696) having a diagnoses histologically proven high-grade CIN (2 or 3) using the primary loop excision. The latter inclusion date (December 2001) was used so that all patients could have had a follow-up of at least 2 years.

Follow-up was done by cervical smear 3, 6, 9, 12, and 24 months after the initial procedure. Subsequently, the patients were referred back to their general practitioner carrying an advice to have a cervical smear performed yearly during 5 years, after which the patient was send back to the population-based screening program for cervical cancer. The follow-up cytology was classified according to the Pap-classification. In case the follow-up cytology indicated a supposed high-grade squamous or glandular lesion (i.e. diagnosed as equal to or more severe than moderate dysplasia or moderate atypia), the patient would return for colposcopic assessment and re-treatment, if necessary.

Cervix and transformation zone were visualized by colposcopy at our outpatient clinic. Assessment of the transformation zone was enhanced using a 3% acetic acid solution. If an abnormality, suggestive for high-grade CIN, was identified on the cervix,

immediate excision of the whole transformation zone would follow using low-voltage diathermy loop excision under local anaesthesia, as previously described by our group.[7]

As commonly used in literature we defined residual lesions as high-grade CIN or carcinoma occurring up to 2 years post-treatment, while recurrent lesions occur from 2 years onwards.

Results

From January 1, 1981 to December 31, 2001, a total of 1696 women underwent a LLETZ for high-grade CIN (2 or 3). The mean age was 34.9 years (standard deviation 7.8 years). The youngest patient was 18 years, while the eldest was 74 years. 80% of the women pertained to the age group of the population screening for cervical cancer (30-60 years), 19% was younger than 30 years and 1% exceeded the age related eligibility for screening. Histological examination of the 1696 excised samples revealed 443 cases of moderate dysplasia, 802 cases of severe dysplasia, and 451 cases of carcinoma *in situ*. Therefore, CIN 2/3 distribution was 26/74%.

The mean follow-up of the total group was 78 months, with a maximum follow-up of 22 years. Approximately 90% of the 1696 women completed a follow-up period of 1 year, 80% a follow-up of 2 years. Of the 361 women failing to complete 2 years of follow-up, 2 had died, 36 had had a hysterectomy, 11 had moved out of our district or abroad, 5 had not yet shown up for their follow-up, 90 had unintentionally been send back to the population-based screening for cervical cancer, and 217 had been lost for follow-up.

A second procedure was performed in 268 cases: 179 re-LLETZ, 48 hysterectomies, 26 biopsies, 11 conisations, and 4 endocervical curettages. In 1 biospy sample and 1 Re-LLETZ specimen insufficient material resulted in indefinite diagnose, whereas in 9 biopsies, 4 cone biopsies, 34 hysterectomies, and 24 re-LLETZ samples no histological abnormalities were found. In 144 cases (8.5%), histology after the second procedure revealed high-grade lesions, 3 patients had an invasive cervical cancer, and low-grade (LG) lesion was detected in 48 cases. High-grade and LG histological distribution per procedure is summarized in Table I.

Hysterectomies showing normal histology (n=34) were performed for various reasons: 4 cases of uterine prolapse, 13 cases of menorrhagia, 2 high-grade follow-up cytology, and 15 for an indeterminate cause. The cone biopsies, showing normal or LG histology (n=5) were performed because of high-grade cytology in follow-up in 2 occasions; the reason for the others remains unclear. The re-LLETZ (63), biopsies (5) and curettages (1), showing no or a low grade histological abnormality, were performed because of involved margins in 2 cases, colposcopic findings in 3 cases, stenosis of the cervix in 5 cases, high-grade cytology in 47 cases, and unknown/low-grade cytology in 27 cases.

In case histology of the second procedures revealed a lesion equal to or more severe than high-grade CIN (n=147), 2 samples recorded involved margins, 10 cases of low-grade follow-up cytology with suspected lesions at colposcopy, and 135 patients had a high-grade cytology somewhere in the follow-up period with a certain sampling date.

Table 1 Distribution of 2nd procedures and abnormal histology (percentages of 268)

	LG lesions	High-grade lesions			
	CIN 1 [*n* (%)]	**CIN 2 [*n* (%)]**	**CIN 3 [*n* (%)]**	**Carcinoma [*n* (%)]**	**Total [*n*]**
Biopsy	7	1	7	1	16
Curettage	1	1	2	0	4
Cone biopsy	1	0	6	0	7
Hysterectomy	0	2	11	1	14
Re-LLETZ	39	39	75	1	154
Total	**48 (17.9)**	**43 (16.0)**	**101 (37.7)**	**3 (1.1)**	**195**

LG: low-grade; CIN: cervical intraepithelial neoplasia; LLETZ: large loop excision of the transformation zone

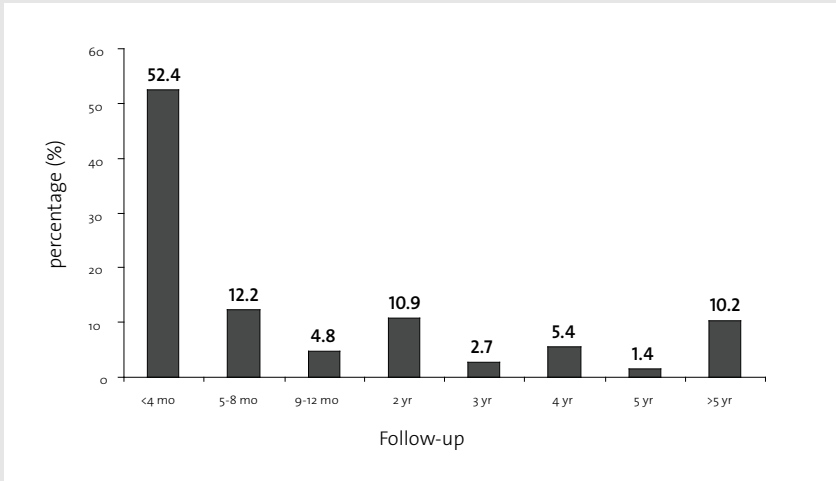
In more than 50% of the 147 patients the residue/recurrence high-grade lesion or invasive cancer was detected within 4 months after the initial procedure, whereas within 2 years following LLETZ 80.3% of all high-grade residual lesions were detected and treated. Moreover, an additional 9.5% was identified between 2 and 5 years after the initial surgery. The remaining 10.2% of recurrences was detected longer than 5 years after LLETZ (Figure 1). The disease-free interval between initial procedure and high-grade recurrence varied from 63 to 159 months. This included one invasive cancer that was detected 137 months subsequent to LLETZ. This patient had attended neither the follow-up regimen nor the population-based screening programme. She had a macro-invasive squamous cell carcinoma of the cervix, FIGO stage IIB, and was treated with chemoradiation. The two remaining cervical malignancies were detected at the first follow-up moment and subsequently treated successfully. Both patients underwent a hysterectomy. Histology revealed malignant micro-invasive growth in the cervix. The resection margins were not involved. The initial LLETZ specimens of the 3 patients having carcinoma had been reassessed and were once again diagnosed as carcinoma *in situ*.

Discussion

The LLETZ technique has been used and studied extensively since the initial reports by Cartier[16] and Prendiville and colleagues.[12] The treatment has shown to be innovative, cost-effective[7,17], time-conserving[17-19], successful[3,7,8,10-12], and causing low morbidity.[3]

The technique of LLETZ allows histological examination of the entire transformation zone, thus facilitating confirmation of diagnosis and detection of involvement of the excision margins. Assessment of these borders is of importance to determine the risk of recurrence, since incomplete excision of the CIN lesion is related to an increased prevalence of neoplasia.[6,8,15,20-22] However, according to Buxton, et al. abnormal cytology is a better prognostic indicator than histologic examination of excision margins.[23] Therefore, pathologists in our unit did not assess involvement of the excision margins. Moreover, larger lesions are removed in multiple fragments, so that orientation of the specimen, and therefore assessment of completeness of excision, is virtually impossible.[24,25]

Figure 1 Detection of 147 high-grade residual or recurrent CIN lesions in follow-up



CIN: cervical intraepithelial neoplasia; mo: months; yr: years

This study describes 22 years of experience in treating high-grade CIN in 1696 women. The average follow-up was 6.5 years, which is 11 months more than the most extended trial described thus far.[8] In case of reappearance of a high-grade CIN, the lesion was detected at the first follow-up moment within 4 months after LLETZ in more than half of the cases. Moreover, 80.3% of all repetitions were identified within 24 months by strictly monitoring the patients; according to literature these are considered the residual lesions. Additionally, 9.5% (n=14) of the recurrent lesions became apparent from the third to the fifth year subsequent to LLETZ. The remaining 10.2% (n=15) high-grade lesions recurred after 5 years of follow-up, showing a variation from 62 to 159 months. Our results show that 119 women have had a second procedure, histologically showing no abnormality or a low-grade cervical lesion.

Our early-onset cytological follow-up is debatable. The effects of tissue regeneration, i.e. reparative changes, often visible subsequent to surgical treatment at the cervix, can result in over-estimative diagnoses. Colgan et al., for instance reported false-positive diagnoses of high-grade CIN or even carcinoma in 3% of the selected smears classified as benign cellular changes with typical reparative change.[26] In order to

reduce this chance of overestimating smears with reparative changes, we have qualified cytology as aberrant if it was diagnosed equal to or more severe than moderate dysplasia or moderate atypia. This in contrast to other studies that have classified all cytology abnormal from mild dysplasia onwards.

As previously suggested, long-term follow-up has to be executed in order to document the pattern of disease recurrence beyond the 5 years of follow-up.[15] The enduring follow-up after LLETZ achieved in this study creates the opportunity to illuminate this issue. According to our data approximately 20% (29/147) of the high-grade repetitive CIN lesions occurred after 2 years of normal follow-up; 9.5% (14/147) until the fifth year, and 10.2% (15/147) after 5 years of follow-up. Although arbitrarily, literature utters that residual lesions occur up to 2 years post-treatment and recurrence lesions from 2 years onwards. Therefore, the approximate 20% repetitive lesions after 2 years are all recurrences, resulting in a residue-recurrence ratio of 4:1.

High-risk Human papillomavirus (hr-HPV) genotypes have great oncogenic potential; hr-HPV infection causes squamous and glandular intraepithelial lesions that can progress to invasive cervical carcinoma. The overall HPV prevalence in cervical cancers worldwide is 99.7%.[27] An effective LLETZ procedure of CIN lesions is suggested to result in the eradication of hr-HPV types.[28] Therefore, persistence of CIN requires perseverance of the hr-HPV infection.[29] Consequently, de novo development of pre-malignancies would require re-infection with the same or other hr-HPV-types. Since, predictive value of a positive high-risk HPV test post treatment was higher than conventional abnormal smear, including hr-HPV tests in the follow-up of women treated for high-grade CIN lesions was suggested.[30] However, it was also reported that high-risk HPV detection in early follow-up after LLETZ has low positive predictive value.[31] Moreover, sensitivity and specificity of the conventional follow-up cytology remain the highest 100% and 96%, respectively.[31] So, currently there is no unambiguous opinion in routine follow-up screening HPV.

Conclusively, this study shows that the majority of repetitive lesions can be detected within the first 4 months following LLETZ. Particularly interesting are the recurrent lesions after 5 years of follow-up, since the pattern of disease recurrence beyond five years has not been described in detail.

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High-risk HPV presence in cervical specimens after a large loop excision of the cervical transformation zone: significance of newly detected hr-HPV genotypes

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Abstract

Large loop excision of the cervical transformation zone (LLETZ) is a well-established treatment for high-grade cervical intraepithelial neoplasia. It has even been postulated that LLETZ is responsible for the elimination of the infectious agent, human papillomavirus (HPV), causing the lesion. Most studies on HPV detection after LLETZ have focussed on the persistence of high-risk (hr-)HPV to identify women at risk for residual or recurrent disease. Therefore, the appearance and or significance of hr-HPV types newly detected after surgical treatment has not been studied extensively so far. The presence of hr-HPV in 85 high-grade squamous cervical LLETZ biopsies and in the first follow-up smear was determined. In 80 (94%) of the LLETZ biopsies hr-HPV was detected in contrast to 30 (35%) hr-HPV positive follow-up scrapes. Twenty of the 80 hr-HPV positive women (25%) had the same hr-HPV genotypes in their follow-up cervical smears as was found in the corresponding biopsies. In the follow-up smear of 13 women a new hr-HPV genotype was detected and HPV 18 was newly detected in 8 of them. The remarkably high presence of newly detected HPV 18 genotypes may argue for a release or re-activation of this virus from proximal layers of the cervical canal incised during surgery.

Introduction

Carcinoma of the cervix is the most common type of cancer in the developing world and the leading cause of death from cancer among women.[1] In women with cervical cancer, malignant cells usually develop from cytologically abnormal tissue that can range from mildly dysplastic ("cervical intraepithelial neoplasia" CIN 1) to moderately or severely dysplastic (CIN 2/3). Women with cervical abnormalities indicative of HPV infection have a much higher incidence risk of cervical cancer than do those with normal cytology.[2,3] Virtually all cervical carcinomas are associated with an infection by one of the high-risk HPV genotypes.[4] Studies towards the oncogenic potential of these HPV types clearly support a causal relationship with cervical cancer carcinogenesis.[5-7] To date, over 120 distinct HPV genotypes have been identified. About 30 of the known HPVs, and even a higher number of variants, have been detected in genital mucosa. These genital HPVs can be divided subsequently into groups based on frequency of association with malignant tumours, and thus presumed oncogenic potential. Thirteen HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) are classified as high-risk types, directly associated with invasive cervical cancers.[8]

Large loop excision of the cervical transformation zone (LLETZ) is a well-established method of treatment of high-grade CIN lesions and has shown to be highly effective.[9,10] It has even been suggested that effective surgical treatment of CIN also eliminates the HPV infection responsible for the lesion.[11-13] Most studies on HPV detection after treatment for high-grade CIN have focussed on either the persistence of hr-HPV in general or type-specific persistence to identify women at risk for residual or recurrent disease.[14-17] The appearance of new hr-HPV types after surgical treatment, however, has not been studied in detail so far.

To obtain more insight into this phenomenon, hr-HPV detection and genotyping was performed both on high-grade squamous cervical biopsies from 85 women treated with a large loop excision of the transformation zone (LLETZ), and on the corresponding first follow-up smear taken within 6 months after treatment.

Materials and methods

All women, visiting the outpatient colposcopy clinic of the Radboud University Nijmegen Medical Centre between April 1997 and October 1999 and treated for high-grade squamous cervical intra-epithelial neoplasia with a LLETZ, were eligible for the study. A high-grade squamous cervical lesion was histologically defined as CIN 2 or worse. All patients were first time referrals from the population-based screening programme for the prevention of cervical carcinoma. The referral cervical scrape indicating a high-grade lesion was performed by the general practioner and was unavailable for HPV assessment. The participants gave written informed consent after approval of the local ethical committee. The LLETZ procedures were undertaken by an experienced gynaecologic oncologist.

The cervix and transformation zone were visualized by colposcopy. Assessment of the transformation zone was enhanced using a 3% acetic acid solution. If an abnormality, suggestive for CIN 2 or worse, was identified on the cervix immediate electrosurgical excision of the whole transformation zone would follow. After local infiltration of the paracervical tissue with 1 mL of 2% lidocainehydrochloride with epinefrine in every quadrant, an ERBE (Tubingen, Germany) ectrosurgical unit was used, together with a wire loop of appropriate size to excise the transformation zone, as described previously.[9,10]

Within 6 months after the LLETZ procedure cervical scrapes were collected using the Cervex brush® (Rovers Medical Devices B.V., Oss, The Netherlands) and processed using a liquid-based approach (ThinPrep®, Cytyc Corp., Marlborough, MA, USA). An experienced pathologist, blinded for the results of the HPV test, performed histopathological and cytological examination.

HPV detection was performed on the LLETZ biopsies as well as on the liquid-based cervical scrapes, using a broad-spectrum short fragment polymerase chain reaction (SPF_{10} PCR) assay as previously described.[18] In case of a positive HPV test, subsequent HPV genotyping was performed via a reverse hybridization line probe assay (LiPA), allowing for simultaneous typing of all hr-HPV genotypes.

The SPF_{10}-LiPA assay has proven to be sensitive, specific, effortless, and reproducible in the assessment of HPV in cervical scrapes and histological samples, and has been clinically validated.[18-21] The assay has also shown to be highly comparable to other frequently used primer-sets.[19,21]

Specimen preparation

A single 3µm thick section of every LLETZ-biopsy specimen was put into a reaction tube and incubated overnight at 56°C in 200µL 10mM Tris-HCl with 1mM EDTA, 0.2% Tween-20 and proteinase K (0,3mg/mL). Proteinase K was inactivated by a 10 min incubation at 100°C for nucleic acid isolation. The sample was centrifuged for 10 min at 11,000 rpm and 10µL were directly used for PCR analysis.

The MagnaPure LC Isolation station (Roche Diagnostics GmbH, Molecular Biochemicals, Mannheim, Germany) was used for nucleic acid isolation of the cervical scrapes in liquid cytology media, was used; 200µL of material was isolated using the Total Nucleic Acid isolation kit (Roche Diagnostics GmbH, Molecular Biochemicals, Mannheim, Germany), as described by the manufacturer. Nucleic acid was resuspended in a final volume of 100µL; 10µL were used for PCR analysis. A water blank control was processed with each batch of 10 samples. After isolation of DNA, samples were tested for the presence of HPV by the SPF_{10}-LiPA HPV detection/genotyping assay.

SPF_{10}-LiPA HPV detection/genotyping

PCR amplification of HPV DNA

Broad-spectrum HPV DNA amplification was carried out using a short PCR fragment assay (SPF_{10}-LiPA detection/genotyping assay system). This assay amplifies a 65-bp fragment of the L1 open reading frame, and allows detection of at least 43 different HPV types.[18-20] SPF_{10}-PCR system was carried out in a final reaction volume of 50mL, containing 10mL of the isolated DNA sample, 10mmol/L Tris-HCL (pH 9.0), 50mmol/L KCl, 2.0mmol/L MgCl2, 0.1% Triton X-100, 0.01% gelatin, 200mmol/L of each deoxynucleoside triphosphate, 15pmol each of the forward and reverse primers tagged with biotin at the 5' end, and 1.5 units of AmpliTaq Gold® (Applied Biosystems, Foster City, Ca, USA). The mixture was incubated for 9 min at 94°C, 40 cycles of 30 s at 94°C, 45 s at 52°C and 45 s at 72°C, with a final extension of 5 minutes at 72°C. Appropriate negative and positive controls were used to monitor the performance of

the PCR method in each experiment. The presence of HPV DNA was determined by hybridization of SPF_{10} amplimers to a mixture of general HPV probes recognizing a broad range of HPV genotypes, in a microtiter plate format, as described previously.[18]

Hr-HPV genotyping by reverse hybridization

The HPV-genotyping assay was performed as described previously.[19] Briefly, equal volumes (10mL each) of the biotinylated PCR products and denaturation solution (400mmol/L NaOH, 10mmol/L EDTA) were mixed in test troughs and incubated at room temperature for 5 minutes, after which 1mL of pre-warmed (37°C) hybridization solution (3x SSC [1x SSC is 0.15mol/L NaCl plus 0.015mol/L sodium citrate], 0.1% SDS) was added, followed by the addition of one strip per trough. Hybridization was performed for 1 hr at 50±0.5°C in a closed water bath with back-and-forth shaking. The strips were then washed twice with 1mL of wash solution (3x SSC, 0.1% SDS) at room temperature for 20 s, and once at 50°C for 30 min. Following this stringent washing, strips were rinsed twice with 1mL of a standard rinse solution. Strips were then incubated on a rotating platform with an alkaline phosphatase-labeled streptavidin conjugate diluted in a standard conjugate solution, at 20–25°C for 30 minutes, after which strips were washed twice with 1mL of rinse solution and once with standard substrate buffer; colour development was initiated by the addition of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium to 1mL of substrate buffer. After 30 minutes incubation at room temperature, the colour reaction was stopped by aspiration of the substrate buffer and addition of distilled water. The 28 oligonucleotide probes which recognize 25 different types (including 13 hr-HPV genotypes) were tailed with poly(dT) and immobilised as parallel lines to membrane strips (Labo Bio-medical products B.V. Rijswijk, The Netherlands). The LiPA strips were manually interpreted using provided reference guide. The samples that tested positive using DNA Enzyme Immuno Assay but showed no results on the LiPA strip were considered to be HPV X-type, i.e., genotypes not available on the LiPA strip.

Every hr-HPV genotype found in the follow-up scrapes, which was not detected in the LLETZ biopsy was considered as a non-identical hr-HPV type and further defined as "new" HPV type. Assessment of hr-HPV genotypes in the follow-up samples, identical to those found in the histological specimens were defined as "identical" types. Statistical analysis was performed by the McNemar's test where appropriate; all test results with a probability (P) of <0.01 were considered to be statistically significant.

Results

Ninety women, treated for a high-grade squamous cervical lesion with a LLETZ, were eligible for the study. In 5 cases no follow-up smear was performed within 6 months after treatment therefore these women were excluded from the study group. The remaining 85 women had a mean age of 36 years (range 26-58 years) and had undergone at least one follow-up smear within 6 months (range 2-6 months) after the LETTZ procedure.

Table I Distribution of hr-HPV types in 80 hr-HPV positive LLETZ biopsies

	hr-HPV genotype									
Infection	16	18	31	33	39	45	51	52	56	58
Single	28	4	4	3	1	1	3	4	1	0
Multiple	17	3	12	4	2	2	3	5	2	6
Total	**45**	**7**	**16**	**7**	**3**	**3**	**6**	**9**	**3**	**6**

Due to multiple HPV infections, the overall percentage of hr-HPV types exceeds 100%

High-risk HPV prevalence in the LLETZ biopsies

In 20 cases (24%) the LLETZ biopsy showed CIN 2 on histopathological examination and 65 cases (76%) had CIN 3 or worse (2 cases of micro-invasive carcinoma). In 80 (94%) of the histopathological specimens, at least one hr-HPV genotype was found. In 4 of 85 (5%) biopsies a single low-risk (lr-) HPV type was detected and in one (1%) case no HPV DNA was detected. This HPV negative case was diagnosed histologically as CIN 3.

Multiple infections with at least one hr-HPV were present in 31 of 80 positive patients (39%), single hr-HPV infections were found in 49 of 80 patients (61%). The most common hr-HPV genotypes (as a single or as part of a multiple infection) detected in the biopsy were HPV 16 (56%), followed by HPV 31 (20%), HPV 52 (11%), HPV 33 and HPV 18 (both 9%), HPV 51 and 58 (both 8%), HPV 39, HPV 45 and HPV 56 (all 4%). An overview is shown in Table I.

High-risk HPV prevalence in the follow-up cervical scrapes

In 55 of 85 (65%) follow-up cervical scrapes, made after the LLETZ procedure, the hr-HPV status was negative. In 8 of these hr-HPV negative cases, the cytological diagnosis revealed a mild disorder defined as atypical squamous (4 cases) or glandular (3 cases) cells of undetermined significance (ASC-US resp. ASG-US) and 1 case as low-grade squamous intraepithelial lesion (LSIL), whereas the remaining 47 cases had a normal cytological. In 30 of 85 (35%) of the follow-up scrapes hr-HPV was detected, which is statistically lower than the 94% hr-HPV positive biopsy samples (McNemar's test, P<0.01). An overview of the results is shown in Table II. In 21 of these 30 hr-HPV positive cases a normal cytological result was found, whereas 4 cases showed ASC-US, two cases LSIL and three cases showed a high-grade squamous intraepithelial lesion (HSIL).

Table II Distribution of hr-HPV types in 30 hr-HPV positive cervical smears after LLETZ

Infection	hr-HPV genotype									
	16	18	31	33	39	45	51	52	56	58
Single	7	4	0	1	1	0	1	1	1	1
Multiple	5	5	3	0	0	0	1	1	0	1
Total	**12**	**9**	**3**	**1**	**1**	**0**	**2**	**2**	**1**	**2**

Due to multiple HPV infections, the overall percentage of hr-HPV types exceeds 100%

Identical hr-HPV genotypes

Twenty of the 80 (25%) women with a hr-HPV DNA positive biopsy showed identical hr-HPV genotypes in their follow-up cervical smear. In 3 of these 20 patients additional new HPV types were detected in the follow-up scrapes (Table III). Cytological examination of the 20 scrapes showed a normal result in 13 cases, ASCUS in 3 cases, LSIL in 2 cases, and finally HSIL in another 2 cases.

New hr-HPV genotypes

In the follow-up smear of 13 of the 85 patients (15%) a new hr-HPV type was detected, which was not present in the LLETZ biopsies of these patients; in 3 cases multiple hr-HPV infections and in 10 cases a single hr-HPV infection were found. HPV 16, HPV 31, HPV 39, HPV 51 and HPV 52 were all newly detected once in the follow-smears. HPV 18, on the other hand, was newly detected in 8 of these 13 women (62%) (Table III). Cytological examination of these 13 cases revealed a normal result in 11 cervical scrapes, ASC-US in one scrape, and high-grade squamous intraepithelial lesion (HSIL) in one scrape.

Table III Thirteen cases with a new hr-HPV genotype in the first follow-up smear after LLETZ

Case	hr-HPV in LLETZ biopsies	hr-HPV after LLETZ[b]	Follow-up (months)	Cytology after LLETZ
1	16	18	6	Normal
2[a]	16	16 18	3	Normal
3	45	18	6	Normal
4	51	31	6	Normal
5	52	16	2	Normal
6	-	18	4	ASCUS
7	16 39	18	3	Normal
8	16 52	18	3	Normal
9	16	18	4	Normal
10[a]	16 58	16 51	3	Normal
11	33 35	18	3	Normal
12	16 52	39	4	HSIL
13[a]	31	31 52	3	Normal

[a] in 3 cases not only a new hr-HPV type but also an identical hr-HPV type was found
[b] new hr-HPV genotypes are underlined

Discussion

Both epidemiological and molecular studies have now clearly established that high-risk human papillomaviruses (hr-HPV) are the causative agents for the development of malignant lesions of the cervix and their precursors (CIN). Although, effective

therapeutic options to treat the viral infection are not yet available, LLETZ has shown an effective therapy for high-grade CIN lesions.[9,10] Moreover, surgical treatment is suggested to eliminate the infection causing the cervical abnormality.[11-13] This study also shows that 65% (55/85) of the women treated with LLETZ harboured no hr-HPV DNA in their follow-up cervical scrapes, suggesting that the surgical treatment eliminated the HPV infection.

However, hr-HPV DNA was still detected in 30/85 follow-up scrapes (35%) taken within 6 months after the treatment. In 20 patients (24%) an identical hr-HPV genotype was found in both the follow-up scrape and the LLETZ biopsy specimen. As suggested in the literature this could reflect non-eliminated persistent hr-HPV infections increasing the risk for residual or recurrent disease. Indeed, residual CIN can be detected in up to 16% of the patients after treatment of CIN. Adding hr-HPV detection could thus be used as a prognostic marker to identify women at risk for residual CIN.[14-17]

However, hr-HPV genotypes were also detected in the follow-up scrapes of 13/85 women (15%) that were not detected in the preceding LLETZ biopsy. These newly detected HPV genotypes were either found as part of a multiple infection, containing also the same hr-HPV genotype found in the LLETZ biopsy (three women), or as a single new hr-HPV genotype different from the hr-HPV genotype found in the biopsy specimen (nine women). In one woman a new hr-HPV genotype was found in the scrape while the LLETZ biopsy showed no evidence of hr-HPV presence.

Although data on newly detected hr-HPV genotypes in women treated for high-grade CIN are rare, this phenomenon has been reported previously. Mann, et al. reported a new HPV genotype in the cervical follow-up smear in 10% of 152 women treated for high-grade CIN after a median follow-up period of 12 months.[13] Bollen and colleagues even found new HPV genotypes in 19% of the follow-up smears in a study comprising 91 women treated with two different treatment modalities for high-grade CIN.[22]

In the present study a remarkably high percentage of newly detected hr-HPV 18 genotypes was observed in cervical smears taken after surgical treatment; in 8 of the 13 women (62%) with new hr-HPV genotypes, HPV 18 DNA was detected. In the hr-

HPV positive LLETZ specimens HPV 18 was detected in only 9%. This latter percentage is comparable to the 7% precalence of HPV 18 published recently in an extensive meta-analysis of 4338 squamous high-grade lesions.[23]

How can we explain this finding? First, the results could be explained due to a coincidental finding related to the relatively small number of newly detected hr-HPVs in the follow-up cervical scrapes influencing the outcome of the analysis. However, as only 7 of the LLETZ biopsies were HPV 18 positive, this explanation, although it cannot be excluded, does not seem plausible. Newly acquired HPV infection of the cervix by sexual intercourse after the LLETZ procedure could also be an explanation for the appearance of new HPV types. However, it is very unlikely that the relatively high appearance rate of specifically HPV 18 could only be explained by sexual transmission, also because of the very low rate of HPV 18 in the LLETZ biopsies as indicated above.

Another possible explanation for the appearance of new HPV types is that after elimination of the lesion, i.e., the HPV-infected epithelium, other types which were not detected previously may fill the niche.[13] In fact, many women are infected with multiple HPV genotypes and genotypes can remain undetectable due to differences in viral load. After eradication of the prominent HPV types with the highest viral loads, HPV types with lower viral loads are no longer dominated by prominent types and will start shedding again[24] becoming detectable after treatment.

But again, how does this may affect the presence of specifically HPV 18?

During the LLETZ procedure the transformation zone is excised and as a result of which the proximal layers of the endocervical canal are incised which may result in a release or re-activation of HPV from these layers of the cervical canal. HPV infections detected after LLETZ may therefore represent HPV genotypes that are more prevalent in the proximal layers of the cervix. Interestingly, from this point of view, it has been demonstrated clearly that although almost 100% of the cervical carcinomas are hr-HPV DNA positive[4], the prevalence of specific hr-HPV genotypes detected in squamous cell carcinomas and adenocarcinomas of the cervix clearly differ indicating a potential malignant tropism of different hr-genotypes. For example, HPV 16 is found in over 60% of cervical squamous cell carcinomas and in

about 20% of the cervical adenocarcinomas, while, HPV 18 is detected in only 10% of the cervical squamous cell carcinomas but in more than 50% of the cervical adenocarcinomas.[5,25-27] As HPV 18 seems to play a more prominent role in the development of cervical adenocarcinomas HPV 18 may have a local preference site in the endocervical canal. After removal of the transformation zone of the cervix by the LLETZ procedure, certain genotypes of HPV in the proximal layers of the cervix, as HPV 18, may become detectable. As HPV 18 seems to play a predominant role in this specific histological type of cervical cancer, studies towards comparison of different cervical sampling devices for ectocervical cell sampling and endocervical cell sampling are warranted, to investigate its potential to detect HPV 18 in the proximal layers of the cervix and to determine the risk of HPV 18 associated abnormalities.

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Evaluation of the SPF$_{10}$-INNO LiPA HPV genotyping test and the Roche LINEAR ARRAY HPV genotyping test

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Abstract

The need for accurate genotyping of human papillomavirus (HPV) infections is becoming increasingly important, since (i) the oncogenic potential among the high-risk HPV genotypes varies in the pathogenesis of cervical cancer, (ii) monitoring multivalent HPV vaccines is essential to investigate the efficiency of the vaccines, and (iii) genotyping is crucial in epidemiologic studies evaluating HPV infections worldwide. Various genotyping assays have been developed to meet this demand. Comparison of different studies that use various HPV genotyping tests is possible only after a performance assessment of the different assays. In the present study the SPF_{10} LiPA version 1 and the recently launched Roche LINEAR ARRAY HPV genotyping assays are compared. A total of 573 liquid-based cytology samples were tested for the presence of HPV by a DNA enzyme immunoassay; 210 were found to be positive for HPV DNA and were evaluated using both genotyping assays (163 with normal cytology, 22 with atypical squamous cells of undetermined significance (ASCUS), 20 with mild/moderate dysplasia, and 5 with severe dysplasia). Comparison analysis was limited to the HPV genotype probes common to both assays. Of the 160 samples used for comparison analysis 129 (80.6%) showed absolute agreement between the assays (concordant), 18 (11.2%) showed correspondence for some but not all genotypes detected on both strips (compatible), and the remaining 13 (8.2%) samples did not show any similarity between the tests (discordant). The overall intertest comparison agreement for all individually detectable genotypes was considered very good (κ-value, 0.79). The genotyping assays were therefore highly comparable and reproducible.

Introduction

Molecular and epidemiologic studies have shown that a persistent infection with high-risk human papillomavirus (hr-HPV) is the most important risk factor for both cervical cancer and its precursors.[1-4] Approximately 40 different HPV types can infect the mucosa of the anogenital tract. Based on their carcinogenicities these anogenital HPV types have been subdivided into low-risk HPV (lr-HPV) types, probable high-risk types and high-risk (hr) HPV types [5], although some controversy remains regarding the probable high-risk genotypes.[6] Almost all squamous cell cervical cancers worldwide harbour hr-HPV types.[7] Moreover, high-risk HPV DNA can be detected in 74% of the pre-malignant low-grade cervical intra-epithelial neoplasia (CIN) and approximately 84% of the high-grade CIN lesions.[8] Consequently, the efficacy of population based screening programmes solely using cervical cytology could benefit from adding hr-HPV testing.[9] Accordingly, many ongoing international research projects assess the feasibility of introducing hr-HPV tests in the available routine screening.

For these screening purposes, several tests have been developed in order to distinguish high-risk HPV infections from no HPV infection. Amongst those are the signal amplification method Hybrid Capture II (hc2, Digene Corp., Gaithsburg, Maryland, USA) and the recently developed target amplification method Roche AMPLICOR® HPV Test (Roche Molecular Systems, Inc., Branchburg, NJ, USA).[10] Although both tests are commercially available and Conformité Européenne (CE) marked, hc2 is currently the only FDA registered HPV screening assay.[11] Both tests differentiate between an infection with one or more of 13 hr-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), and no hr-HPV infection; a "hr-HPV plus/minus" screening. Although these tests are not designed to detect the recently described probable hr-HPV, or any lr-HPV infections, some cross-reactivity outside of the spectrum of 13 hr-HPV genotypes has been reported for the hc2 assay.[12] Neither hc2 nor AMPLICOR HPV assays allow identification of specific genotypes[13] nor do they have the ability to identify infections involving multiple genotypes.

However, recent studies have provided evidence for a difference in oncogenic potential between the different hr-HPVs[14], arguing for the importance of HPV genotyping in addition to the "hr-HPV plus/minus" screening. Outside of the clinical setting, HPV genotyping is a key-characteristic of studies evaluating the epidemiology

of HPV infections worldwide. Although a number of HPV genotyping assays have been used in such studies a reliable comparison between the diagnostic and epidemiological data generated is difficult, since data on the inter-test comparisons between the different genotyping assays are limited.

The SPF_{10}-INNO LiPA assay is capable of amplifying up to 43 different genotypes and providing type-specific genotype information for 25 different HPV genotypes simultaneously, has been extensively tested, and has proven to be highly sensitive and specific.[8,15] The Roche LINEAR ARRAY (LA) HPV Genotyping Test (Roche Molecular Systems, Inc., Branchburg, NJ, USA) is a recently launched new HPV genotyping assay able to genotype 37 HPV types, concurrently assessing human ß-globin. The full spectrum of HPV genotypes amplified by the PGMY primer system[16] used in the Roche LINEAR ARRAY HPV Genotyping Test has not been assessed beyond the probed 37 genotypes. In essence, both assays could be used for genotyping analysis.

This study was designed to compare these two well-known and commonly used commercially available genotyping assays with HPV DNA positive samples.

Materials and Methods

Cervical scrapes were obtained from 573 women attending the Department of gynaecology for routine cervical screening. Specimens were collected using the Cervex-Brush® (Rovers Medical Devices B.V., Oss, The Netherlands) and processed using a liquid-based cytology medium (ThinPrep®, Cytyc Corp., Marlborough, MA, USA) that provides monolayer distribution for cytological assessment. Moreover, it offers the opportunity to isolate DNA for various HPV detection assays. This method has received U.S. FDA approval for clinical use.[17,18]

Specimen preparation

For isolation of DNA from cervical scrapes in liquid based cytology medium, the MagNAPure LC Isolation station (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was used; 200µL of material was isolated using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim Germany), as described by the manufacturer. With each set of 28 cervical scrape samples 4 negative controls (distilled water) were used to monitor the DNA isolation procedure and to assess contamination. Nucleic acid was resuspended in a final volume of 50µL; 10µL were used for each of the various PCR analyses.

SPF_{10}-INNO LiPA HPV detection and genotyping (DNA enzyme immunoassay [DEIA] and LiPA)

(i) PCR amplification of HPV DNA

Broad-spectrum HPV DNA amplification was performed using a short PCR fragment assay (SPF_{10} HPV PCR, Labo Bio-medical products B.V. Rijswijk, The Netherlands). This assay amplifies a 65-bp fragment of the L1 open reading frame, and allows detection of at least 43 different HPV types.[8,19] SPF_{10} PCR system was used in a final reaction volume of 50mL, containing 10mL of the isolated DNA sample and 40mL PCR mix, which contained 10mmol/L Tris-HCL (pH 9.0), 50mmol/L KCL, 2.0mmol/L $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200mmol/L of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 15pmol each of the forward and reverse primers tagged with biotin at the 5' end, and 1.5 units of AmpliTaq Gold® (Applied Biosystems, Foster City, CA, USA). Activation of AmpliTaq Gold for 9 minutes at 94°C, was followed by 40 cycles of 30 s at 94°C, 45 s at 52°C and 45 s at 72°C, with a final extension of 5 minutes at 72°C. Appropriate negative and positive controls were used to monitor the performance of the PCR method in each experiment.

(ii) HPV detection by DEIA

The presence of HPV DNA was determined by hybridization of SPF_{10} amplimers to a mixture of general HPV probes recognizing a broad range of high-risk, low-risk and possible high-risk HPV genotypes in a microtiter plate format, as described previously.[8,15] All HPV DNA-positive samples (by SPF_{10} DEIA) were genotyped using the INNO-LiPA HPV genotyping assays and the Roche LINEAR ARRAY® HPV Genotyping Test as described below. Twenty randomly selected DEIA-negative samples, that had previously been tested negative by Roche AMPLICOR® HPV Test[10] were also assessed using both genotyping assays.

(iii) HPV genotyping by reverse hybridization using the INNO-LiPA HPV genotyping system

The 28 oligonucleotide probes that recognize 25 different types (see table 1) were tailed with poly(dT) and immobilised as parallel lines to membrane strips

Table I Distribution of HPV-genotypes in the LiPA and LA assays

Oncogenic Potential[5]	HPV Genotype	Detected in[a]:	
		SPF_{10}-LiPA	LA
High-risk	16	X	X
	18	X	X
	31	X	X
	33	X	X
	35	X	X
	39	X	X
	45	X	X
	51	X	X
	52	X	X
	56	X	X
	58	X	X
	59	X	X
	68	X[b]	X
	73	X[b]	X
	82		X
Probable high-risk	26		X
	53[c]	X	X
	66	X	X
Low-risk	6	X	X
	11	X	X
	34	X	
	40	X	X
	42	X	X
	43	X	
	44	X	
	54	X	X
	55		X
	61		X
	62		X
	64		X
	67		X
	69		X
	70	X	X
	71		X
	72		X
	74	X	
	81		X
	83		X
	84		X
	IS39		X
	CP6108		X

[a] X: detected; LiPA: SPF_{10}-INNO-LiPA test; LA: Roche LINEAR ARRAY HPV Genotyping test; [b] LiPA does not distinguish between HPV 68 and HPV 73, since both types are detected by a single probe; [c] Oncogenic potential of HPV 53 is controversial.[6]

(Labo Bio-medical products B.V. Rijswijk, The Netherlands). The HPV genotyping assay was performed as described previously.[15] The LiPA strips were manually interpreted using the reference guide provided. The samples that tested positive using the DNA Enzyme Immuno Assay but showed no results on the LiPA strip were considered to be HPV X-type, i.e., genotypes not available on the LiPA strip.

LINEAR ARRAY HPV Genotyping Test

The LINEAR ARRAY (LA) HPV Genotyping Test (Roche Molecular Systems, Inc., Branchburg, NJ, USA) is a new qualitative *in vitro* test for the determination of 37 anogenital HPV DNA genotypes (table 1). The LA test was applied to all samples that tested positive for HPV by DEIA and to 20 randomly selected DEIA-negative samples.

(i) PCR amplification of HPV DNA

The LA test uses biotinylated PGMY primers to amplify a 450-basepair fragment within the polymorphic L1 region of the HPV genome. The PGMY amplification system has been described previously.[16] The PGMY primers are present in the 'master mixture' (containing: buffer, nucleotides [dATP, dCTP, dGTP, and dUTP], $MgCl_2$, and <0.02% AmpliTaq® Gold DNA polymerase) and amplify HPV DNA from 37 HPV genotypes including 13 high-risk types (Table I). Amplicons incorporate dUTP, allowing the use of AmpErase® enzyme (urasil N-Glycosylase) which is included in the master mixture to prevent PCR carryover contaminations. Capture probe sequences are located in polymorphic regions of L1 bound by these primers. An additional primer pair targets the human ß-globin gene (268 bp amplicon) to provide a control for cell adequacy, extraction and amplification.

PCR was performed in a final reaction volume of 100µL, containing 50µL HPV master mixture, 40µL PCR water and 10µL isolated DNA. The mixture was incubated for 2 minutes at 50°C and 9 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 1 minute at 55°C and 1 minute at 72°C, with a final extension at 72°C lasting from 10 minutes to a maximum of 1 hour. The provided HPV positive and negative controls were used with each set of 10 samples to assess the performance of the reaction.

(ii) Hybridization and Detection

Following amplification, the HPV and human ß-globin amplicons were denatured by immediately adding 100µL denaturation solution to each PCR tube. Hybridization

and HPV genotyping were performed as described by the manufacture (Roche Molecular Systems, Inc., Branchburg, NJ, USA). The strips were manually interpreted using the LINEAR ARRAY HPV Reference Guide, by reading the individual types down the length of the strip. Samples that were both SPF_{10}-DEIA and LA-ß-globin positive, yet were not reactive to any of the genotype probes on the LA strip, were considered "LA negative".

Design of the study

Previously the samples had been assessed in an analysis comparing only high-risk HPV types detected by the Roche AMPLICOR® HPV test and the INNO-LiPA HPV detection and genotyping assay.[10] Since the present study compares two genotyping assays, only the DEIA HPV positive samples and 20 randomly selected DEIA (and Roche AMPLICOR®) HPV negative samples were assessed. In order to have the most accurate comparison between the two genotyping tests, only the HPV genotypes identified by both assays (i.e. lr-HPV 6, 11, 40, 42, 54, and 70; possible hr-HPV 53 and 66; hr-HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) were considered for direct comparison of the individual HPV genotypes (Table I). These will be termed assay-common genotypes. High-risk HPV genotypes 68 and 73 were not taken into account for individual comparison, since these types are identified by a single probe in the LiPA assay and can thus not be distinguished. Moreover, the classification of HPV 53 as possible high-risk is currently disputed. When comparing the two genotyping assays, results were termed concordant, compatible or discordant, based on the following definitions. If the analyses yielded identical assay-common genotypes in both tests the results were termed concordant. Results were termed compatible if one or more additional assay-common genotypes were not detected by either one of the assays. Genotyping results were termed discordant if there were no similarities in the assay-common genotypes between the two tests. Assay results for HPV genotypes uniquely identified by each of these two assays (i.e. assay-unique HPV genotypes 34, 43, 44, and 74 only detected by the LiPA and the assay-unique HPV genotypes 26, 55, 61, 62, 64, 67, 69, 71, 72, 81, 82, 83, 84, IS39, and CP6108 solely detected by the LA) were not considered in determining concordant, compatible, or discordant status.

From all compatible and discordant samples a re-extracted DNA sample was randomly retested in a blinded approach in a discrepancy analysis using both genotyping assays. Eleven concordant samples (6 single infections, 4 double and 1 triple infections) and six double negative (i.e., DEIA-positive, LiPA X-type and LA negative) samples were used as positive and negative controls for both inter- and intra-assay performance control.

All HPV tests were performed by investigators unaware of the results of the comparative HPV detection or genotyping tests.

Statistics

All data were analyzed using SPSS version 12.0.1. for Windows. Agreement was measured by absolute agreement and Cohen's kappa statistics, a measure of the agreement between two methods that is in excess of that due to chance.

Results

In total, 218 of the 573 DNA samples tested positive by SPF_{10} DEIA. These were considered suitable for analysis using the SPF_{10} LiPA and the LA HPV Genotyping assays. Eight samples were excluded from further analysis: 4 showed negative b-globin results in the LA test and from 4 other samples insufficient material was available to perform adequate assessment. Twenty randomly selected DEIA negative control samples were negative in both genotyping assays and were thus not taken into consideration for further analysis. Of the 210 DEIA positive samples 163 (77.6%) indicated normal cytology. Atypical Squamous Cells of Undetermined Significance (ASC-US) were detected in 22 samples (10.5%), mild/moderate dysplasia was observed in 20 samples (9.5%), and 5 samples (2.4%) showed severe dysplasia.

Of the 210 DEIA-positive samples tested using both genotyping assays, 40 samples were excluded since either one of the tests was negative whereas the comparative test detected an assay-unique genotype or LA was negative and LiPA showed an X-type (Table II).

In 132 of the remaining 170 samples, all detected genotypes that could have been identified by both assays. Of these samples harbouring only assay-common genotypes, 87/132 (65.9%) were concordant, 24 (18.2%) were compatible, and 21 (15.9%) showed discordant results (Table III). Finally, in 38 cases, assay-unique

genotypes were detected in addition to assay-common genotypes. Of these samples 25 (65.8%) had concordant results, 12 (31.6%) were compatible and one (2.6%) was discordant. In the final analysis of 170 samples these 38 samples were retained. The additional assay-unique genotypes found in these 38 samples were not taken into consideration. The outcome of the concordant, compatible, and discordant cases is described in detail below.

Table II Distribution of 40 excluded samples that either showed only assay-unique genotypes or were HPV DNA positive but genotype negative (i.e. LiPA X-type)

LA result	SPF_{10}-LiPA		Total
	LiPA X-type (No.)	Assay-unique genotype (No.)	
Negative	9	7	16
Assay-unique genotype	24	0	24
Total	33	7	40

X-type: HPV DNA positive sample (DEIA) without results on LiPA strip

Table III Overview of the 170 included samples with assay-common genotypes

Assay-unique genotypes	Concordant (No.)	Compatible (No.)	Discordant (No.)	Total
None	87	24	21	132
LiPA	3	0	0	3
LA	20	12	1	33
LiPA and LA	2	0	0	2
Total	112	36	22	170

Concordant cases

Of the 112 concordant cases (25 with and 87 without assay-unique genotypes) 69 (61.6%) contained a single HPV genotype and the remaining 43 samples contained multiple genotypes. Thirty-two samples (28.6%) harboured two different genotypes, 8 samples (7.1%) contained 3 HPV genotypes, and 3 samples (2.7%) contained 4 genotypes. One or more high-risk genotypes were detected in 86.6% (97/112) of these samples, whereas 7 samples (6.3%) only contained low-risk genotypes and 8 samples (7.1%) also harboured probable hr-HPV genotypes.

Compatible cases

All 36 compatible cases were multiple infections. The LiPA assay did not detect a total of 41 genotypes in 30 separate clinical samples. In 23 cases, 1 type was missed; in 5 cases, 2 types were missed; and in 2 cases, 4 types (thirteen low-risk, 1 possible high-risk and 27 high-risk genotypes were not detected by the LiPA test). The LINEAR ARRAY assay on the other hand did not detect 12 genotypes in 8 separate samples. In 6 cases, 1 type was missed; in 1 case, 2 types; and in 1 case, 4 types (2 low-risk, 1

Table IV Overview of the 36 compatible and 22 discordant samples

Oncogenic Potential	Genotype	No. of specific genotypes not detected			
		Compatible samples		Discordant samples	
		LiPA	LA	LiPA	LA
High-risk	16	7			1
	18	2	1		
	31	2	2		
	33	1			2
	35				1
	39	3			
	45	2		1	
	51		1		4
	52	1	1		2
	56		3	1	1
	58	3			
	59	5			
	68/73	1	1		2
Probable high-risk	53		1	1	3
	66	1			2
Low-risk	6				2
	11	1	2		
	42	4		1	
	54	8		2	1
	Total	41	12	6	21

possible high-risk and 9 high-risk HPV types). Table IV gives an overview of the individual types not detected. Fifteen of the 16 cases in which LiPA missed a hr-HPV type were multiple hr-HPV infections and tested positive for another high-risk type which was also detected in the LA.

Discordant samples

In 22 (12.9%) of the 170 samples considered, no similarity was observed in the genotypes found in the two tests. These were predominantly single infections. An overview of the individual discordant cases is given in Table IV. Twenty-seven genotypes were discrepant between the two assays in 22 different samples. The LA test did not detect 13 hr-HPV, 5 probable hr-HPV and 3 lr-HPV types, that were found to be positive in the LiPA assay. The LiPA assay on the other hand failed to detect 2 high-risk, 1 probable high-risk and 3 low-risk types, which were all found positive on the LA strip.

Table V Kappa values and the p-values of McNemar's test for individual HPV genotypes detectable by both assays[a]

Oncogenic Potential	Genotype	No. of genotypes found positive by:			κ-value (95% CI)[b]	P-value (McNemar's test)
		LiPA	LA	LiPA and LA		
High-risk	16	39	45	38	0.874 (0.788-0.959)*	0.08
	18	14	15	13	0.887 (0.760-1.014)*	1.00
	31	13	13	11	0.833 (0.672-0.995)*	0.62
	33	10	9	8	0.833 (0.645-1.020)*	1.00
	35	9	8	8	0.938 (0.817-1.059)*	1.00
	39	7	9	7	0.869 (0.687-1.050)*	0.48
	45	5	8	5	0.761 (0.492-1.029)$	0.25
	51	16	11	11	0.799 (0.626-0.973)$	0.07
	52	23	21	20	0.896 (0.795-0.997)*	0.62
	56	12	9	8	0.747 (0.528-0.965)$	0.37
	58	8	11	8	0.833 (0.646-1.020)*	0.25
	59	6	11	6	0.692 (0.426-0.958)$	0.07
Probable hr	53	20	17	16	0.848 (0.718-0.979)*	0.37
	66	9	8	7	0.814 (0.606-1.023)*	1.00
Low-risk	6	11	9	9	0.894 (0.748-1.040)*	0.48
	11	4	3	2	0.563 (0.072-1.053)+	1.00
	40	0	0	0		
	42	2	7	2	0.434 (-0.055-0.923)+	0.07
	54	9	18	8	0.562 (0.311-0.812)+	0.02
	70	6	6	6	1.000 (1.000-1.000)**	

[a] The results of 112 concordant, 36 compatible and 22 discordant samples after initial analysis are shown
[b] CI; confidence interval
Strength of agreement considered: **perfect, *very good, $good, +moderate

The genotypes which were detectable by both assays of all 170 samples (112 concordant, 36 compatible and 22 discordant) were individually compared as summarized in Table V. The overall strength of agreement between the two assays for the individual genotypes was considered good (κ = 0.792). Although HPV 16 was detected in 45 samples using the LA test and in 39 samples using the LiPA agreement between the tests was considered very good with a κ-value of 0.874. The agreement between the two assays for the other high-risk and probable high-risk genotypes varied between "good" and "very good". The agreement between the two tests for the low-risk genotypes was "moderate" to "perfect". The agreement for HPV 54 was moderate since LiPA and LA shared 8 samples harbouring the low-risk genotype whereas LA detected it in 10 additional samples. Also, the agreement for lr-HPV's 11 and 42 was moderate, while HPV 70 was detected in equal amounts by both assays. Low-risk HPV 40 was not detected in either one of the test, thus no agreement could be calculated. The difference in detection of lr-HPV 54 was statistically significant ($P<0.05$; McNemar's test). Although the differences for hr-HPV 16, 51 and 59, and lr-HPV 42 between the assays were large, they were considered not quite statistically significant ($P>0.07$ McNemar's test). In the individual comparison of the other genotypes no statistically significant differences were detected.

Discrepancy analysis

The compatible (n=36) and discordant (n=22) samples were reanalysed using the two genotyping assays in a discrepancy analysis. DNA was re-extracted from these 58 compatible/discordant samples. As interassay test controls 11 previously concordant (6 single and 5 multiple infections) and 6 previously double negative samples (LiPA X-type and LA negative) were also included, these samples were used for method performance assessment only. All 6 double negative samples remained negative and all 11 concordant samples appeared identical in both second genotyping assays. These internal controls were not further considered in the discrepancy analysis. Of the 58 discrepant samples 10 were ß-globin negative in LINEAR ARRAY and were also negative by LiPA. Of these 10 samples, 6 had been concordant and 4 had been discordant; these 10 samples were excluded from the discrepancy analysis.

The crude initial and discrepancy analysis results of the remaining 48 samples are shown in Table VI. Of the 30 compatible samples from the initial analysis 18 remained compatible after discrepancy analysis, while 8 appeared concordant and 4 discordant in a comparison of the second genotyping assays. Of the 18 discordant samples from the first test run, 9 remained discordant in the second analyses between LiPA and LA, whereas 4 appeared genotype concordant and 5 were concordant as LiPA X-type, LA negative. Thus, comparing the second LiPA and LA test, yielded 17 concordant, 18 compatible and 13 discordant results.

Intra-assay comparisons taking these 48 samples and the 17 control samples in both initial and discrepancy analysis into account, show highly comparable results for both assays (Table VII).

In conclusion, of the 160 samples considered for final analysis 80.6% (129/160) showed identical results, 11.2% (18/160) appeared compatible and 13 samples (8.2%) were discordant.

Discussion

Based on this study, we can conclude that the SPF_{10}-INNO LiPA and the LINEAR ARRAY HPV genotyping assays are highly congruent for the genotypes detectable in both assays. Moreover, manageability of both the SPF_{10}-INNO LiPA and the LINEAR ARRAY assay is highly comparable, as is to a large extent the total runtime required for both assays, including amplification and preparation of all of the reagents.

Generally, a separate screening is needed preceding genotyping in order to assess a sample's HPV DNA positivity, i.e., an HPV plus/minus screening. An advantage of the LiPA is the usage of the same amplicon for both detection of 43 different lr-, probable hr-, and hr-HPV genotypes and genotyping of 25 different HPVs. For the LA, a pre-screening test with the PGMY primers is available using a generic HPV probe labelled with digoxigenin in a microtiter plate-based assay as recently described.[20] Without the need for further amplification, this amplicon can be directly used for the LINEAR ARRAY genotyping assay. However, the efficiency of such a combination has not been studied. The recently launched HPV Roche AMPLICOR® test for HPV plus/minus

Table VI All genotyping and comparison results of the 35 initially compatible and discordant samples assessed discrepancy analysis

Initial comparison	HPV genotype (initial analysis)		Discrepancy comparison	HPV genotype (discrepancy analysis)	
	LiPA_1	LA_1		LiPA_2	LA_2
Compatible	35	33, 35	Concordant	33, 35	33, 35
Compatible	51	16, 39, 51	Concordant	51	51
Compatible	18, 33	18, 31, 33	Concordant	18, 33	18, 33
Compatible	33	16, 33	Concordant	33	33
Compatible	68/73	58, 73	Concordant	68/73	73
Compatible	39	16, 39	Concordant	39	39
Compatible	52, 53	52, 53, 54, 67	Concordant	52, 53, 54	52, 53, 54, 67
Compatible	35, 39, 70	16, 35, 39, 70, 81	Concordant	35, 39, 70	35, 39, 70, 84
Compatible	16	16, 59	Compatible	16	16, 59
Compatible	6, 51	6, 16, 18, 39, 51, 66	Compatible	6	6, 16, 18, 39, 51, 66
Compatible	51, 52, 53, 59	45, 51, 52, 53, 59, IS39	Compatible	53	42, 51, 52, 53, 59, IS39
Compatible	6, 33	6, 33, 58, 59, 72	Compatible	6, 31, 33, 58, 59	6, 33, 58, 59, 72
Compatible	6, 16, 52	6, 16, 42, 52	Compatible	6, 16, 52	6, 16, 42, 52
Compatible	52	16, 52	Compatible	6, 16, 52, 56	16, 52
Compatible	6	6, 59	Compatible	6	6, 59
Compatible	31, 70	31, 54, 62, 70	Compatible	6, 31, 70	62, 70
Compatible	54	54, 73	Compatible	54	54, 73
Compatible	16	11, 16, 59, 81	Compatible	16	11, 16, 59, 81
Compatible	56, 66, 68/73	39, 52, 56, 66, 68	Compatible	56, 66, 68/73	52, 56, 66, 68
Compatible	16, 52	16, 52, 54	Compatible	16, 52	16
Compatible	53	42, 53, IS39	Compatible	53	42, 51, 53, 59, IS39
Compatible	53, 66	16, 53, 66	Compatible	66	53, 66
Compatible	31, 33, 53	33, 42, 45, 53, 54, 59, 61, 83	Compatible	31, 33, 45, 53, 59	33, 42, 45, 53, 54, 59, 61, 83
Compatible	56, 58	54, 56, 58, 62	Compatible	56, 58	54, 56, 58, 62
Compatible	54, 56	54	Compatible	54, 56	54
Compatible	16, 31, 53, 58	16, 18, 53, 54, 62, CP6108	Compatible	16, 18, 31, 53, 58	16, 53, 54, 58, 62, CP6108
Compatible	33	33, 54	Discordant	33	54
Compatible	56, 66	66, 67	Discordant	56, 66	67
Compatible	56, 59	59	Discordant	X-type	59
Compatible	51, 53	51, 53, 54, 62	Discordant	51, 53	62
Discordant	6	negative	Concordant	6	6
Discordant	6, 53	negative	Concordant	6, 53	6, 53
Discordant	X-type	53	Concordant	X-type	negative
Discordant	16	negative	Concordant	16	16
Discordant	X-type	45, 61, 83	Concordant	45	45, 61, 83
Discordant	53	negative	Concordant	X-type	negative
Discordant	52	negative	Concordant	X-type	negative
Discordant	53	negative	Concordant	X-type	negative
Discordant	52	54	Concordant	X-type	negative
Discordant	66	negative	Discordant	66	68
Discordant	35	negative	Discordant	35	negative
Discordant	56	negative	Discordant	56	negative
Discordant	X-type	42	Discordant	X-type	42
Discordant	68/73	negative	Discordant	68/73	negative
Discordant	51, 66, 68/73	negative	Discordant	51	negative
Discordant	X-type	56	Discordant	X-type	56
Discordant	51	negative	Discordant	51	negative
Discordant	51	negative	Discordant	51	negative

Table VII Intra-assay comparison overview of the 65 samples re-analysed in the discrepancy analysis, including the 17 control samples concordant in all four assays

Test compared[a]	Concordant (No.)	Compatible (No.)	Discordant (No.)	Total
1st LiPA vs 2nd LiPA	48	11	6	65
1st LA vs 2nd LA	43	16	6	65

[a] 1st: initial comparison; 2nd: discrepancy comparison

screening is not meant for a LA screen. It could also be used as pre-test, but the assay only detects high-risk HPV types.[10]

In the initial comparison, i.e., prior to the discrepancy analysis, LiPA did not detect 27 high-risk genotypes in 30 compatible cases. Evidently, all the cases involved were multiple infections, i.e., containing two or more HPV types. Apparently, if an infection encompasses multiple genotypes the SPF_{10}-INNO LiPA assay is less sensitive than the LA. After finding analogous results using the LiPA assay, Van Doorn, et al. propounded the idea of PCR competition between genotypes in mixed infections and suggested a combined testing algorithm using broad-spectrum and type-specific PCRs for HPV 16 and HPV 18 (L.J. van Doorn, A.C. Molijn, B. Kleter, W.G.V. Quint and B. Colau, Abstr. 22nd IPV Conference., abstr. N-01, 2005). The complexity of assessing multiple genotypes was addressed previously.[21] Amplification and identification of two genotypes present in equimolar amounts are likely possible. However, 'primer competition' between genotypes might occur in case one particular genotype is present in molar excess, out competing the other one.[21] In the present study this is demonstrated by the samples harbouring multiple infections which were not identically genotyped by both assays. Also, LA detected hr-HPV 16 in 7 samples that were LiPA HPV 16 negative; after the second LA, however, 5 samples no longer showed HPV 16. Moreover, in a previous study Van Doorn and colleagues detected HPV 16 and HPV 18 using type-specific PCR in samples negative to these genotypes (but not for other genotypes) using general primer sets.[21] In the present study we observed similar results (data not shown). Although viral load was not determined in the present study, low-copy-number samples have previously shown more discrepancy in intralaboratory and interlaboratory comparison.[22]

The LA assay is unable to distinguish hr-HPV 52 from other high-risk genotypes (33, 35, and 58). This can be inconvenient in future studies using the LINEAR ARRAY, since hr-HPV 52 is prevalent in approximately 5% of the HPV-positive women with normal cytology[23] and causes 2.2% of all cervical cancers.5 In 19 samples of the present study hr-HPV 52 positivity could not be excluded based on LA genotyping. However, in these cases the comparative LiPA tests did not detect this specific genotype. Two samples were considered LINEAR ARRAY HPV 52 positive based on the LiPA results.

Among the 22 discordant cases, the number of hr-HPV genotypes detected by the LINEAR ARRAY was not higher than the number detected by LiPA. All but three of these samples were single infections, predominately HPV 33, HPV 51, and HPV 52. A higher inclusivity level has been observed for some high- and low-risk HPV genotypes, particularly hr-HPV 33 and hr-HPV 56, when the PGMY amplification system is used (see product insert for the CE-marked LINEAR ARRAY HPV Genotyping Test, European market). The inclusivity level equates to the lowest concentration (copies/mL) that shows a 100% positive hit rate in a replicate of 6 tests or the concentration that is the probit predicted 95% positive hit rate. This could explain some of the differences between the two assays observed in our study. Thus the LA seems to be less sensitive than the LiPA if a sample has a single infection with some specific HPV genotypes that are poorly amplified by PGMY. Even though the majority of samples was cytologically classified as normal, proper HPV assessment, including genotyping, remains essential particularly in healthy women with normal cytology[10], especially since Wallin and colleagues observed a strong concordance between the HPV type found in baseline smear with normal cytology and the eventual type found in histological samples of invasive cancers.[24] In the present study hr-HPV 51 was missed by LA in 4 of the discordant cases; this genotype accounted for approximately 0.9% of all squamous-cell cervical cancer in previous studies.[5] Curiously, the inclusitivity level for HPV 51 is lower than the level for HPV16 using PGMY primers, suggesting a highly sensitive detection (see product insert CE-marked LINEAR ARRAY HPV Genotyping Test European market). The observed difference in HPV 51 detection between the two assays cannot be explained by a lower efficiency of LINEAR ARRAY PGMY primer.

After discrepancy analysis of the compatible and discordant cases, both LiPA and LA detected more concordance (Table VI). Some previously undetected genotypes for example appeared in the second test run, and vice versa. This could be due to low

copy numbers or sampling, as DNA re-extracts were used for the analysis. Also it could possibly indicate the suggested competition between genotypes present in more or lesser molar excess. However, results from a discrepancy analysis should generally be handled with care and interpreted carefully. Discrepancy analyses are not perfect, since an analysis is easily biased in favour of the new test, and hard and fast rules do not exist.[25] Moreover, the interpretation of results that cannot be dichotomised (i.e. concordant, compatible and discordant) is less straightforward.

Failing to detect genotypes will lead to underestimation of the prevalence of certain genotypes and causing false-negative results. Studies concerning (i) epidemiology of HPV, (ii) HPV vaccination/surgical treatment trials, and (iii) cervical cancer screening and triage, especially will be negatively affected by this. In epidemiologic studies genotyping is compulsory in order to evaluate type-specific HPV DNA prevalence among infected women[26], to assess geographic heterogeneity in HPV type distribution[23] and to study type-specific HPV concordance between sexual partners.[27] The importance of suitable algorithms for HPV detection and genotyping in addition to the introduction of type-specific antiviral therapies or monovalent vaccines, was already addressed by Koutsky and colleagues.[28] Moreover, current extensive trials testing multivalent vaccines, comprising multiple commonly occurring HPV types, demand accurate, unequivocal, and sensitive methods and algorithms detecting and, specifically genotyping HPV.[28-30] These algorithms are also compulsory for clinical trials monitoring surgical treatment of HPV induced CIN lesions[13,31] or monitoring of persistent infections in consecutive smears, because persistence has been identified as important risk factor.[32,33] Finally, according to Snijders and colleagues, adding general hr-HPV testing could be beneficial for the efficacy of the population based screening programmes for cervical cancer.[9] Castle and colleagues however observed that ASC-US women infected with the hr-HPV 16 had a 2-year cumulative absolute risk for developing ≥ CIN3 of 32.5% compared to the 8.4% risk developing ≥ CIN3 for other high-risk HPV types.[14] This underlines the potential importance of assessing the specific genotype causing the HPV infection. Triaging patients using cytology and genotyping assays might have a cost benefit over cytology combined with hr-HPV testing alone. The existence of triage management of ASC-US women in the United States solely depends on an accurate genotyping test.[34] Both tests assessed in the present study could be suitable as triage test.

In addition to accurate genotyping, the appropriate detection of multiple infections seems to be an important application of tests when they are implemented into any format of population based screening for the prevention of cervical cancer, especially since the presence of multiple human papillomavirus genotypes in a single sample -suggesting repetitive exposure- is suspected to be associated with an increased risk for progressive disease.[35] Moreover, mixed infections appear to be more frequent than previously expected; 35% of the HPV-positive samples and more than 50% of human immunodeficiency virus-positive women are infected with multiple HPV types.[36,37] Multiple infections were less prevalent in cervical carcinomas.[15]

In conclusion, both genotyping assays are handled equally well and have shown to be highly comparable. All of the HPV genotypes detected in either one or both of the assays, regardless the analytical or clinical sensitivity and specificity of the tests, should not be trivialized, since their natural behaviours and cancerous potentials in both single and mixed infections remain ambiguous.

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Biological behaviour of CIN lesions is predictable by multiple parameter logistic regression models

Chapter 6

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Abstract

Progression and regression of pre-malignant cervical lesions cannot be predicted using conventional cyto- or histomorphological parameters. However, markers as HPV or makers indicating proliferation, genetic instability and chromosomal aberration may be of predictive value assessing short-term biological behaviour of cervical intraepithelial neoplasia (CIN). In this paper we have studied the usage of logistic regression models with Ki-67 labelling index (LI), chromosome index (CI) and aneusomy for chromosome 1 in cervical smears to predict progressive and regressive behaviour of CIN lesions. Retrospectively, the intake-smears of 42 women showing regression in follow-up and of 31 women showing progression in follow-up were assessed. A multi-parameter logistic regression model containing the parameters Ki-67 LI, CI#1, and the fraction of cells with 4 copies of chromosome 1 per nucleus appeared to be the best predicting model, overall correct classification of 93.2% (AUC 0.96±0.02). After cross-validation, the model correctly classified 66 of 73 samples (90.4%). Moreover, the model predicted biological behaviour perfectly assessing the smear taken subsequently to the intake-smear of 46 women. Although measuring parameters indicating proliferation and chromosome 1 aberration is laborious, this study demonstrates that short-term pro- and regressive behaviour is highly predictable using a model combing these parameters. We also showed that in the triage management of high-risk HPV positive women with minimally abnormal smears applying a model as such can be useful.

Introduction

It is generally accepted that cervical intraepithelial neoplasia (CIN) precedes squamous cell carcinoma of the uterine cervix.[1] According to the classification of the International Society of Gynecological Pathologists, these pre-malignant CIN lesions are classified solely by histomorphological criteria, i.e. nuclear atypia, presence, frequency and localization of mitotic figures and the loss of polarity of the nuclei. CIN is hereby subdivided in low-grade CIN 1 (mild dysplasia), and CIN 2 (moderate dysplasia) and CIN 3 (severe dysplasia and carcinoma *in situ*).

High-grade CIN (CIN 2 and 3) can be successfully treated by large loop excision of the transformation zone of the cervix (LLETZ).[2-4] Unfortunately, substantial over-treatment of low-grade CIN is the penance for the accessibility of this procedure. For achieving adequate and well-deliberate treatment, knowledge on the pro- and regressive behaviour of squamous intraepithelial cervical lesions is compulsory. Östör showed in an extensive literature review that CIN 1 will progress to CIN 3 in only 10% of the cases and to invasive cancer in 1%.[5] CIN 3 lesions on the other hand will advance to invasive carcinomas in 12%, whereas spontaneous regression will occur in 33%.[6] However, ambiguity persists about the exact timescale of pro- and regression of “any-grade” cervical intraepithelial lesion. Moreover, neither cyto- nor histomorphological markers of cervical intraepithelial abnormalities can predict the biological behaviour of CIN.

Genital infection with high-risk human papillomavirus (hr-HPV) is considered the most important factor in the carcinogenesis of cervical cancer.[7] This significance is verified by the observations that almost all cervical cancers harbour hr-HPV genotypes[8], and that 74% of CIN 1 and approximately 84% of high-grade CIN lesions harbour high-risk genotypes.[9] The key event of HPV-induced oncogenesis is the integration of viral-DNA in the human genome.[10] Frequently, hr-HPV16 DNA integrates near common fragile sites of the host genome[11], and this is believed to occur somewhere in the gradual process of progression of a CIN lesion. Integration results in the disruption of the viral E2 region, inducing an over-expression of viral E6 and E7 oncoproteins. Successively, these oncoproteins interfere with two crucial mitosis-regulating pathways of the host cell, the p53-pathway and pRb-pathway, respectively. Normally the p53-pathway induces growth arrest or apoptosis while pRb regulates

the passage through the cell cycle. Accordingly, E6/E7-induced inactivation of these pathways results in hyperproliferation (E7 related) and genetic instability, numerical and structural chromosome aberrations and immortalization (all E6 related) (reviewed by Zur Hausen, 2002).[12]

Structural aberrations as deletions, translocations and inversions in cervical carcinomas have been reported in chromosomes 1, 3, 6, 9, 11, and 17.[13] E6-induced structural rearrangements of chromosome 1 have been described in 90% of the cervical cancers.[14] Moreover, chromosome index (CI) -defined as the mean number of chromosome copies per nucleus- for chromosome 1 shows a significant positive correlation with CIN grading.[15,16]

Monoclonal antibody MIB1 recognizes the proliferation-associated Ki-67 antigen.[17] The Ki-67 labelling index (percentage of MIB1 positive cells) has been propound a promising alternative method for classification of CIN lesions.[18-20]

As opposed to cyto- or histomorphological markers of a CIN lesion, the parameters related to aberrations of chromosome 1 and those related to cell proliferation are suggested to have predictive value regarding the natural behaviour of CIN. The present study was designed to elucidate whether univariate and multivariate linear logistic regression models using Ki-67 labelling index, chromosome index for chromosome 1 and aneusomy for chromosome 1 assessed in cervical smears were able to predict progressive and regressive behaviour of CIN lesions. According to previous studies only women positive for high-risk HPV are at risk for the progression of a CIN lesion and should thus be followed more closely, hence the results of the predictors were assessed considering hr-HPV positive samples cytologically indicating borderline and mild dysplasia (BMD).

Materials and methods

From a cohort of 800 women, all women referred to the Department of Obstetrics and Gynaecology of the Radboud University Nijmegen Medical Centre having two consecutive smears indicating atypical squamous cells of undetermined significance (ASC-US) or mild dysplasia, or one smear indicating moderate dysplasia were eligible for this study. All patients underwent colposcopy within one month of the intake. Preceding the colposcopy procedure a new cervical smear ("initial smear") was obtained using the Cervex-Brush® (Rovers Medical Devices B.V., Oss, The Netherlands). The remnants of the cervical smear were fixed with Unifix® and processed into AgarCyto blocks as previously described[21], allowing for multiple analysis. In case colposcopy was suggestive for high-grade CIN immediate excision of the transformation zone (LLETZ) would follow as previously described.[2] These patients were not included in the present study. All referred patients whose colposcopy did not indicate a high-grade CIN were followed using cervical cytology.

Table I Classification of the two study groups

Group	Selection criteria	Controls	No
I	Initial smear: ASC-US, mild or moderate dysplasia Follow-up: two successive normal smears or two grades less severe than the initial smear	Lesions with a negative short-term follow-up with a low risk to progress towards CIN 3 **"Regressive lesions"**	42
II	Initial smear: ASC-US, mild or moderate dysplasia Treated by LLETZ ≥13 weeks and diagnosed CIN 3	Lesions with a positive short-term follow-up, corresponding with a high risk to progress into CIN 3. **"Progressive lesions"**	31

BNC: borderline nuclear changes

In case two consecutive follow-up smears would indicate normal cytology or 2 grades less severe than the initial smear, the lesions were considered non-progressive or regressive. Women with an initial smear diagnosed ASC-US, mild or moderate dysplasia having a histologically proven CIN 3 lesion were considered progressive. In both progressive and regressive group the follow-up period required to be at least 3 months. These two groups were used to obtain classifiers to facilitate the development of a logistic regression model for assessing the biological behaviour of

CIN lesions (table 1). In the present study the classifiers were considered reliable if they had the ability to predict a negative short-term follow-up correctly within a high percentage of the women who did not develop CIN 3 in follow-up (group I). On the other hand, the classifiers would predict a positive short-term follow-up correctly in very high percentages of the women who developed a CIN 3 lesion in the follow-up (group II).

In order to test the classifiers, the model was supposed to predict initial smears as progressive in 7 under-diagnosed women who had had a LLETZ performed indicating a CIN 3 lesion, but whose initial smear taken 6 weeks prior to LLETZ procedure was under-diagnosed as moderate dysplasia (test group). Moreover, classifiers that correctly predicted the biological behaviour would perform as good or even better if applied to cervical smears taken subsequent to the initial smears whereupon the model is based, i.e. closer to the endpoint of pro- or regression. Therefore, 46 patients from groups I and II who had a subsequent follow-up smear taken after the initial smear were selected from the local pathology database and assessed in a similar way as the initial smears. These samples were used to validate the performance of the model.

Since cervical cancer screening programmes can benefit most from any triage of minimally abnormal cervical smears, the logistic regression model best predicting biological behaviour was applied to the HPV positive minimally abnormal smears, the borderline and mild dysplasia (BMD) smears.

HPV genotyping

For HPV detection a highly sensitive short fragment polymerase chain reaction (SPF_{10}-INNO LIPA HPV genotyping assay, Labo Bio-medical products B.V. Rijswijk, The Netherlands) was performed on a section of the AgarCyto cellblock. A serial 6µm thick tissue section was put into a reaction tube and incubated overnight at 56°C in 200µL of 10mM tris-HCL with 1mM EDTA, 0.2% Tween-20, and proteinase K (0.3mg/mL). Proteinase K was inactivated by 10 min incubation at 100° C. The sample was centrifuged for 10 min at 11,000 rpm and 10µL was directly used for PCR analysis. HPV DNA was amplified using the SPF_{10} PCR primer set. This assay amplifies a 65-bp fragment of the L1 open reading frame, and allows detection of at least 43 different HPV types. Each experiment was performed with separate positive and negative PCR controls. Samples positive for HPV DNA were assessed using reverse hybridization by a line probe assay (LiPA), allowing for simultaneous typing of 25 different HPV genotypes. This procedure for HPV detection and genotyping has been previously validated and considered highly sensitive.[9,22-24] In this present study HPV genotyping of the samples was only used in order to differentiate between high-risk HPV infection and no-HPV infection.

Immunohistochemistry

Detection of Ki-67 in AgarCyto cervical smear samples was performed using a standard immunocytochemical procedure, described in detail previously.[25] Four µm thick paraffin sections of the tissue samples were mounted onto polylysinecoated slides and dried overnight at 58°C. The sections were dewaxed in xylene and endogenous peroxidase was blocked using H_2O_2 in methanol for 15 minutes and the slides were rinsed three times in phosphate-buffered saline (PBS; pH 7,4) for 5 minutes. The slides were placed in a citrate buffer (0,01M; pH 6,0), heated in a household microwave oven (3 min at 850W until boiling; followed by 10 min at 180W. The sections were allowed to cool down to room temperature (RT) and washed in PBS (10 minutes). The primary antibody was diluted in PBS and incubated overnight at 4°C. All following antibodies were diluted in PBS with 1% bovine serum albumin (BSA; Sigma, St. Louis, Missouri, USA) and incubated for 30 min at RT. All intermediate wash steps were performed in PBS. Ki-67 was detected by MAb MIB1 (1:50; Dianova, Hamburg, Germany), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse (1:100; DAKO SA, Glostrup, Denmark). The slides were developed with 0.05% diaminobenzidine (DAB; Sigma, St. Louis, Missouri, USA) with 0.15% H_2O_2 in PBS for 5 min at RT. Specimens were counterstained with Mayer's hematoxylin, dehydrated in ethanol and xylene and finally mounted in Permount (Fisher Scientific; Fair Lawn, NJ, USA). The Ki-67 labelling index is defined as the fraction of Ki-67 positive nuclei. This labelling index was assessed in at least 100 non-overlapping nuclei.

***In situ* hybridization**

DNA-probe pUC1.77 for the centromere region of chromosome 1 was labelled by nick translation with biotin-16-dUTP according to the supplier's instructions (Boehringer; Mannheim, Germany). The hybridizations protocol applied to AgarCyto sections have been previously described in detail.[25] In short, AgarCyto sections were consecutively

dewaxed, blocked for endogenous peroxidase, and pre-treated with 1M NaSCN for 10 min at 80°C. Protein digestion was performed using 4000U/mL pepsin (Sigma; St. Louis, Missouri, USA) in 0.2M HCl for 5 min at 37°C and the sections were dehydrated through an alcohol series and air-dried. DNA probe (2ng/µL) was dissolved in 15µL hybridization mix containing 60% formamide, 2x standard saline citrate (SSC), pH 7.0, 10% dextran sulphate (Sigma; St. Louis, Missouri, USA), and 50ng/µL herring sperm DNA (Boehringer; Mannheim, Germany). The probe mix was applied to the sections, covered with a cover slip, and sealed with rubber cement. Probe and target DNA were heat-denatured simultaneously for 10 min at 80°C and hybridized overnight at 37°C in a moist chamber. Cover slips were removed by immersing the slides at 42°C in 2xSSC, pH 7.0. Post-hybridization washes at 42°C were carried out twice for 5 min in 60% formamide/2x SSC, pH 7.0, and twice for 5 min in 2x SSC, pH 7.0. The slides were rinsed in phosphate buffered saline (PBS)/0.05% Tween-20 (PBST). Hybridised DNA probes were detected by immunohistochemically using mouse anti-biotin (1:100; DAKO SA, Glostrup, Denmark), biotinylated horse anti-mouse, and peroxidase-ABC as described for immunochemistry. Evaluation of ISH signals of non-overlapping and morphologically good preserved nuclei was performed as previously described.[16] At least 100 nuclei per sample were assessed.

The chromosome index is defined as the mean number of chromosome copies per nucleus in the sample of measured nuclei. The CI measured in non-truncated diploid nuclei has a theoretical value of 2. In truncated nuclei, the CI measured in dipoloid cells, will be always smaller than 2. As pointed out in an earlier study, an aberrant CI was defined as any value exceeding 1.4 (mean value CI + 2.58 x SD, obtained from normal cervical epithelium in control subjects).[15,16] In this study the chromosome index for chromosome 1 (CI#1) was assessed.

Statistics

Between group I and II independent t-tests were performed to assess differences in the means of the various parameters measured. In order to select a subset of features to discriminate between the patients in group I and II a forward likelihood-ratio stepwise logistic regression analysis was performed. Logistic regression analyses were also used to find the best multi-parameter linear predictor to allocate women in these groups, as is given by the formula: $\eta = a_o + a_1 \cdot x_1 + \ldots + a_n \cdot x_n$ in which a_i are the regression coefficients of the corresponding predictor x_i which are selected using forward stepwise logistic regression analysis. The conditional probability that a low-grade cervical lesion will progress to a CIN 3 if the value of the linear predictor η (as described above) is known, is given by the formula: $P(progression|\eta) = \frac{e^n}{1+e^n}$ In case the progression probability is ≥0.5 the sample is classified progressive, a probability <0.5 means the sample is classified regressive. The same samples were used to construct as well as to evaluate the performance of selection criteria.

The quality of the multi-parameter linear models' probabilistic judgements was assessed using several quantitative methods: the Brier score, the area under the "receiver operating characteristic" (ROC) curve and the Hosmer and Lemeshow Test. The Brier score (B) is the mean-squared-error of chance expectancy, measuring the difference between a predicted probability of an event (P_i) and its corresponding observed response (Y_i) for the ith observation, expressed as 0 or 1 depending on if the event has occurred or not $B = \frac{1}{n}\sum_{i=1}^{n}(P_i - Y_i)^2$.

So, a Brier score value near zero corresponds to a nearly perfect prognosis by the model.[26] The area under the ROC curve is a graphical plot of the sensitivity vs. (1 – specificity) for a binary classifier system as its discrimination threshold is varied. The curve represents the probability that a randomly chosen progressive sample is correctly rated with greater suspicion than a randomly chosen non-progressive sample. The greater the area under the curve, the better the prediction model. The Hosmer and Lemeshow test is a commonly used test for the goodness-of-fit based on grouping predicted probabilities into deciles and performing a χ^2-test for the mean predicted probability against the observed fraction of events.[26] The higher the P-values of the χ^2-test the better the fit.

Classification results from this procedure may therefore be too optimistically biased. The leave-one-out cross-validation method was used in the logistic regression analyses to reduce this bias. Using this method one observation is omitted from the analytical process and the response for that observation is predicted using a model derived from the remaining n-1 observations.[26] The statistical analyses were performed with SPSS version 12.0.1 for Microsoft Windows.

Results

From a cohort of 800 women, 73 were allocated meeting the criteria defined in table I. The mean age at initial smear was 37.0(±9.4) years. Thirty-five of the 73 initial smears showed moderate dysplasia (47.9%), 14/73 (19.2%) of the women had a smear indicating mild dysplasia and in 24/73 (32.9%) the initial cytology was diagnosed ASC-US. Details of the distribution of the initial cytology per group are given in table II. Twenty-one of the 24 women having ASC-US (87.5%) showed regression towards normal epithelium in the consecutive smears, while progression to CIN 3 appeared in 3/24 cases (12.5%). Eleven of the 14 women (78.6%) initially having mild dysplasia regressed to a normal smear, while progressive behaviour was observed in 3/14 cases (21.4%). Finally, of the 35 women demonstrating moderate dysplasia in their initial smear 4/35 (11.4%) regressed to a normal smear, 6/35 (17.1%) regressed towards an ASC-US smear and, 25/35 (71.4%) showed progression.

Table II Cytological classification of the initial smears in the two groups assessed

Initial smear	Total	Group	
		I [n (%)]	II [n (%)]
ASC-US	**24**	21 (50)	3 (12.5)
Mild dysplasia	**14**	11 (26.2)	3 (21.4)
Moderate dysplasia	**35**	10 (23.8)	25 (59.5)
Total	**73**	**42**	**31**

ASC-US: atypical squamous cells of undetermined significance

Table III Mean and SD for the biomarkers in the **initial** smear which appeared to be most suited for logistic regression and (multi)variate analysis

Parameter	Group I (N=42) Mean±SD	Group II (N=31) Mean±SD	P-value Student t-test	Test group (N=7) Mean±SD
Ki-67 LI	0.19±0.15	0.43±0.15	<0.001	0.59±0.14
CI#1	1.32±0.21	1.86±0.35	<0.001	2.11±0.33
Fr 4 copies #1	0.01±0.03	0.04±0.05	<0.01	0.04±0.06

LI: Labelling index; CI#1: chromosome index for chromosome 1; Fr 4 copies #1: fractions of cells with 4 copies of chromosome 1 per nucleus; SD: standard deviation

The indicated follow-up time between initial smears and subsequent normal or two grades less severe smears (group I) and between initial smears and LLETZ procedures (group II) was at least 3 months. However, the actual mean (±SD) follow-up time in months for the two groups was prolonged, group I 8.7(±6.1) months and group II 5.9(±5.0) months.

High-risk HPV detection

Forty-four of the 73 initial cervical smears (60.3%) harboured high-risk HPV genotypes, in the remaining 29 (39.7%) no hr-HPV types were detected using the SPF_{10}-LiPA assay. Considering the initial cytological results 11/24 (45.8%) ASC-US smears, 7/14 (50%) mild dysplasia smears and 26/35 (74.3%) moderate dysplasia smears contained hr-HPV. Using the presence or absence of a hr-HPV infection to classify women in respectively group II (progression) and group I (regression) yielded a poor overall classification percentage of 63.0% (46/73), 52.4% (22/42) was correctly classified in group I and 77.4% (24/31) in group II.

Ki-67 Labelling Index and numerical chromosome aberrations

The mean values and standard deviations of the Ki-67 LI, the fraction of cells with 4 copies of chromosome 1 per nucleus, and of the chromosome index for chromosome 1 (CI#1) for groups I and II are given in table III. In group II the fraction of MIB1 positively stained nuclei (e.g. Ki-67 LI) was more than 2 times greater than in group I ($P<0.001$, Student t-test). Also the CI#1 was significantly greater in group II than in group I ($P<0.001$, Student t-test). Moreover, compared to group I the fraction cells with 4 copies of chromosome per nucleus was significantly greater in group II ($P<0.01$, Student t-test).

Logistic regression model

Using forward likelihood-ratio stepwise logistic regression analysis with Ki-67 LI, CI#1, and the fractions of cells with 0, 1, 2, 3, 4, 5, 6, 7, and 8 copies of chromosome 1 per nucleus a linear predictor (η_1) based on three parameters was identified (data

not shown). The chromosome index for chromosome 1 (CI#1) was the best discriminating biomarker, followed by Ki-67 labelling index and the fraction of cells with 4 copies of chromosome 1 per nucleus (Fr4ISH). This led to the following logistic regression models; $\eta_{(1)}$ was based on the best discriminating biomarkers respectively Ki-67 LI, CI#1 and Fr4ISH, bivariate linear prediction model $\eta_{(2)}$ was based on Ki-67 LI and CI#1. Univariate linear prediction models $\eta_{(3)}$ and $\eta_{(4)}$ were solely based on CI#1 and Ki-67 labelling index, respectively.

$\eta_{(1)}$ = -19.432+10.680·Ki-67 LI+11.044·CI#1-57.511·Fr4ISH
$\eta_{(2)}$ = -11.479+8.766·Ki-67 LI+5.559·CI#1
$\eta_{(3)}$ = -10.853+6.893·CI#1
$\eta_{(4)}$ = -3.712+10.955·Ki-67 LI

The results of the tests validating the models, i.e. Hosmer and Lemeshow Test, the area under the receiver operating characteristics (ROC) curve (AUC) and the Brier score, are listed in table 4. The highest AUC score and the lowest Brier score indicate that model $\eta_{(1)}$ is the best prediction model. Applying the models to the initial cervical smears of group I and group II multivariate logistic regression model $\eta_{(1)}$ generated correct predictions of >90% in both groups, whereas the other 3 models ($\eta_{(2)}$- $\eta_{(4)}$) predicted overall correct group membership substantially less, table IV. This was especially true for the samples in the progressive group II.

Reducing optimistically biased results using the leave-one-out cross-validation procedure in model $\eta_{(1)}$ led to correct classification of 27/31 (87.1%) progressive samples and 39/42 (92.9%) regressive samples, all other models performed less good, table IV.

Validating model $\eta_{(1)}$ using the additional moderate dysplasia samples of the test group, led to correct classification of all 7 samples. Thus, using regression model $\eta_{(1)}$ all smears in the additional group were correctly classified progressive.

Subsequent smears

Since the cervical smears following the initial smear are taken closer to the imaginary endpoint of pro- or regression, the parameters indicating proliferation and chromosomal aberrations will have shown development toward the final diagnoses.

Therefore, the classifiers described in our logistic regression model should perform as good or even better if applied to these consecutive cervical smears.

Table IV Percentage correctly classified per group using the logistic regression models $\eta(1)$-$\eta(4)$ and the results of the test validating the models

				Percentage correctly classified			
Logistic regression model	**H&L Test**	**ROC curve (AUC±SE)**	**Brier score**	**Group I (N=42)**	**Group II (N=31)**	**Cross validation Group I**	**Cross validation Group II**
$\eta(1)$	0.572	0.96±0.02	0.07	95.2%	90.3%	92.9%	87.1%
$\eta(2)$	0.622	0.94±0.03	0.12	92.9%	80.6%	90.5%	80.6%
$\eta(3)$	0.544	0.91±0.04	0.14	95.2%	74.2%	92.9%	74.2%
$\eta(4)$	0.713	0.87±0.04	0.26	78.6%	67.7%	78.6%	67.7%

H&L Test: Hosmer and Lemeshow test; ROC: Receiver operating characteristics; AUC: area under the ROC curve; SE: standard error

Using the results of the biomarkers assessed in the selected 46 subsequent smears (see also Materials and Methods section), we were once more able to assess and validate the logistic regression models. Applying the initial samples of the 46 selected smears to the best predicting model $\eta_{(1)}$, the percentages correctly classified samples were comparable with the percentages in the total group of 73 samples (93% vs 95% and 88% vs 90% for group I and II, respectively). The overall correct classification in these 46 initial smears using the other models was substantially less. Using the biomarkers assessed in the subsequent smears led to correct prediction of all members of the two groups using the prediction models $\eta_{(1)}$, $\eta_{(2)}$, and $\eta_{(3)}$, table V.

HPV positive borderline and mild dysplasia (BMD) smears

In total 38 initial smears indicating BMD were assessed in the present study, 24 ASC-US (i.e. borderline) smears and 14 mild dysplasia smears. Eighteen of the BMD smears (47.4%) were hr-HPV positive, 4 of the hr-HPV positive BMD smears showed progression. Of the 20 hr-HPV negative BMD smears 18 regressed and two showed progression. Applying logistic regression model h(1), to these BMD smears predicted

progression and regression correctly in all hr-HPV positive samples, 4/18 (22.2%) and 14/18 (77.8%), respectively. One of the 20 (5%) hr-HPV negative BMD samples was classified progressive, while actually being regressive. The two hr-HPV negative BMD smears, which showed progression, were correctly classified progressive by the model.

Table V Percentage correctly classified by (multi-)parameter logistic regression models $\eta_{(1)}$-$\eta_{(4)}$ based on initial and subsequent smear

Logistic regression model	Initial smear		Subsequent smear	
	Group I (N=29)	Group II (N=17)	Group I (N=29)	Group II (N=17)
$\eta(1)$	93.1%	88.2%	100%	100%
$\eta(2)$	89.7%	82.4%	100%	100%
$\eta(3)$	48.3%	88.2%	100%	100%
$\eta(4)$	75.9%	76.5%	100%	94.1%

Discussion

This study shows the possibility to predict biological behaviour of cervical lesions using a logistic regression model based on Ki-67 labelling index and numerical aberrations for chromosome 1 assessed in cervical smears. Taking all samples into account, our model containing the proliferation marker Ki-67 LI, the chromosome index of chromosome 1, and the fractions of cells with 4 copies of chromosome 1 per nucleus predicted 40/42 (95.2%) of the regressive lesions and 28/31 (90.3%) of the progressive lesions correctly. Overall, the behaviour of the cervical lesion of more than 93% of the women referred to the gynaecologist as selected in this study could be correctly predicted using the parameters assessed from the smears obtained at intake. In only 5/73 patients the model did not predict the behaviour of the cervical lesion correctly; 2 women were predicted progressive while being regressive (i.e. false positive), leaving 3 patients as false negative (predicted regressive while being progressive). If patient management would be solely based on the best predicting model the false negative results would have possibly lead to under-treatment. After reducing optimistically biased results using the leave-one-out cross-validation procedure, the biological behaviour of 66/73 (90.4%) samples was correctly predicted. Validating the model using the 46 follow-up smears taken subsequent to the initial smear, led to correct prediction of all 17 progressive and all 29 regressive samples. Since the 46 smears were obtained closer to the endpoint of pro- or regression, the parameters assessed were indeed involved in progression. The initial smears of the 7 additional moderate dysplasia samples (test group) were also predicted correctly progressive by the model. Since, the time to progression was short (i.e. <6wks), these initial samples ought to be considered cytologically under-diagnosed CIN 3.

High-risk HPV is associated with an increased risk in the development of cervical carcinoma. Supposing regressive or progressive behaviour of the lesions assessed in this study was predicted solely based on the absence or presence of hr-HPV genotypes, respectively, only 63% (46/73) of the samples would have been correctly classified; 22 (of 42) hr-HPV negative samples were predicted regressive and 24 (of 31) hr-HPV positive samples progressive, whereas 27.4% (20/73) would have been incorrectly predicted to be progressive and 9.6% (7/73) would have been incorrectly predicted to be regressive. This suggests that hr-HPV testing is inappropriate as a prediction marker for progressive behaviour and that this parameter can be merely valued as a risk indicator in case of screening patients susceptible to developing high-grade CIN and/or cervical cancer. Previously, high negative predictive values of HPV DNA tests for identifying high-grade CIN and cancer and the questionable clinical relevance of a single positive hr-HPV test have been extensively reviewed and debated in literature.[27,28]

Previous studies have already shown a significant positive correlation between structural rearrangements of chromosome 1 and CIN lesions[15,16,29,30] and between MIB1 and CIN lesions.[18,19,31] This is the fist study successfully assessing chromosome indexes and Ki-67 labelling index simultaneously in serial sections of cervical smears. Although MIB 1 is suggested an excellent neoplasm proliferation marker and aneusomy for chromosome 1 seems to be a promising surrogate marker for the prediction of biological behaviour of low-grade CIN lesions, the combination of parameters appears an even better method predicting CIN behaviour. Large-scale implementation, e.g. population based screening programmes might however be

restricted. Especially since assessments of Ki-67 LI and of rearrangements of chromosomes are time-consuming, require expertise, are expensive, and need to be performed under strict standardised conditions and laboratory settings. Moreover, the thickness of the dissected sample is influenced by various uncontrollable factors, e.g. barometric pressure. Since, the chromosome index measured in truncated nuclei is dependent on the fraction of the nuclear volume that is enclosed in the tissue section, reliable measurement of CI requires standardised thickness of the assessed sections.[32] Although, all parameters in this study were measured in AgarCyto blocks, assessing the parameters in liquid based cytology (LBC) samples is also possible. MIB-1 for instance has lately shown to be of promising value as surrogate marker in a cervical cancer screening setting using LBC.[33] In addition to a clinical use our multiple-parameter logistic regression model can be used for the validation of and correlation to new biomarkers which might be discovered in the future and are associated with progressive or regressive behaviour of CIN.

However, the model could also be used more practically, i.e. in the triage of women with minimally abnormal cervical cytology, like ASC-US and mild dysplasia (i.e. the borderline and mild dysplasia (BMD) smears). Approximately 5% of the women participating in the population-based screening for cervical cancer are diagnosed having a BMD smear. The management of patients having these minimal abnormal cervical smears has been a clinical problem for a long time.[34] At this moment women with two consecutive BMD smears are referred to the gynaecologist for colposcopic assessment. Since, cytology is not able to predict the pro- or regressive behaviour and HPV DNA testing alone has a too low positive predictive value for identifying high-grade CIN and cancer, additionally assessing HPV in the triage of repetitive BMD smears is recommended by the American Society for Colposcopy and Cervical Pathology (ASCCP) and other (inter)national medical societies. This triage will improve the management of BMD women at risk for developing cervical cancer.[35-37] Further triaging the BMD HPV positive smears using the progression makers described in our model, could improve management even more. Indeed, in the present study 18 of the 38 BMD (47.4%) smears were hr-HPV positive, which is consistent with other studies.[37,38] So, using the proposed hr-HPV triage only 50% of the minimally abnormal cervical smear would be referred to the gynaecologist. In our follow-up 4/18 hr-HPV positive BMD smears (22.2%) showed progression, while the remaining 14 hr-HPV positive smears showed regression. Using the multiple parameter prediction model ($\eta_{(1)}$) all progressive lesions were predicted correctly and only 1 observed regressive lesion was misclassified. This suggests that our prediction model works very well and that subsequent to the hr-HPV triage in borderline and mild dysplasia the hr-HPV positive smears can be assessed using Ki-67 LI and rearrangements of chromosome 1 to reliably predict the behaviour of the lesion. In this (small-numbered) study it would mean that of a total number of 38 BMD smears only 4 (10%) should have been referred to the gynaecologist since the model correctly predicted progression. However, of the 20 hr-HPV negative BMD smears 2 showed progression. According to the ASCCP triage guidelines for hr-HPV in BMD smears these women would not have been referred to the gynaecologist, nevertheless our model did predict these progressive lesions correctly. Since, the general dogma states that progression to ultimately cervical cancer will only occur in hr-HPV positive lesions, the behaviour of the lesions in these two women should however be considered non-progressive.

Our data demonstrate that short-term progressive and regressive behaviour is highly predictable. In case high-risk HPV is detected in a borderline and mild dysplasia smear, one should consider to determine the biological behaviour of the cervical lesion by using proposed the logistic regression model with Ki-67 LI, chromosome 1 aberration parameters. This could significantly improve patient management.

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Detecting and genotyping hr-HPV in cervicovaginal self-obtained samples using dried fluid spots: new possibilities for cervical cancer screening

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Abstract

High-risk human papillomavirus (HPV) testing in cervical cancer screening has a beneficial effect in patient management and can increase the success rate of population-based screening programmes. Women not participating in these programmes have a higher risk of developing cervical cancer. The introduction of cervicovaginal self-sampling might increase the participation rate and can thus reduce cancer incidence. This study assesses the possibility to use self-sampled genital smears subsequently applied to specific filter papers (Dried Fluid Spots) allowing easy storage and transport. Fifty women obtained a self-sampled genital smear, which was applied to a filter paper and assessed for (probable) high-risk HPV genotypes by the HPV SPF_{10} Line Blot 25 assay. The HPV results were compared to a cervical smear taken by a trained physician. Twenty-five (50%) of all self-obtained samples were positive for (probable) high-risk HPV. Of these samples 23 also tested positive in the physician obtained smear. Twenty-four of the 50 samples (48%) were negative to (probable) high-risk HPV genotypes in both self-collected and physician smear. The overall agreement between self- and physician obtained sample was 96% (κ-value: 0.92). This study shows that HPV detection and genotyping in self-obtained genital samples that are subsequently applied to Dried Fluid Spots is very well possible. Moreover, this method shows a high overall agreement with HPV detection and genotyping in physician-obtained cervical smear. Compared to other self-sampling devices the Dried Fluid Spot method is not dependent on liquid storage methods that are potentially inflammable, hazardous and are not always allowed regular mailing.

Introduction

The clinical value of human papillomavirus (HPV) testing is increasingly recognized.[1] If used as primary screening method, HPV assessment has a higher sensitivity and a higher negative predictive value, but a lower specificity for the detection of pre-invasive disease than cervical cytology.[2,3] In the U.S., the Food and Drug Administration (FDA) has authorized high-risk HPV (hr-HPV) assessment in women aged 30 and older for primary screening, in addition to cytological screening, and for the triage of ASC-US smears. The Dutch Pathology association (NVVP) has recently approved additional hr-HPV testing in all follow-up smears after the detection of a first-time borderline or mild dysplasia (BMD) smear. So, high-risk HPV testing already has a beneficial effect in patient management. Most likely this will increase in case hr-HPV assessment replaces cytology as primary screening tool.

Improving the sensitivity of cervical cancer screening methods through the implementation of hr-HPV testing may increase the success rate of population-based screening programmes. Maximizing the participation-rate could improve this success even more.[4] In the Dutch screening programme approximately 70% of the invited women are actually taking part.[5] Tragically, half of the cervical carcinomas are diagnosed in the remaining group of non-responders.[6,7] Cervical cancer incidence would decrease significantly if these non-responders can be reached, providing that they do participate.[4] Women do not respond or participate for various reasons; being afraid of the procedure or the possible diagnosis, having a too busy schedule, or general unawareness. Genital self-sampling could be an easy accessible, user-friendly and timesaving alternative for the physician-based collection of cervicovaginal material.

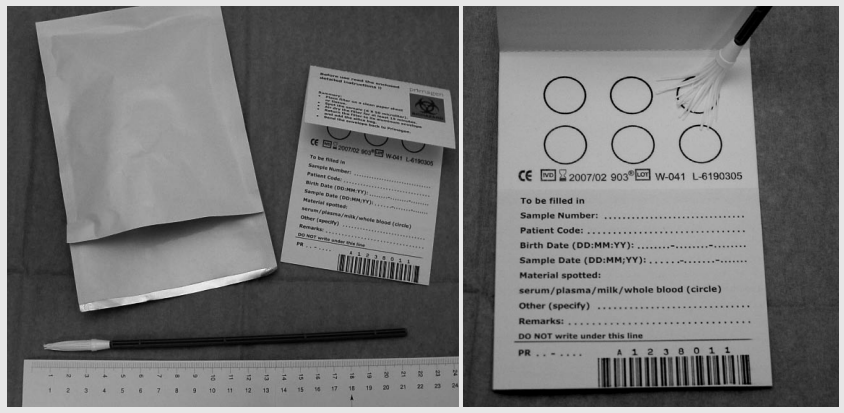
Material from vaginal lavages or self-sampling brushes is highly representative for the cervical HPV status.[8-14] In a review, Brink and colleagues described a study in which 1.6% of the population-based screening non-responders with a valid and hr-HPV positive self-sample test, appeared to have a high-grade cervical intraepithelial lesion.[15] This is significantly higher than the high-grade CIN prevalence observed in a population-based randomized controlled trial for implementation of hr-HPV testing in cervical screening, i.e., the Dutch POBASCAM trial.[16] These results not only show that women who do not participate in a well-organised screening programme

indeed have a higher risk of developing cervical cancer[9,11,15], but also that hr-HPV testing on self-sampled materials might be a promising opportunity to increase the efficiency of population-based screening programmes worldwide.[8,15] Moreover, non-responding women do actually participate in self-sampling projects, leading to an increased participation rate of population based screening programmes and thus a higher rate of success.

The vast majority of studies assessing self-sampling have used methanol-buffered solutions or other liquid transport media in liquid based cytology (LBC) vials, i.e. PreservCyt® solution, and ThinPrep®. Since these samples can be inflammable, hazardous, and infectious, careful handling is required and in The Netherlands regular mailing is not allowed. This severely hampers the introduction of liquid based cervicovaginal self-sampling methods.

In this study we have assessed the possibility to use self-sampled genital smears applied on filter paper, i.e., Dried Fluid Spots (Figure 1) allowing easy storage and transport for HPV DNA testing. The self-sampling material was compared to a cervical smear obtained for diagnostic purposes by a trained physician in the outpatient clinic.

Figure 1 Original Primagen Dried Fluid Spot filter paper with 6 separate spots including return envelope and Viba-brush self-sampling brush. In this study only 1 spot per paper was used per patient



Materials and Methods

The participants were recruited between May and September 2006 at the Department of Obstetrics and Gynaecology of the Radboud University Nijmegen Medical Centre. The patients had initially been referred to the gynaecologist because of two subsequent smears indicating borderline or mild dysplasia (BMD). They had had a colposcopic examination and were followed using an expectative management, allowing spontaneous regression of the lesion.[17] In this expectative management study setting, the patients were cytologically assessed every 6 months for 2 years at our outpatient clinic. Smear specimens were collected using the Cervex-brush® (Rovers Medical Devices B.V., Oss, The Netherlands), and processed using a liquid-based approach (Thinprep®, Cytyc corp. Boxborough MA, USA) that provides monolayer distributions for cytological assessment and the possibility of isolating DNA for HPV detection and genotyping assays. This method has received approval for clinical use from the U.S. FDA.[18,19]

All patients were informed and the participants willing to participate were asked to sign a written informed consent. A specific illustrated explanation was developed to instruct the women on how to obtain a genital self-sample. Briefly, a Viba-brush® (Rovers Medical Devices B.V., Oss, The Netherlands) was inserted 10 cm into the vagina and rotated 5 times. Subsequently the brush was applied to a specific filter paper (Primagen Holding B.V., Amsterdam, The Netherlands), Figure 1. The Dried Fluid Spot was dried to air, placed in an envelope, and sent to the Department of Medical Microbiology for further processing and HPV assessment. After self-sampling, a vaginal speculum was inserted and the physician obtained a cervical smear using a Cervex-brush® (Rovers Medical Devices B.V., Oss, The Netherlands) that was rinsed in a Thinprep® vial (Cytyc corp. Boxborough MA, USA). Regular liquid-based cytological (LBC) examination was performed at the Department of Pathology and 0.5mL LBC medium was used for HPV assessment at the Department of Medical Microbiology. In order to have the samples assessed anonymously and in a blinded approach, all self-obtained samples and cervical LBC samples were sent to the laboratory with a unique code that could only be encoded by the principal investigator.

Specimen preparation LBC

For isolation of DNA from cervical scrapes in liquid-based cytology medium, the MagNAPure LC Isolation station (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was used; 500µL of material was isolated using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim Germany), as described by the manufacturer. With each set of 28 cervical scrape samples 4 negative controls were included. Nucleic acid was resuspended in a final volume of 50µL; 10µL were used for PCR.

Specimen preparation DFS

The dried fluid spots (DFS) were punched out of the filter paper using a sterilised perforator specifically designed for the DFS papers. DNA was isolated using the QIAGEN® DNeasy Tissue Kit (QIAGEN Inc, Valencia, CA, USA), as described by the manufacturer. Subsequently, HPV DNA assessment was performed identically as for the liquid based cytology specimens, as described below.

SPF_{10} Line Blot 25 HPV detection and genotyping

Broad-spectrum HPV DNA amplification was performed using a short PCR fragment assay (HPV SPF_{10} Line Blot 25, Labo Bio-medical products B.V. Rijswijk, The Netherlands).

Table 1 High-risk HPV detection for corresponding genital self-sampled smears and MD obtained cervical smears

MD-sampling		Self-sampling		Agreement, κ-value and 95% CI
		Positive	Negative	
Normal cytology	Positive	8	0	
	Negative	3	19	
	Total	11	19	90%, 0.77 (0.53-1.02)*
BMD cytology	Positive	9	1	
	Negative	0	6	
	Total	9	7	93.8%, 0.87 (0.63-1.12)†
>BMD cytology	Positive	4	0	
	Negative	0	0	
	Total	4	0	100%, 1.00 (1.00-1.00)‡
Overall	Positive	21	1	
	Negative	3	25	
	Total	**24**	**26**	**92% 0.84 (0.69-0.99)†**

Strength of agreement considered *good, †very good, ‡perfect

This assay amplifies a 65-bp fragment of the L1 open reading frame, and allows detection of at least 43 different HPV types.[20,21] The presence of HPV DNA was determined by hybridization of SPF_{10} amplimers to a mixture of general HPV probes recognizing a broad range of high-risk (hr), low-risk (lr) and possible hr-HPV genotypes in a microtiter plate format, as described previously.[21,22]

Twenty-eight oligonucleotide probes which recognize 25 different types were tailed with poly(dT) and immobilised as parallel lines to membrane strips (Labo Bio-medical products B.V. Rijswijk, The Netherlands). The HPV genotypes detectable are hr-HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68/73 and 2 probable hr-HPV types (53 and 66). The HPV genotyping assay was performed as described previously.[23] The LiPA strips were manually interpreted using the provided reference guide. Since HPV assessment in screening and triage is solely based on (probable) high-risk genotypes, the comparison in this study is focussed on these genotypes.

As a quality control for the presence of DNA and absence of PCR inhibitors in the isolated material a ß-globin PCR was performed, as recently described by Snijders and colleagues.[24,25]

In all HPV 16, 18, 31, and/or 33 positive samples the viral load for the individual genotypes was assessed using real time PCR methods as previously described.[24,25] We have indicated the viral load as copies per scrape.

Statistics

All data were analyzed using SPSS version 12.0.1 for Windows. Agreement was measured by absolute agreement and Cohen's kappa statistics, a measure of the agreement between two methods that is in excess of that due to chance.

Results

Fifty women were included in this study. The median age of the women was 35 years (range, 22 to 56). All women were initially referred to the gynaecologist for 2 subsequent equivocal smears, i.e., borderline and mild dysplasia (BMD). The LBC cervical smears taken by the physician showed normal cytology in 60% (30/50) of

the cases, BMD cytology in 16/50 (32%) cases, and in 4/50 cases (8%) the smears appeared to be more severe than BMD.

Of the 50 self-collected genital samples 25 (50%) tested positive for (probable) high-risk genotypes by the HPV SPF_{10} Line Blot 25. Of these 25 samples 23 also tested positive for (probable) hr-HPV in the cervical smear obtained by the physician. Twenty-four of the 50 samples (48%) were negative to (probable) high-risk HPV genotypes in both self-collected and physician smear. In 22 of the 50 (44%) cervical smears obtained by the physician hr-HPV types were detected, 21 cases also tested hr-HPV positive in the self-obtained sample. The overall agreement between self-sampling and the cervical smear taken by the physician for high-risk HPV was 92% (κ-value: 0.84), Table I. Taking both probable high-risk (i.e. 53 or 66) and hr-HPV genotypes into account the overall agreement increased to 94% (k-value: 0.88), Table II.

Table II Probable high-risk and hr-HPV detection for corresponding genital self-sampled smears and MD obtained cervical smears

MD-sampling		Self-sampling		Agreement, κ-value and 95% CI
		Positive	Negative	
Normal cytology	Positive	9	0	
	Negative	2	19	
	Total	11	19	93.3%, 0.85 (0.65-1.05)*
BMD cytology	Positive	10	1	
	Negative	0	5	
	Total	10	6	93.8%, 0.86 (0.60-1.12)*
>BMD cytology	Positive	4	0	
	Negative	0	0	
	Total	4	0	100%, 1.00 (1.00-1.00)†
Overall	Positive	23	1	
	Negative	2	24	
	Total	**25**	**25**	**94% 0.88 (0.75-1.01)***

Strength of agreement considered *very good, †perfect

The agreement of HPV DNA detection between the self-obtained and the physician obtained sample increased with the severity of the lesion detected in the LBC. For the probable high-risk and the high-risk genotypes together the agreement was 93%, 94% and 100% in the 30 smears with normal cytology, the 16 samples with BMD cytology and the 4 samples showing cytology more severe than BMD, respectively. Regarding the defined hr-HPV genotypes the agreement was 90%, 94% and 100%, respectively.

Table III Distribution of HPV-genotypes in genital self-sampled smears and cervical smears taken by the physician

Oncogenic Potential	HPV Genotype	Infection frequency	
		Self-sampling	MD-sampling
High-risk	16	5	5
	18	2	2
	31	5	4
	33	0	2
	35	0	0
	39	1	1
	45	0	0
	51	3	1
	52	3	4
	56	2	2
	58	1	1
	59	2	1
	68/73*	2	2
Probable high-risk	53†	2	1
	66	4	3

*HPV SPF_{10} Line Blot 25 does not distinguish between HPV 68 and HPV 73, since bothtypes are detected by a single probe; †Oncogenic potential of HPV 53 is controversial

The frequencies of the probable high-risk and high-risk HPV genotypes detected by both methods are summarized in Table III. Taking the samples of all 50 women into account, including those negative to probable high-risk and hr-HPV DNA, 40 samples (80%) were concordant, 7 (14%) were compatible, and 3 (6%) samples were discordant. Of the 40 concordant samples 24 showed no (probable) high-risk HPV DNA in both self- and physician obtained samples. The remaining 16 concordant samples were hr-HPV positive and showed identical genotypes in the two samples, see samples no. 1-16 in Table IV. The seven compatible sample-sets, i.e., showing one or more comparative HPV genotypes between the samples, were multiple infections

when considering both samples. In 6 self-obtained and 5 MD-obtained samples a hr-HPV type was detected, see Table IV samples no. 17-22. In 3 cases the MD-obtained sample did contain a specific hr-HPV genotype that was found in the self-obtained sample, and in three other cases the self-obtained sample did not contain a hr-HPV type. In one compatible sample-set only probable hr-HPV types were detected. In the three discordant cases a hr-HPV genotype was detected in either one of the samples, sample no. 23-25 in Table IV. In two samples the MD-smear did not contain a hr-HPV type in contrast to the self-obtained sample. Whereas in one sample-set only the MD-obtained sample contained a high-risk genotype.

The average ß-globin of the self-obtained samples was 4.5E+4 (±3.0E+4), whereas the average ß-globin of the samples taken by the physician was 6.4E+3 (±6.1E+3). This difference was statistically significant P<0.001 (Unpaired t-test).

Table IV gives a summary of the 25 sample-sets that contained at least one high-risk HPV genotype in either one or both samples, 16 concordant samples, 6 compatible, and 3 discordant sample-sets. In case a sample contained HPV 16, 18, 31, and/or 33 the viral loads per scrape per genotype are given. Although numbers of samples with a viral load were too small for proper statistical analysis, the loads show little variance between the self-obtained and the MD obtained sample in case the viral loads could be detected. In five samples however, the viral load was too low for detection using the real-time PCR method and could thus not be detected. In four cases it concerned a genotype which was only detected in either one of the samples and in 1 case it concerned HPV 31 which was detected in both genital-self and MD obtained sample.

Discussion

Several studies have already shown that cervicovaginal self-sampling is highly representative for the HPV status of the cervix.[8-14] Despite a difference in the self-sampling methods, all these earlier studies have used a liquid sample storage and transport medium. Due to legislations the logistics for these potentially hazardous liquid-based techniques will be difficult to organize and therefore expensive. Implementation of liquid-based at-home self-sampling of population-based screening non-responders might thus be delayed or even impossible.

The present study shows that HPV detection and genotyping in self-obtained genital samples that are subsequently applied to dried fluid spots (DFS) is very well possible. Moreover, this method shows a high overall agreement with HPV detection in physician-obtained cervical LBC. All but three DFS hr-HPV positive samples were also hr-HPV positive in the MD-smear, whereas only one DFS hr-HPV negative sample appeared to be hr-HPV positive in the MD-smear. Leading to a overall agreement between the two methods of 92%, and 94% taking the high-risk and probable hr-HPV genotypes into account.

The HPV SPF$_{10}$ Line Blot 25 test has previously shown good concordance with various other assays.[23,26,27] However, this assay is known for its high analytical sensitivity level, i.e. the test result is positive even if a little amount of HPV copies is present. This feature might explain the undetectable viral load in some samples found positive for certain HPV genotypes by the assay. Since, a high analytical sensitivity is not equal to a high clinical sensitivity or clinical relevance[28], genital self-sampled dried fluid spots ought to be assessed using other commercially available HPV detection and genotyping tests with lesser analytical sensitivity as well, e.g. Hybrid Capture II. Although the viral load could not be determined for all genotypes, the agreement between the samples in this study would be higher when excluding the samples with a known undetectable viral load, 96% κ-value of 0.92 (95% CI 0.81-1.03).

Also high concordance between specific HPV genotypes assessed in the self-sample and the cervical smear by the physician signifies that the samples self-obtained by the patients are representative for the genotypes that infect the cervix. Moreover, the amount of ß-globin found was significantly higher in the self-obtained samples (t-test, P<0.001), implying that limited cell count and adequacy in self-obtained samples is no limitation. The higher ß-globin might be due to the fact that the cervico-vaginal self-sampling brush with unknown volume was directly applied to the DFS, whereas the smear obtained by the physician was rinsed in 20 mL LBC and homogenised before analysis, as the procedure requires. The difference could also be explained by the fact that 500 µL homogenised LBC is used for DNA isolation, while the complete DFS paper was processed.

In four of the 7 compatible samples additional genotypes were detected in the self-obtained samples. These additional types could arrive from the vaginal epithelium

which might be infected with other HPV genotypes. Especially self-sampling with brushes is susceptible to obtaining cells different than cervical epithelial cells. The impact of these vaginal or non-cervical localized genotypes in the pathogenesis of cervical dysplasia is unknown.

Table IV Overview of the sample-sets containing at least one hr-HPV genotype in either one or both samples including viral load for HPV 16, 18, 31, and/or 33

	HPV genotype (HPV SPF$_{10}$ Line Blot 25 genotyping test)		Concordant compatible discordant	Viral load (16, 18, 31, or 33) (copies/scrape)			
	Self-sampling	MD-sampling		Self	MD	Self	MD
1	31	31	Concordant	1.5 • 10^7	8.1 • 10^7		
2	52	52	Concordant				
3	18	18	Concordant	2.7 • 10^7	7.4 • 10^6		
4	68/73	68/73	Concordant				
5	18	18	Concordant	2.0 • 10^6	1.7 • 10^4		
6	56, 66	56, 66	Concordant				
7	16, 53	16, 53	Concordant	5.9 • 10^5	1.1 • 10^5		
8	16	16	Concordant	2.7 • 10^6	3.5 • 10^5		
9	16	16	Concordant	7.9 • 10^4	1.6 • 10^6		
10	58	58	Concordant				
11	56	56	Concordant				
12	39	39	Concordant				
13	59	59	Concordant				
14	52	52	Concordant				
15	68/73	68/73	Concordant				
16	51	51	Concordant				
17	31	31, 52	Compatible	7.7 • 10^4	9.6 • 10^2		
18	16, 59, 66	16	Compatible	5.0 • 10^4	2.8 • 10^5		
19	51, 66	66	Compatible				
20	16	16, 31	Compatible	5.0 • 10^5 (HPV 16)	1.2 • 10^4 (HPV 16)		LND (HPV 31)
21	31	31, 33	Compatible	LND (HPV 31)	LND (HPV 31)		1.9 • 10^4 (HPV 33)
22	31, 52	52	Compatible	LND			
23	31	-	Discordant	LND			
24	51	-	Discordant				
25	-	33	Discordant		LND		

LND: viral load not detectable

The dried fluid spots used in this study are neither hazardous nor inflammable; applying genital scrapes on these filter papers can solve storage and transportation problems. Since this technique could be applicable to at-home self-sampling, it could imply an incentive for decreasing non-compliance of the population-based screening. Especially women not participating due to fear might be persuaded to actually participate. Moreover, since self-sampling can be done by the patient and only the women who are persistently hr-HPV positive ought to be examined, less physicians and MD-time is needed, which will eventually reduce costs.

Dried fluid spots (i.e. dried blood) have already been used for decades in the postnatal screening of certain congenital disorders and diseases. Dried blood spots have also been successfully used in studies detecting and genetically characterizing measles virus strains.[29] Additionally, at-home collection for HIV testing using DFS has been considered feasible and acceptable in a high-risk cohort[30], but also viral load and genotypic-resistance assessments in HIV-positive patients appear to be possible. The air-dried samples are stable at room temperature for months up to years and various studies have demonstrated conserved stability of virus particles under suboptimal conditions using filter papers.[29,31,32] Cervical or genital samples obtained in developing countries could therefore be spotted on DFS filter paper and send to designated central laboratories for analysis. This could simplify the introduction of organised HPV-based cervical screening programmes in developing countries as well.

Regarding to cytological assessment the existing self-sampling methods have repetitively shown to be no alternative for the classic Pap smear.[8,11] Also, the DFS self-sampling method is unsuitable for cytological cervical cancer screening.

In conclusion, HPV detection and genotyping on self-sampled cervicovaginal samples using a Viba-brush®, and dried fluid spots is very well possible and the results are highly representative for the cervical HPV status. The dried fluid spot technique is non-hazardous and the samples are allowed regular mailing. This suggests that the DFS method might be applicable to at-home self-sampling in the population-based screening non-responders, for the introduction of a primary HPV-based cervical cancer screening, and for establishing cervical cancer screening programmes in developing countries.

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General discussion

Reviews in Medical Virology 2007, in press (combined with chapter 1)

Managing abnormal cervical cytology

In developed countries well-organised cervical cancer screening programmes, based on Pap (Papanicolaou) smears, have resulted in a significant decrease in the incidence of cervical carcinomas. For decades, however, the management of cervical cytological abnormalities has been subject for debate. Thereby, important issues are addressed such as (i) the progressiveness of cervical lesions and (ii) factors influencing the success rates of organised screening-programmes.

(i) The majority of all cytological abnormalities detected through screening are not progressive in behaviour and will not lead to cervical carcinoma. However, those susceptible to malignant progression should be detected in an early, pre-malignant stage. Previous studies suggest that additional biomarkers like human papilloma-virus (HPV) can be valuable in the early-detection of the pre-malignancies that are truly progressive. Indeed, in chapter 6 of this thesis we have shown that a logistic regression model based on a combination of biomarkers indicative for proliferation and numerical chromosome aberrations (i.e., Ki-67 labelling index and chromosome indexes, respectively), can correctly classify the biological behaviour in a high percentage of HPV-positive equivocal smears.

(ii) The success rate of screening-programmes on the other hand highly depends on the patients' participation rate, the experience of physicians and cytologists, and the sensitivity and specificity of the tests used. Improving these features will ultimately lead to a higher success rate. Improving patient information on cervical cancer, its precursors, its risk factors, and the necessity of early detection, for instance, could encourage the screening programmes' non-responders to participate.[1] Whereas, training of involved medical professionals will increase their experience and awareness, and subsequently the overall success rate of the programmes. The interpretation of cervical cytology however remains highly subjective and susceptible to disturbing factors such as fatigue. The low sensitivity of conventional cytological tests for the detection of high-grade CIN can be increased through the introduction of liquid-based cytology (LBC). This technique has also shown to significantly reduce unsatisfactory smears, it is faster and more efficient, and the interpretation of LBC is associated with fewer screening errors.[2,3] Furthermore, it offers the opportunity for other diagnostic analyses, e.g., HPV and P16 assessment.

The success rate of screening-programmes could benefit even more by assessing high-risk HPV (hr-HPV) in cervical smears. After all, the clinical value of HPV DNA testing in the management of cervical cancer is increasingly being acknowledged[4], and the sensitivity of hr-HPV testing for detecting high-grade CIN or worse is 20-40% higher compared to cytology. Specificity however lags behind, being 5-10% lower than that of cervical cytology[5,6], so HPV-positive women could be falsely judged having a high-grade CIN based on HPV detection alone.

Only recently the Dutch Pathology association (NVVP) followed the health-care policies from other countries by approving additional high-risk HPV testing. All smears persistently (i.e., after a 6 months interval) showing borderline and mild dysplasia (BMD), and, even the BMD smears that have normalized after 6 months, are subjected to hr-HPV detection, see also the flowchart of the Dutch Pathology association (Figure 3) in the first chapter of this thesis. In case of a negative hr-HPV test, the patient suffering a persistent BMD smear is not referred to the gynaecologist, whereas, the patient with the normalized smear can return to the regular cervical screening programme. This triage is solely designed for the follow-up management of equivocal smears from the population-based screening-programme and not for first-time equivocal smears within the screening programme. The main reasons for this 'negative triage' of follow-up smears are to reduce the number of repeated cytological smears and the colposcopy referrals.

Implementation or integration of immediate HPV detection in the screening-programme is not yet issued. Hence, a major question to be addressed in the near future is whether hr-HPV testing should be used as an immediate triage-tool for first-time BMD cytology in the regular population-based screening, thus without awaiting the repeated cytology after 6 months. Since logistical and technical issues will not be the major problem in any highly developed country, sophisticated analyses deliberating costs, effectiveness, and health-care benefits of such an immediate hr-HPV triage ought to answer this question.

After implementation of hr-HPV testing in addition to cytological screening, a population-based screening programme entirely based on hr-HPV detection is suggested to be the next rational step. Major concern regarding primary and exclusively HPV testing is the expected higher referral rate for colposcopy due to its

low specificity. Therefore high-risk HPV positive women should be reassessed after 6 months and only in case of hr-HPV persistence the women will undergo a cytology triage. Considering primary HPV-testing, cervicovaginal self-sampling should be addressed as well. Various studies have shown that hr-HPV detecting in self-obtained samples is highly concordant to HPV assessments in cervical smears. Implementing these techniques has shown to encourage a large group of non-responders to participate in the population-based cervical cancer screening.[7]

Regardless the primary or triage position of HPV assessment in the population-based screening policy, the importance of genotyping genital HPV infections has to be addressed as well. Distinguishing equivocal smears that are HPV 16 positive from smears positive for any other hr-HPV genotype, for instance, is already regarded essential since, HPV 16 positive equivocal smear have a 30-fold higher risk for developing high-grade CIN or worse than those infected with any other hr-HPV genotype.[8] Moreover, the association between hr-HPV persistence and high-grade CIN development is based on genotype specific persistence, consequently a 'hr-HPV on/off' detection does not sufficiently cover the need and adequate HPV genotyping is necessary. Also, the significance of infections harbouring multiple genotypes - suggesting repetitive exposure and association with an increased risk for progressive disease- remains undetermined and can merely be challenged using assays that are able to detect and distinguish multiple genotypes in a sample in a single run. Chapters 4 and 7 of this thesis specifically studied and compared the presence of HPV genotypes in various cervical samples and could not have been performed without an accurate genotyping test. All these issues are considered prospects for HPV genotyping. At the 23rd International Papillomavirus conference it was shown that in addition to the clinical use for genotyping assays, epidemiological studies - essential in addressing the burden of HPV infections- can only be performed using accurate and reproducible genotyping assays.[9] The value of individual genotyping assays can merely be assessed through comparative studies. In chapter 5 of this thesis two commercially available genotyping assays (SPF_{10} INNO LiPA and the Roche LINEAR ARRAY) were compared and considered highly equivalent.

For both HPV detection assay and genotyping assay the clinical and the analytical relevance are going to play a significant role in the ultimate decision which test to use for future screening and/or triage purposes.[10]

Nevertheless, even if the ultimate hr-HPV test or genotyping assay, with high sensitivity, specificity, and positive and negative predictive value, is used in addition to the ideal algorithm, merely the developed countries -since money and logistics are available- will take advantage of this improvement in patient management. The benefits that can be yielded from less significant and cheaper advances in healthcare are relatively higher in developing countries than in the developed ones. In India, Kenya, and Peru for instance three lifetime hr-HPV tests could reduce the lifetime risk of cervical cancer by 60%. Whereas shortening the cytology-screening interval from 3 to 2 years in well-developed countries would lead to a reduction in lifetime risk of cancer from 80% to approximately 88%. The costs of this latter improvement would however be 20-fold higher per year of life saved compared to the first development.[11]

Another example of less significant, low cost, and easy applicable development in cervical cancer prevention is the use of genital self-sampling as described in chapter 7 of this thesis. The self-sampling method using dried fluid spots has shown to be highly concordant to HPV assessments in cervical smears. In developed countries this and other methods could encourage population-based screening non-responders to participate in a more anonymous setting and might increase the availability of cervical cancer prevention in developing countries.

Secondary prevention in the form of a well-organised cervical cancer-screening programme is, and probably will however remain unavailable in most of the developing countries. Therefore, in addition to various therapeutic interventions (i.e. tertiary prevention), prophylactic vaccination (primary prevention) may provide a convenient alternative conquering HPV associated genital neoplasia, hereby refraining from an organised screening programme.

HPV vaccination

Early data already suggested that L1 virus-like particles (VLP) vaccines can potentially reduce worldwide cervical cancer rates. In all phase II trials the different intramuscular administered vaccines available for testing were well tolerated and no serious vaccine-related adverse events were reported.[12-15] The vaccines have thus met the

demands of an ideal vaccine that is generally administered to individuals who would have never had developed cervical cancer even without the vaccine. It concerns a monovalent hr-HPV 16 vaccine[14], a bivalent vaccine against hr-HPV 16 and 18[12,13] and a quadrivalent vaccine against lr-HPV 6 and 11 and hr-HPV 16 and 18.[15] The generated immune responses are robust, durable, of 20-100 times greater magnitude than those caused by a natural infections. Persistent HPV infections were prevented in 89-100%, whereas the effectiveness against incident infections in young women was approximately 90% for HPV 16 and 18 (see for review Quint 2006).[16] In HPV positive women the HPV vaccine does not seem to speed viral clearance (A. Hildesheim, R. Herrero, Abstr. 23rd IPV Conference, abstr. PL1-1, 2006). The quadrivalent vaccine also seems to prevent abnormal cytology, CIN lesions, and even pre-malignant vaginal and vulvar lesions (J. Paavonen, Abstr. 23rd IPV Conference, abstr. PL1-2, 2006).

Recently, a significant cross protection against other high-risk HPV genotypes, i.e. HPV 31 and 45 has been observed.[13] Since HPV 16 and 18 together account for 69.8-77.1% of the cervical cancers worldwide, cross protection of multivalent vaccines could lower the worldwide incidence of cervical cancer by an additional 8%.[16] Phase III studies however should definitively elucidate these questions. Although, the protectiveness of the vaccine is promising and has shown to be effective for at least 48 months[13], true long-term protection and effectiveness, re-boost strategies, and cost-effectiveness need to be studied in large-scale trials with long-term follow-up. Based on the published randomized controlled trials regarding the HPV vaccines, health policy makers recommended vaccinating young adolescents prior to the onset of their sexual active life, in order for a vaccination programme to become successful. Taira and colleagues calculated that in case i) the HPV 16/18 vaccine reduces the chance of an infection by 90%, ii) only 12 year old girls are vaccinated, and iii) the conventional screening is continued, the risk of developing cervical cancer would decrease by 89%.[17] In contrast, the lifetime risk for cervical cancer is reduced by 58-70% in the current 5-yearly Dutch screening protocol.[1,18] Broadening the vaccination age will reduce HPV related disease even more, but will also increase costs. Providing the per-woman cost of the vaccine is less than $25, mathematical models have predicted that prophylactic vaccination will be cost-effective, and might even be cost saving.[19] In countries with well-organised secondary prevention programmes, screening policies should be modified considerably in order to generate

a cost-effective cervical cancer prevention management after the introduction of HPV vaccines.

Besides effectiveness of vaccines, approval of national governments and registration authorities are important issues to be addressed when introducing the vaccine. Also, ethical considerations will presumably be more pronounced in case of a vaccination for a STD. Despite extensive research on the effectiveness of the various vaccines, and the approval of the quadrivalent vaccine for women aged 9-26 by both the FDA and the European Commission, neither the Dutch Minister of Health nor the Dutch National Institute for Public Health and the Environment (RIVM) have yet taken position concerning this issue (personal communication).

As the HPV vaccines will be implemented as a primary prevention for cervical cancer, the suggested vaccination policies are predominantly focussed on girls and young women. However, since most young adolescents are not yet aware of their sexual preferences, i.e. hetero-, homo-, or bisexual, vaccination programmes should also consider targeting boys before HPV-exposure.[20] According to Meijer and colleagues, the prevention of penile lesions through vaccination would result in a considerable reduction of viral transmissibility to sexual partners, consequently lowering HPV related disease (C.J. Meijer, M.C.G. Bleeker, F.J. Voorhorst, P.J.F. Snijders, Abstr. 23rd IPV Conference, abstr. PS 26-4, 2006). Moreover, in addition to cervical cancer, hr-HPV 16 and 18 are also the most frequently detected genotypes in anal carcinomas, anal sexual activity is practised in at least 33% of the heterosexual and 80% of the male homosexual couples, and the incidence of anal carcinoma is rising.[20] Although less frequently occurring, HPV 16 may also be aetiologically important in tonsillar cancerous development[21], and may represent an alternative pathway in carcinogenesis to the established factors of tobacco smoke and alcohol (ab)use.[22]

Sexual activities and sexual preferences are in general non-beloved and frequently avoided conversation and discussion issues. This is presumably why HPV knowledge and the awareness of the association with cervical cancer is extremely low in the general population.[23] In contrast to the quadrivalent vaccine which is marketed as a vaccine against HPV, the bivalent vaccine will be promoted as a primary prevention of cervical cancer. Giving recipients full information about the vaccine's purpose will also necessitate teaching them about sex, the sexual viral transmissibility, and the

oral and genital cancer risks. Educating health-care workers is highly necessary as well (S. Moorthy, Abstr 23rd IPV Conference, abstr PS1-6, 2006).

The post-vaccination era of cervical cancer screening

The introduction of HPV vaccines will have a global impact as a preventive strategy for cervical cancer.[6] Ultimately, successful prophylactic vaccines will make secondary preventative efforts by means of cervical screening redundant, and thus cause reallocation of money from secondary prevention to primary prevention. But even with a high vaccination rate, a reduction of cervical cancer burden is not expected for at least 20 years. Moreover, although two prophylactic vaccines have shown cross-protection for other HPV genotypes than originally designed for, protection does not include all high-risk genotypes. On the contrary to the 100% efficacy found in the vaccination phase II studies, Franco and colleagues recently suggested that the protection for the targeted types cannot be expected absolute.[6] Taken the above and the vaccine non-responders into account, screening for pre-malignant CIN cannot be discontinued after the introduction of the first generation vaccines.

On June 8, 2006 the U.S. FDA approved Merck's quadrivalent vaccine GARDASIL™ for 9- to 26-year old girls and women. The European Commission licensed the same vaccine, now by Sanofi Pasteur MSD (a joint venture between Sanofi Pasteur and Merck & Co.), on September 22 for the 25 member countries of the EU. GlaxoSmithKline's bivalent Cervarix™ will most likely follow late 2007 targeting the same age group. Consequently, it will take decades before the true effects in this entire cohort can be verified. And since group immunity will definitely not occur, women aged 27 and above can -at the start of the vaccination programme- never benefit directly from the programme, they will be compelled to the regular cervical screening programmes. These women might also benefit from "catch-up" immunization even if they have already been infected with a genotype included in the vaccine. Large trials in women aged 24 and older are currently conducted and will address these issues.

Clearly the conventional population-based cervical cancer-screening programmes will not be abolished in the short run. But concerning the future, these programmes ought to be thoroughly re-evaluated, an aspect which was largely neglected in the studies regarding prophylactic HPV vaccinations. In the post-vaccination era a decrease in the prevalence of cervical abnormalities will ultimately lead to a decrease of the positive predictive value of cervical cytology. This will successively cause an increase in false-positive diagnoses in women who screen positive, resulting in unnecessary referrals[6] and increased anxiety.[24] Addition of HPV testing could be beneficial for the efficacy of the existing approach of many screening programmes[10], in pre- and post-vaccination eras. But adding an extra assay and a vaccine will be too costly for public-health budgets in numerous countries. Whether primary HPV assessment is efficient and cost-effective in an HPV vaccinated population, should be one of the research priorities for the near future.

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Summary

Chapter 1

Cervical cytological pathology is not uncommon. Prevention of cervical cancer by detection of the disease in an early and pre-malignant stage is practised globally either through population-based screening programmes or more optimistically non-organised ones. High-grade cervical intraepithelial neoplasia (CIN) detected by cervical cytological screening are extensively visualised by colposcopy and successively treated by, for instance, large loop electro-surgical excision of the transformation zone (LLETZ). Persistent infections with certain high-risk human papillomavirus (hr-HPV) genotypes play a significant, but not determinative, role in cervical cancer carcinogenesis. The HPV-induced oncogenesis is suggested to be a multi-step process in which the integration of viral DNA in the human genome is the established key-event. Next to the assessment of markers indicative for cell-proliferation and parameters related to aberration of chromosome aberration, are the detection and genotyping of HPV infections in cervical smears also suggested to become highly established tools in assessing the risk of progression of cervical abnormalities. Apart from the above, features such as the clinical and analytical sensitivity of a HPV test and the sense and feasibility of genital self-sampling are discussed in chapter 1.

Chapter 2

Genital infections with human papillomavirus (HPV) are amongst the most common sexually transmitted diseases (STD) worldwide. In the vast majority of adolescents and young adults an HPV infection is transient and will not lead to detected cervical abnormalities. Another highly prevalent STD, *Chlamydia trachomatis*, is more prevalent in subfertile women than in the general population and the leading cause of tubal factor subfertility. One could hypothesise that since the prevalence of *C. trachomatis* is higher in subfertile women, these women are more likely to have HPV-induced cervical abnormalities compared to the general population. Chapter 2 describes a retrospective case-control study assessing the smears of 669 cases, i.e. women visiting the fertility clinic for *in vitro* fertilisation (IVF), and 77055 women attending the population-based screening programme for cervical cancer, i.e. the controls. Significantly more cases had an abnormal cervical smear compared to the controls. The chance that a high-grade cervical lesion is detected is a woman eligible for *in vitro* fertilisation is twice as high compared to a woman in the general population. Since cytological abnormalities are induced or enhanced by highly prevalent STDs that are related to promiscuous precarious sexual activity, taken a cervical smear, as part of the fertility screening in women, should be common practice, most certainly before they undergo expensive IVF treatment.

Chapter 3

Early treatment of cervical intraepithelial neoplasia (CIN) significantly reduces the risk of invasive cancerous progression of the lesion. One of the most widely used methods effectively treating high-grade CIN is the electro-surgical large loop excision of the transformation zone (LLETZ). This procedure has a high success rate, while maintaining anatomy and function of the cervix. Chapter 3 gives an overview of 22 years experience in treating high-grade CIN and the subsequent cytological follow-up as performed at our outpatient clinic. The vast majority of the women with a high-grade cervical lesion have shown to be adequately treated, since the follow-up of at least 2 years showed no repetitive lesion. In less than 10% of the women a high-grade repetitive lesion was detected and subsequently treated. In 80% these repetitive lesions recurred within 2 years and were therefore considered residual CIN. The remaining 20% repetitive high-grade CIN were regarded as recurrent lesions. More than half of the recurrent lesions appeared more than 5 years postoperatively, suggesting that adequate and long-term cytological follow-up is compulsory. Although not assessed in the present study, previous studies have recommended using hr-HPV detection as a more sensitive follow-up tool.

Chapter 4

Chapter 3 has already shown that the LLETZ is a well-established treatment for high-grade CIN. It has also been postulated that LLETZ is responsible for the elimination of HPV, the infectious agent causing CIN. Most studies on HPV detection after a LLETZ procedure have merely focussed on the persistence of high-risk (hr-)HPV to identify women at risk for residual or recurrent disease. The appearance and or significance of new hr-HPV types detected after treatment has not been extensively studied. The presence of hr-HPV in 85 high-grade squamous cervical LLETZ biopsies and in the first follow-up smear was determined. In 94% of the LLETZ biopsies hr-HPV was detected compared to 35% hr-HPV positive follow-up scrapes. Twenty of the 80 hr-HPV positive women (25%) had the same hr-HPV genotypes in their follow-

up smears as was found in the corresponding biopsies. In the follow-up smear of 13 women a new hr-HPV genotype was detected and HPV 18 was newly detected in 8 of them. The remarkably high presence of newly detected HPV 18 genotypes may argue for a release or re-activation of this virus from proximal layers of the cervical canal incised during surgery.

Chapter 5

Accurate genotyping of human papillomavirus (HPV) is important in order to i) monitor the efficacy of multivalent vaccines and surgical treatment, ii) study the epidemiology of HPV infections worldwide, and iii) assess the oncogenic potential of high-risk HPV genotypes. Various HPV detection and genotyping assays have been developed to meet this demand. In chapter 5 the Roche LINEAR ARRAY a recently developed genotyping assay based on a well-known primer set (PGMY 09/11) is compared to a more established and highly sensitive genotyping assay (the SPF_{10}-INNO LiPA). The samples used for comparison showed identical results in both assays in 81% of the cases. In 11% of the samples the tests showed comparable but not identical results, whereas in 8% no resemblance was observed. The differences found could be attributable to a variation in analytical sensitivity for certain genotypes in the assays used or to low copy numbers in particular samples. Despite the differences, the two assays can be regarded highly comparable and reproducible.

Chapter 6

High-grade pre-malignant cervical lesions are effectively treated by LLETZ (chapter 3 and 4). However, not every high-grade CIN would have progressed towards cervical carcinoma if left *in situ*. Biological behaviour, i.e. progression and regression, of CIN cannot be predicted using conventional cytology or histology. Since HPV integration into the human genome marks the onset of carcinogenesis, parameters as HPV or makers indicating proliferation, genetic instability and chromosomal aberration may be of predictive value assessing the progressive and regressive behaviour of CIN. In chapter 6 logistic regression models based on the progression marker Ki-67 labelling index (MIB1) and chromosome aberration parameters (chromosome index and aneusomy) for chromosome 1 are used to study the biological behaviour of CIN lesions. Patients referred to the gynaecologist having two consecutive equivocal smears were assessed for this study. In the in-take smears of 42 women showing regression and 31 women showing progression (maximal CIN 3) in the follow-up the above-mentioned parameters were assessed. A multi-parameter logistic regression model containing the parameters Ki-67 labelling index, chromosome index for chromosome 1, and the fraction of cells with 4 copies of chromosome 1 per nucleus appeared to be the best predicting model, classifying 93.2% of the samples correctly (AUC 0.96±0.02). If applied to women with a borderline or mild dysplastic smear who are hr-HPV positive the logistic regression model correctly triaged the ones showing progression. So, short-term pro- and regressive behaviour is highly predictable using a model combing these parameters. Although laborious, the model can be useful further triaging hr-HPV positive women with equivocal smears, reducing colposcopy referral rate even more.

Chapter 7

Previous research has undoubtedly shown that hr-HPV testing in cervical cancer screening has a beneficial effect in patient management. Moreover, it is suggested to increase the success rate of population-based screening programmes. Since women who do not participate in these programmes have a higher risk of developing cervical cancer. The introduction of cervicovaginal self-sampling might increase the participation rate and can thus reduce cancer incidence. In chapter 7 we have assessed the possibility to detect and genotype (probable) hr-HPV-DNA using self-sampled genital smears that were subsequently applied to specific filter papers. These filter papers allow easy storage and transport. The HPV results were compared to a cervical smear taken by a trained physician using the HPV SPF_{10} Line Blot 25 assay. The overall agreement between self- and physician obtained sample was 96% (κ-value: 0.92). This study shows that HPV detection and even genotyping in self-obtained genital samples using filter papers is very well possible. Compared to other self-sampling devices the filter paper method is not dependent on liquid storage methods that are potentially inflammable, hazardous and are not always allowed regular mailing.

Chapter 8

The last chapter focuses on the importance of the early-detection and the management of abnormal cervical cytology, the problems associated with it (sensitivity and specificity issues), and possible solutions for these problems in the

near future (implementing additional or new bio parameters). Moreover, this chapter briefly issues the introduction of the recently approved HPV vaccines and the impact this might have on HPV associated cancers. These vaccines will most certainly have a global impact as a primary preventive strategy for cervical cancer. This emphasizes the importance of cervical cancer screening in the post-vaccination era.

Samenvatting

Hoofdstuk 1

Afwijkende cervix cytologie is een omvangrijk probleem. Over de gehele wereld wordt getracht baarmoederhalskanker (cervix kanker) te voorkomen door de ziekte in een vroeg en nog goedaardig stadium te detecteren. Dit geschiedt veelal in een door het land/de regio georganiseerd preventief bevolkingsonderzoek, maar ook door minder grootsopgezette, meer opportunistische detectie worden afwijkingen van de cervix gevonden. De niet-kwaadaardige cervix afwijkingen, ook wel hoog-gradige cervicaal intra-epitheliale neoplasiën (CIN) genoemd, worden meestal opgespoord door middel van uitstrijkjes, vervolgens uitgebreid in kaart gebracht door middel van een kolposcopisch onderzoek en indien noodzakelijk behandeld door een diathermische lisexcisie van de overgangszone. Aanhoudende infecties met bepaalde hoog-risico humaan papillomavirus (HPV) genotypen spelen een belangrijke, maar niet alles bepalende, rol in de carcinogenese van het cervixcarcinoom, ofwel de ontwikkeling van baarmoederhalskanker. De door HPV geïnduceerde carcinogenese is met zekerheid een proces met meerdere elkaar deels overlappende fasen. Hierbij is voor de versmelting (integratie) van het virale genetische materiaal met het humane materiaal de een hoofdrol weggelegd. Naast diverse testen (b.v. celproliferatie markers en parameters welke afwijkingen in chromosomen kunnen bepalen) welke indirect de voortgang van de carcinogenese kunnen nagaan, zullen methoden om HPV te detecteren en te genotyperen in de nabije toekomst zeer belangrijk worden om een progressie risico-inschatting van bepaalde cervix afwijkingen te maken. Naast het hierboven geschrevene, zullen aspecten als klinische en analytische gevoeligheid van HPV testen, en de zin en mogelijkheden van genitale HPV zelfsampling aan de orde komen in hoofdstuk 1.

Hoofdstuk 2

Genitale humaan papillomavirus (HPV) infecties zijn een van de meest voorkomende seksueel overdraagbare aandoeningen (SOA) wereldwijd. Bij de meerderheid van de adolescenten en jong volwassenen is deze infectie van voorbijgaande aard en zal ze niet leiden tot detecteerbare afwijkingen van de baarmoederhals. Een andere veel voorkomende SOA is *Chlamydia trachomatis*. *Chlamydia* wordt vaker gevonden bij subfertiele vrouwen dan bij vrouwen in de algemene bevolking en is in veel gevallen de veroorzaker van verklevingen in de eileiders hetgeen leidt tot “tubafactor

subfertiliteit". Hypothetisch kan het zijn dat gezien de hoge prevalentie van *C. trachomatis*, subfertiele vrouwen vaker HPV geïnduceerde cervix afwijkingen hebben in vergelijking met vrouwen uit de algemene bevolking. Hoofdstuk 2 beschrijft een retrospectieve studie waarbij de uitstrijkjes van 669 subfertiele vrouwen in aanmerking komend voor *in vitro* fertilisatie (IVF) en 77055 vrouwen uit het bevolkingsonderzoek screening voor baarmoederhalskanker werden onderzocht. Het blijkt dat de subfertiele vrouwen beduidend vaker een afwijkend uitstrijkje hadden dan de vrouwen uit de algemene bevolking. De kans dat een hoog-gradige cervicale intraepitheliale neoplasie wordt gevonden in een vrouw die in aanmerking komt voor IVF is twee maal zo hoog als voor een vrouw in de normale bevolking. Cytologische cervix afwijkingen worden geïnduceerd dan wel bevorderd door veel voorkomende SOA's welke op hun beurt worden veroorzaakt door onveilig en promiscue seksuele activiteiten. Het is derhalve raadzaam om bij elke vrouw die wordt gescreend in het kader van een oriënterend fertiliteitonderzoek een uitstrijkje te maken, vooral bij degenen die kostbare IVF behandelingen zullen ondergaan.

Hoofdstuk 3

Vroegtijdige behandeling van CIN reduceert de kans op progressieve ontwikkeling van deze afwijking tot cervixcarcinoom aanzienlijk. Een veel gebruikte behandelingsmethode is de diathermische lisexcisie van de transformatiezone (Eng.: Large Loop Excision of the Transformation zone, LLETZ). Deze methode is erg succesvol en behoudt de anatomie en functie van de cervix. Hoofdstuk 3 laat een overzicht zien van 22 jaar ervaring met de LLETZ behandeling van hoog-gradige CIN en de cytologische follow-up van de behandelde patiënten. Gezien het lage percentage herhaalde afwijkingen in de gehele groep met een minimale follow-up van 2 jaar, blijkt dat de meerderheid van de behandelde patiënten een adequate behandeling hebben ondergaan. Slechts in 8.5% van de patiënten werd een herhaalde afwijking gevonden en wederom behandeld. Ongeveer 80% van deze afwijkingen ontstond binnen twee jaar na de initiële LLETZ procedure en kan daarom als residu laesie worden beschouwd. De overige 20% kan worden betiteld als zijnde een recidief afwijking. Meer dan de helft van deze recidieven ontstond meer dan 5 jaar postoperatief, hetgeen suggereert dat langdurige strikte follow-up onontbeerlijk is. Eerdere en latere studies hebben overigens aangetoond dat de bepaling van hr-HPV in de follow-up gebruikt kan worden als gevoeligere marker voor de detectie van residu/recidief CIN.

Hoofdstuk 4

In hoofdstuk 3 is reeds naar voren gekomen dat de LLETZ een veel gebruikte en beproefde methode is voor de behandeling van hooggradige cervicale afwijkingen. Er wordt gesuggereerd dat LLETZ verantwoordelijk is voor het elimineren van HPV. Waar de meeste studies hebben gekeken naar de aanwezigheid van hoog-risico HPV na behandeling teneinde vrouwen op te sporen die een verhoogd risico zouden hebben op residue of recidief laesie, hebben wij voornamelijk het voorkomen van nieuwe HPV typen na LLETZ bestudeerd. In totaal werden 85 hoog-gradige CIN laesies en hun follow-up controle uitstrijkjes geanalyseerd op het voorkomen van HPV. Bij 20 patienten werd hetzelfde HPV genotype gevonden in laesie en follow-up uistrijk, terwijl bij 13 personen een nieuw HPV type werd ontdekt. Hierbij werd in maarliefst 8 uitstrijkjes HPV 18 gevonden. Dit opmerkelijke gegeven pleit voor het vrijkomen of re-activeren van het virus uit proximale lagen van het cervicale kanaal geincideerd tijdens de LLETZ behandeling.

Hoofdstuk 5

Het correct kunnen genotyperen van het humaan papillomavirus is van belang ten einde 1) de doeltreffendheid te kunnen bepalen van multivalente vaccins en chirurgische behandelingen van CIN, 2) de epidemiologische verdeling van HPV over de wereld gedegen te kunnen bestuderen, en 3) het oncologische potentieel van hoog-risico HPV genotypen te kunnen observeren. Diverse HPV testen zijn ontwikkeld om aan deze eis te voldoen. In hoofdstuk 6 wordt de LINEAR ARRAY van Roche een recent ontwikkelde genotyperingstest, welke is gebaseerd op een bekende primerset (PGMY 09/11) vergeleken met een bestaande en hooggevoelige genotyperingstest (de SPF_{10}-INNO LiPA). Bij 81% van de monsters werden identieke resultaten gevonden tussen de twee testen, in 11% waren er grote, maar niet identieke, overeenkomsten, en in 8% werd er geen enkele overeenkomst tussen de testen ontdekt. De gevonden verschillen zouden kunnen worden verklaard door een variatie in analytische sensitiviteit voor bepaalde genotypen in een test of door een lage virale load in bepaalde monsters. Ondanks de gevonden verschillen kunnen de twee testen statistisch gezien worden aangemerkt als erg goed vergelijkbaar en reproduceerbaar.

Hoofdstuk 6

Hoog-gradige premaligniteiten van de cervix kunnen effectief worden behandeld door middel van een LLETZ (zie hoofdstuk 3). Niet elke onbehandelde hoog-gradige

CIN laesie zou echter een progressieve ontwikkeling tot cervixcarcinoom hebben doorgemaakt. Biologische gedrag, i.e. progressie en regressie, van CIN kan niet worden voorspeld door conventionele markers als cytologie en histologie. Integratie van HPV DNA in het humane genoom markeert het begin van de carcinogenese. Daarom zouden parameters als HPV of markers die duiden op cel proliferatie, genetische instabiliteit en chromosomale afwijkingen van voorspellende waarde kunnen zijn bij het beoordelen van progressief en regressief gedrag. In hoofdstuk 7 wordt een logistisch regressiemodel gebruikt om het biologisch gedrag van CIN te bestuderen. Het model is gebaseerd op de progressiemarker Ki-67 labelingsindex en parameters voor chromosoom 1 afwijkingen (chromosoom index en aneusomie). Al de genoemde parameters werden in de inclusie uitstrijkjes van 73 patiënten bepaald. Deze patiënten werden verwezen naar de gynaecoloog in verband met het hebben van twee licht afwijkende uitstrijkjes. Van deze patiënten lieten er 42 in de follow-up regressie zien en 31 progressie (maximaal CIN 3). Het opgestelde model met de parameters Ki-67 labelingsindex, chromosoom index voor chromosoom 1 en de fractie cellen met 4 kopieën van chromosoom 1 in de nucleus bleek biologisch gedrag het best te voorspellen. Het gedrag van ruim 93% werd correct voorspeld (oppervlakte onder de ROC-curve 0.96±0.02). Indien het model enkel werd toegepast bij hr-HPV positieve vrouwen met een klasse 2 of klasse 3a geringe dysplasie uitstrijkje dan werden progressie en regressie eveneens correct voorspeld. Korte termijn pro- en regressief gedrag is dus goed voorspelbaar gebruik makend van een model met de aangegeven parameters. Het model kan erg het aantal verwijzingen voor kolposcopisch onderzoek aanzienlijk verlagen indien het naast de triage met hr-HPV wordt toegepast bij vrouwen met licht afwijkende uitstrijkjes.

Hoofdstuk 7

Het staat onomstotelijk vast dat hoog-risico HPV testen in baarmoederhalskankerscreening van voordeel kan zijn in de management van de patienten. Vrouwen die niet deelnemen aan de screening hebben een verhoogd risico op het krijgen van baarmoederhalskanker. De introductie van cervicovaginale zelfsampling kan het aantal deelnemers vergroten en zo de incidentie van baarmoederhalskanker verlagen. Hoofdstuk 7 beschrijft een studie waarin we hebben gekeken naar de mogelijkheid van HPV genotypering in zelf afgenomen uitstrijkjes die vervolgens op speciale filtreerpapiertjes worden gebracht. Deze zelfsampling monsters werden vergeleken met de HPV inhoud van de uitstrijkjes genomen door een arts. De overeenkomst tussen de samples was 96%. Detectie en zelfs genotypering van HPV gebruik makend van zelfsampling en filtreerpapiertjes is dus goed mogelijk en het materiaal blijkt overeenkomstig met dat van het “officiele” uitstrijkje. In vergelijking met andere bekende methoden van zelfsampling is de filtreerpapier methode onafhankelijk van vloeistoffen die vaak licht ontvlambaar en potentieel infectieus zijn en niet altijd via de reguliere weg verstuurd mogen worden.

Hoofdstuk 8

Het laatste hoofdstuk bespreekt het belang van de vroege opsporing en de behandeling van afwijkende cervixcytologie, de sensitiviteit- en specificiteitsproblemen die hiermee samengaan en de mogelijke oplossingen voor deze problemen (het implementeren van additionele of nieuwe parameters). Daarnaast komen in dit hoofdstuk de recent geintroduceerde HPV vaccins ter sprake. Omdat deze vaccins vrijwel zeker een rol gaan spelen in de primaire preventie van baarmoederhalskanker, wordt het belang van baarmoederhalskankerscreening in het post-vaccinatie tijdperk eveneens uiteengezet.

Abbreviations

ALTS	ASCUS-LSIL Triage Study
ASC-US	Atypical squamous cells of undetermined significance
BMD	borderline and mild dysplasia
BNC	Borderline nuclear changes
CI	Chromosome index
CIN	Cervical intraepithelial neoplasia
DEIA	DNA enzyme immuno assay
DFS	Dried fluid spots
DNA	Desoxyribo nucleic acid
E	Early region (E1-E7 in HPV genome)
et al.	et alii, and others
HPV	Human Papillomavirus
hr-HPV	High-risk Human Papillomavirus
HSIL	High-grade squamous intraepithelial lesion
IVF	*In vitro* fertilisation
L	Late region (L1 and L2 in HPV genome)
LA	LINEAR ARRAY
LBC	Liquid based cytology
LiPA	Line probe assay
LLETZ	Large loop excision of the transformation zone
lr-HPV	Low-risk Human Papillomavirus
LSIL	Low-grade squamous intraepithelial lesion
NA	Nucleic acid
NPV	Negative predictive value
ORF	Open reading frames
PCR	Polymerase chain reaction
PPV	Positive predictive value
pRb	Retinoblastoma protein
PSP	Population-based screening programme
SCJ	Squamo-columnar junction
STI	Sexually transmittable infection
TZ	Transformation zone

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Curriculum Vitae

Op 16 januari 1975 werd Dennis van Hamont in Moedervreugd te Goirle geboren.

Na aanvankelijk een carrière als Paus te hebben geambieerd, is Dennis via de Norbertus MAVO, HAVO en VWO (beide Theresialyceum te Tilburg) Bewegingswetenschappen gaan studeren aan de Universiteit Maastricht (prof. dr. Harm Kuipers en dr. Hans Keizer). Tijdens zijn verblijf in Nieuw Zeeland voor een wetenschappelijke stage aan Otago University in Dunedin (dr. Nancy Rehrer), ontmoette hij Carolina, met wie hij binnenkort zal trouwen.

Na vier maal te zijn uitgeloot voor Geneeskunde, was het de vijfde keer raak. Aan de Katholieke Universiteit Nijmegen kon in maart 2000 een lang gekoesterde droom in vervulling gaan. Zo werd met de nodige vrijstellingen in januari 2003 het doctoraal examen Geneeskunde gehaald.

Reeds tijdens het co-schap Gynaecologie werd hij door dr. Leon Massuger (toen nog dr.) gestrikt voor de wetenschap. Direct na het behalen van het artsexamen aan het Universtair Medisch Centrum St Radboud, startte Dennis in september 2004 als artsonderzoeker via de afdelingen Medische Microbiologie (dr. Willem Melchers) en Verloskunde en Gynaecologie (inmiddels prof. dr. Leon Massuger) aan een onderzoeksproject wat uiteindelijk heeft geleid tot dit proefschrift.

Sinds oktober 2006 is hij als AIOS (arts-assistent in opleiding tot specialist) Gynaecologie werkzaam in het Catharina Ziekenhuis te Eindhoven (opleider dr. Tom Hasaart) binnen het opleidingscluster Nijmegen (opleider mw. prof. dr. Didi Braat).

De paranimfen

Irene van der Avoort
Maaike van Ham