

**Combination of cytological and molecular methods for
improvements in cervical cancer prevention**

PhD Dissertation

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Abstract

To date, there is strong evidence that cytology-based screening programs have resulted in a significant decrease of the incidence of and mortality from cervical cancer, however, substantial over-treatment of non-invasive intraepithelial lesions results from the low specificity of traditional cytological screening. To address these issues, we have developed a risk-adapted multimodal cervical screening and management protocol at the Institute of Pathology in Bonn-Duisdorf including a combination of liquid-based cytology, PCR-based dynamic HPV genotyping and DNA cytometry, and introduced it in the routine clinical practice for the first time in Germany. By using this protocol, the major aims of the present project were: 1) to characterise cytomorphological and molecular basic features of HPV infection, (2) to define association between non-classical HPV-related cytomorphological signs and molecular markers of HPV infection from the DNA to protein level, (3) to determine the prevalence of rare and new HPV genotypes as well as HPV types with undefined oncogenic potential in cervical pre-cancer and cancer, (4) to define prognostic markers for early CIN (cervical intraepithelial neoplasia) behaviour and for post-treatment surveillance and (5) to evaluate the effect of our risk-adapted multimodal cervical cancer screening and management strategy (the Bonn-protocol) in a routine screening population of about 30.000 women from the Bonn-region in West-Germany. Our results are expected to increase our understanding of the molecular biology and associated functional-morphological signs of HPV infection during cervical carcinogenesis and tumour progression. More importantly, we attempt to investigate how this knowledge could be transferred into the daily diagnostic practice to improve early detection and prognostication of HPV-related cervical (pre)-cancer.

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Table of contents	Page
I. Introduction.....	1
I. 1. The history of the cervical cancer screening.....	1
I. 2. Papillomaviruses and cancer: historical context.....	4
I. 3. Human Papillomaviruses (HPV).....	6
I.4. HPV pathogenesis.....	9
I. 5. The development of HPV induced intraepithelial lesions.....	12
I. 6. Carcinoma of the uterine cervix.....	13
I. 7. Cytology-based cervical screening.....	14
I. 8. HPV tests in cervical cancer screening programmes.....	16
I. 9. Clinical significance of HPV genotyping.....	17
II. Material and methods.....	19
II. 1. IN GENERAL.....	19
a) Patients and samples.....	19
b) Procedures.....	19
1) Cytology.....	19
2) Histology.....	21
3) PCR-based HPV DNA detection and genotyping.....	21
4) DNA image cytometry.....	22
5) In situ hybridisation.....	22
6) Immunohistochemical detection of HPV L1 capsid protein.....	24
7) HPV mRNA detection.....	24
II. 2. IN PARTICULAR.....	25
II. 2. 1. Studies for characterising cytomorphological and molecular basic features of HPV infection.....	25

a)	Patients and specimens.....	25
b)	HPV detection and typing by PCR.....	28
c)	Statistics.....	28
II. 2. 2.	HPV genotype distribution and spectrum of cervical diseases induced by low-risk and undefined-risk HPVs: Implications for patient management.....	29
a)	Screening population and procedures	29
b)	Cytology.....	29
c)	Histology.....	30
d)	PCR-based HPV DNA detection and genotyping by sequence analysis.....	30
e)	Statistical analysis.....	30
II. 2. 3.	Validity of combined cytology and human papillomavirus (HPV) genotyping with adjuvant DNA-cytometry in routine cervical screening.....	31
a)	Screening population and procedures.....	31
b)	Cytology.....	31
c)	Histology.....	31
d)	PCR-based HPV DNA detection and genotyping by sequence analysis.....	32
e)	DNA image cytometry.....	32
f)	Screening algorithm and patients management.....	32
g)	Statistics.....	34
III.	Results.....	35
III. 1.	Cytomorphological and molecular basic features of HPV infection.....	35
III. 1. 1.	Diagnostic value of minor non-classic HPV-related cytological	35

alterations.....	
III. 1. 2. Molecular basic features of HPV infection.....	41
a) Immunohistochemical expression of the HVP L1 capsid protein and HPV DNA in situ hybridisation (CISH).....	41
b) HPV caused chromosomal imbalance [fluorescence in situ hybridisation (FISH)].....	43
c) Diagnostic and prognostic value of the detection of E6/E7 mRNA transcripts in cervical cytology up to PapIVa (HSIL/ in situ carcinoma).....	44
III. 2. HPV genotype distribution and the spectrum of cervical lesions induced by low-risk and undefined-risk HPVs.....	47
III. 3. Efficiency of a risk-adapted multimodal screening and management strategy (the Bonn-protocol) for cervical cancer prevention.....	53
1. Global results of cytology, histology, HPV testing and DNA ploidy for the whole screening population in the year 2002.....	53
2. Comparison of relative test performances and correlations between cytology, HPV testing, DNA ploidy and histology.....	59
3. Accuracy of single tests and combinations in the multimodal screening setting.....	62
IV. Discussion.....	63
IV. 1. Cytomorphological and molecular basic features of cervical HPV infection....	64
IV. 2. HPV genotype distribution and the spectrum of HPV-induced cervical lesions.....	71
IV 3. Efficiency of risk-adapted multimodal screening and management strategy for cervical cancer prevention.....	76
V. Literature.....	79
VI. List of Publication.....	91

I. Introduction

I.1. The history of the cervical cancer screening

The Pap test is one of the greatest success stories in the history of medicine. Before the era of the Papanicolaou smear, carcinoma of the uterine cervix was a leading cause of cancer related deaths among women. By detecting cervical abnormalities that can be treated before they develop to invasive cancer, Pap test screening has correlated with a remarkable reduction in the morbidity and mortality of the disease.

In the 19th century, it was believed that cervical cancer started as a node in the stroma, which arose from stromal cells, displaced epithelial cells, embryonal rests or even wandering blood cells (57). 1852, Charles Robin recognised the epithelial nature of carcinoma (104).

In the early 20th century, it was proposed that cervical cancer have an incipient or non-invasive stage (101, 130). Although the concept of precancerous lesion was slow to be accepted, it gradually became known that cervical cancer could be cured, if it would be found early.

In 1925, Hans Hinselman invented the colposcope as a tool for detecting early invasive cancer. The colposcope illuminates and magnifies the exocervix, making early cancer visible as a small ulcer or a small exophytic lesion.

In 1928, Walter Schiller devised a test for cervical cancer that involved painting the cervix with iodine solution. In theory, healthy cells stain dark owing to glycogen content, while abnormal cells remain unstained. Unstained “Schiller-positive” areas were biopsied. Schiller proved that carcinoma of the cervix could be diagnosed before invasion has occurred and that when adequately treated, preinvasive cancers were nearly always curable (116).

Before the era of the Pap test, carcinoma of the cervix was documented by either full thickness biopsy or curettage. These methods were problematical owing to risks of surgery, time and expense, as well as patient acceptance, which precluded their use as screening tests.

In the 1920s, George Nicholas Papanicolaou made the discovery that made him famous. First he was making his studies on a guinea pig colony using them for his research in reproductive endocrinology. He examined vaginal smears microscopically. In 1917, he published two papers on the use of vaginal cytology as an indicator of a stage of the estrous cycle in guinea pigs. His biological assay spread all over the world and initiated a golden age in reproductive endocrinology and Papanicolaou became “the father of modern endocrinology”.

In 1923, he began studying the hormonal maturation of the human vaginal mucosa. Because uterine cancer was so common then it was inevitable, that Papanicolaou would detect malignant cells. Papanicolaou presented his “New diagnosis on cancer” in 1928 but his findings had no impact at the time. At that time, histologic techniques were being perfected and cytology methods were on the wane. It took more than 12 years until Papanicolaou published his cytologic cancer detection method in 1941 (95). Then in 1943, the monograph “Diagnosis of uterine cancer by the vaginal smear” became the turning point in cancer screening (96).

Papanicolaou was not alone, not even the first, in his discovery. In 1854, Lionel Smith Beale in his book: “The microscope and its application to clinical medicine” described cancer cells during microscopy of the vaginal smear. In 1886, C Friedlaender used the cytologic method to diagnose cervical cancer, but recommended confirmation by examination of a

biopsy. Thus, vaginal smears have been examined and exfoliated cancer cells have been detected, long before Papanicolaou (62).

Although is generally credited for the invention of the cervical cancer screening test by cervical cytology, O'Dowd and Philipp believe that Dr. Aurel Babeş, of Romania, was the true pioneer in the cytologic diagnosis of cervical cancer. Babeş's 1927 work, however, was published in the "Proceedings of the Bucharest Gynecological Society", and it is unlikely that Papanicolaou was aware of it (93).

Shortly after Papanicolaou's publication 1943, more studies confirmed the value of the cytologic method (6, 71, 72). In 1947 J Ernest Ayre introduced the method known today as the conventional Pap smear, using a spatula to scrape cells directly from the cervix, which were then smeared on a glass slide (5, 7).

By the late 1940s, cytology laboratories were opening and by the 1950s Pap test screening was becoming widespread in the USA and Western Europe. In the 1960s, about 30% of women in USA had at least one Pap test, but by the 1990s more than 90% of adult women had been screened at least once (53).

In theory, cervical cancer should be completely preventable, but in practice, cervical cancer will never been completely eradicated, even where there are well organised screening programs. Some of the problems are: reaching all women, including high-risk, low income women; consistently obtaining satisfactory, representative samples; achieving and maintaining high laboratory standards; and providing appropriate clinical follow-up of women with abnormal results (63).

One of the difficulties was and is the low sensitivity of the Pap test alone. To improve the cervical cancer screening, The Bethesda System was developed in 1988, a standardised protocol for reporting of gynecological cytology. Then in the late 1980s and 1990s, liquid-based processing of cytology samples, computer assisted screening and human papillomavirus-testing were established and until today there is an ongoing research in these fields.

I. 2. Papillomaviruses and cancer: historical context

Papillomaviruses co-evolved with humans over a span of million years. Lesions in animals, now known to be due to papillomaviruses, have been recognised for more than a millennium. Celsus (25 BC-50 AC) documented skin warts in the first century AC. Genital warts or condylomas were also described by ancient Greek and Roman writers and were associated with sexual contact (119).

1898, M'Faydan and Hobday transmitted dog warts using cell-free extracts. Ciuffo used 1907 the same method to produce human warts by self-inoculation. In 1933, Richard Shope described the first papillomavirus in the cottontail rabbit (124). The Shope papillomavirus was shown to be capable of causing cancer (105, 106). Peyton Rous, in 1911, has already established that infectious agents could cause cancer. He described a sarcoma in chickens caused by what later became known as the Rous Sarcoma Virus (107).

In 1922, in Basel, Lewandowsky and Lutz described a rare hereditary condition: epidermodysplasia verucciformis, without thinking of an infectious component of the disease. Twenty years later it was found to be caused by a papillomavirus infection. (146).

Virus particles were first identified ultrastructurally in skin warts in 1949 (131) and in venereal warts in the 1960s (76).

Between 1974 and 1976 zur Hausen and co-workers started to postulate and analyse a possible role of HPV in cervical cancer (143, 144). In 1976, Meisels and Fortin published two reports outlining that the appearance of koilocytes in cervical smears indicates a presence of a papillomavirus infection (74). They also suggested that it might be possible to differentiate between benign warty lesions that do not progress to cervical cancer and precursor lesions that do progress to cervical cancer. This idea was supported by the identification of papillomavirus particles in mild dysplastic lesions of the cervix (38).

In 1980, Gissman first reported partial characterisation of an HPV from genital warts, which was designated HPV6 (47).

In 1983 and 1984, HPV 16 and HPV 18 were first isolated from cervical cancer, respectively (41, 20).

Modern techniques such as PCR, have confirmed an extremely strong relationship between HPV and cervical cancer. Beginning in the 1980s, scientific investigation began unravelling the molecular biology of human papillomavirus and the actual cellular mechanism of cervical infection and carcinogenesis (146).

The first epidemiologic study of HPV infection was published by de Villiers and co-workers in 1987. This was followed by a large scale of epidemiologic studies that provided solid evidence that HPV is the primary risk factor for cervical cancer (84, 86, 87).

In 1995 the World Health Organisation officially recognized certain human papillomaviruses (specifically, HPV types 16 and 18) as human carcinogens (55).

HPV has now been found guilty of causing cervical cancer beyond doubt, but it is not sufficient by itself to cause cancer and it needs more than one cofactor. (18).

I. 3. Human Papillomaviruses (HPV)

Papillomaviruses are oncogenic DNA viruses. They are small about 55-60 nm in diameter, non-enveloped, and have an icosahedral structured capsule. They infect cutaneous and mucous epithelia in a variety of animal, including humans. There are more than 300 distinct types of papillomaviruses (PVs), including about 200 different types of human PVs of which at least 70 types infect the anogenital tract. PVs are strict species-specific; they do not infect any other host than their natural one (111).

Phylogenetic studies suggest that PVs normally evolve together with their mammalian and bird host species, do not change host species, do not recombine, and have maintained their basic genomic organisation for over 100 million years. All PVs form the family “Papillomaviridae”. Major branches of the phylogenetic tree of all PVs are considered as “genera”. Minor branches are considered as “species” and represent unite PV types that are genomically distinct without exhibiting known biological differences. This taxonomy is recognised by the International Committee on Taxonomy of Viruses (ICTV). One hundred eighteen papillomavirus (PV) types have been completely described, and a yet higher number of presumed new types have been detected according to preliminary data (37).

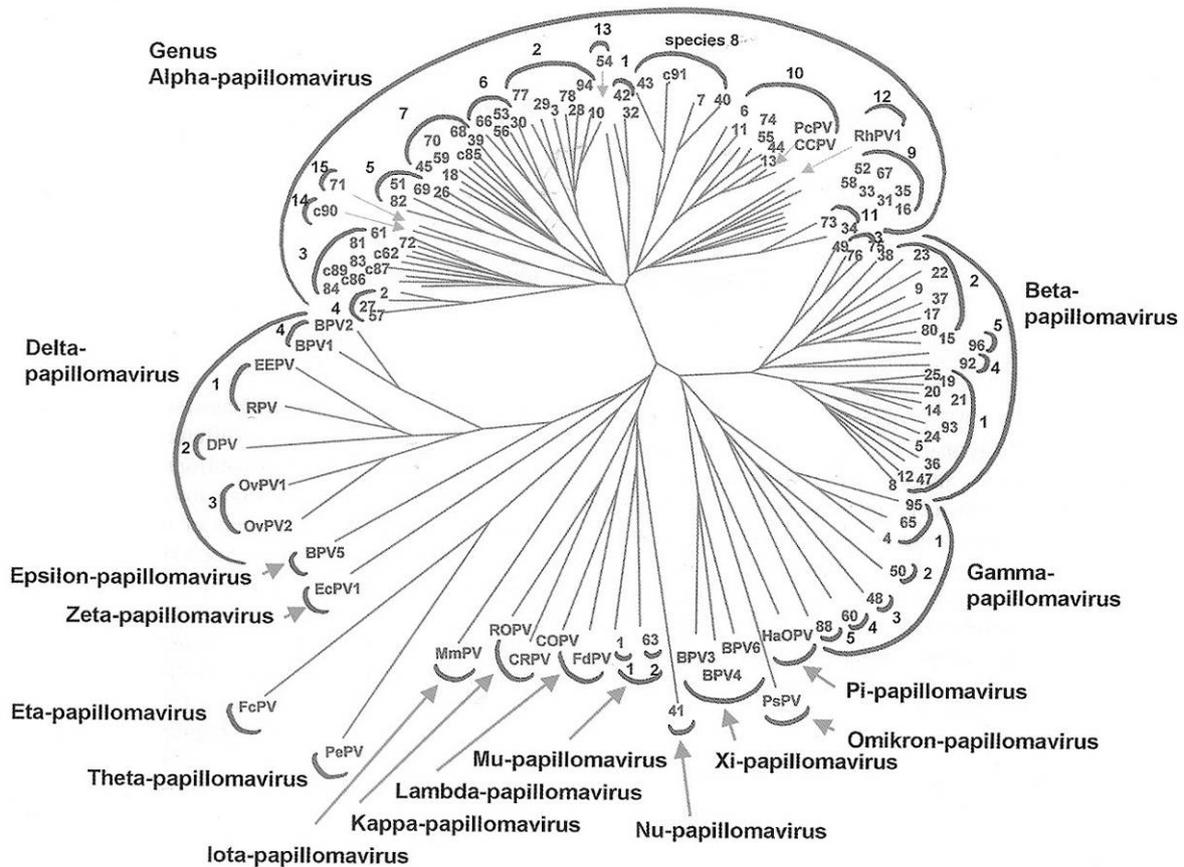


Figure 1: Phylogenetic tree of 118 papillomavirus types (de Villiers et al. 2004)

Despite their heterogeneity, all PVs have a very similar genomic structure. The viral genome is a double stranded circular DNA of approximately 8000 bases (8kb).

There are three regions which can be divided into the E region coding for early proteins (E1 - E7), the L region coding for late proteins (L1, L2) and a non-coding region called long coding region (LCR).

E1-E7 are responsible for the pathogenicity of the virus, the L region is coding for late structural proteins and the LCR contains the cis-elements necessary for replication and transcription of the viral genome.

The E1 and E2 genes are involved in viral replication and genome maintenance.

E1 has helicase activity that catalyzes the unwinding of DNA duplex. It also brings the DNA polymerase to the origin of replication, where the E1 and E2 proteins will initiate the replication. E2 also acts as a transcription repressor of the HPV E6 promoter (44).

Although the E4 protein is a product of early gene expression, it is considered to be a late protein with production and localisation in the cytoplasm of the upper epithelial layers just prior to full viral assembly and play an important role in the maturation and replication of the virus. The E4 protein also induces the collapse of the cytoplasmic cyokeratin network in human keratinocytes (137).

The E5 gene product interacts with various transmembrane proteins and receptors such as receptors of epidermal growth factor (EGF), platelet-derived growth factor (PDGF β) and colony stimulating factor and has transforming activity in infected cells (60).

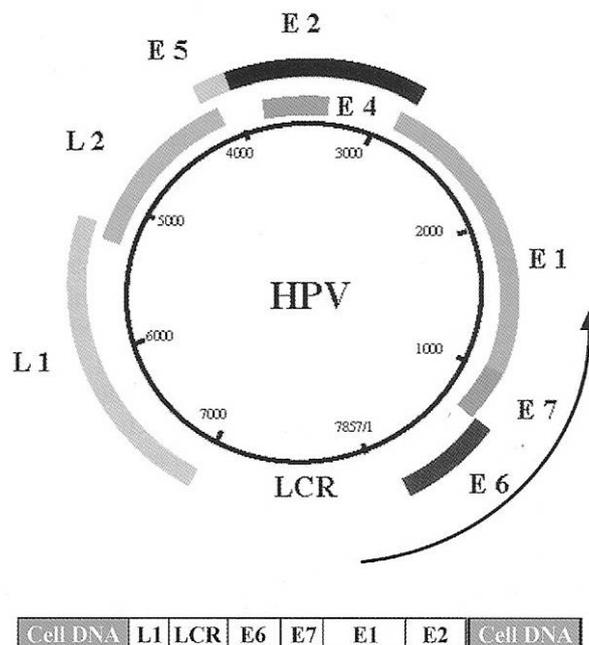


Figure 2: The genomic organisation of a typical papillomavirus (Saveria Campo, 2006)

E6 and E7 genes encode for oncoproteins allowing replication of the virus, immortalisation and transformation of the host cells. Both E6 and E7 proteins can bind to multiple cellular targets. E6 binds, via the cellular protein E6-AP, to the tumour suppressor gene product p53 and its related pocket proteins, p107 and p130. The first interaction results in rapid ubiquitin-dependent proteolytic degradation of p53, which prevents cells from undergoing p53-mediated apoptosis. A consequence of E7-pRb interaction is interference with cell cycle control. In combination, the E6-p53 and E7-pRb interactions seem to compromise the accuracy of mitosis. In addition hrHPV E6 can activate the telomere-lengthening enzyme telomerase independent of p53 binding and E7 can induce abnormal centrosome duplication through a mechanism independent of inactivation of pRb and its family members (125).

The late genes L1 and L2 encode the structural proteins of viral particles that are expressed at the final stages of viral production. The L1 protein comprises 80% of total viral protein and is highly conserved among different papillomavirus species. The minor capsid protein encoded by L2 has more sequence variations (44).

HPVs are distributed into high-risk, low-risk and unknown-risk types linked to their potential to induce high grade lesion and cancer (85).

I. 4. HPV pathogenesis

HPVs are epitheliotropic by nature and their life cycle is closely linked to the terminal differentiation of the squamous cells.

In the cervix, initial infection occurs in the basal cells through small abrasions in the tissue or during squamous metaplasia in the transformation zone when basal cells are exposed. HPV can remain latent or adopt replication in the nucleus.

During latent infection, the virus infects epithelial stem cells in the basal layer and remains as a silent passenger in them.

During productive infection, different phases can be distinguished. First the viral genes E7 and E6 push the basal and parabasal cells into cell cycle result in proliferation. These proliferating cells maintain episomal virus DNA in low copy number. Then amplification of the virus DNA begins in the intermediate cell layers and the viral genes E1-E4-E5 are expressed. In the upper layer of the squamous epithelium expression of the late genes L1 and L2 and production of the capsid proteins occurs. The episomal viral DNA gets enveloped and the infectious virions get released from the superficial cell layer (132).

The virus replicating cells display the characteristic morphology of low grade squamous intraepithelial lesion (LSIL) like koilocytosis and minor nuclear changes.

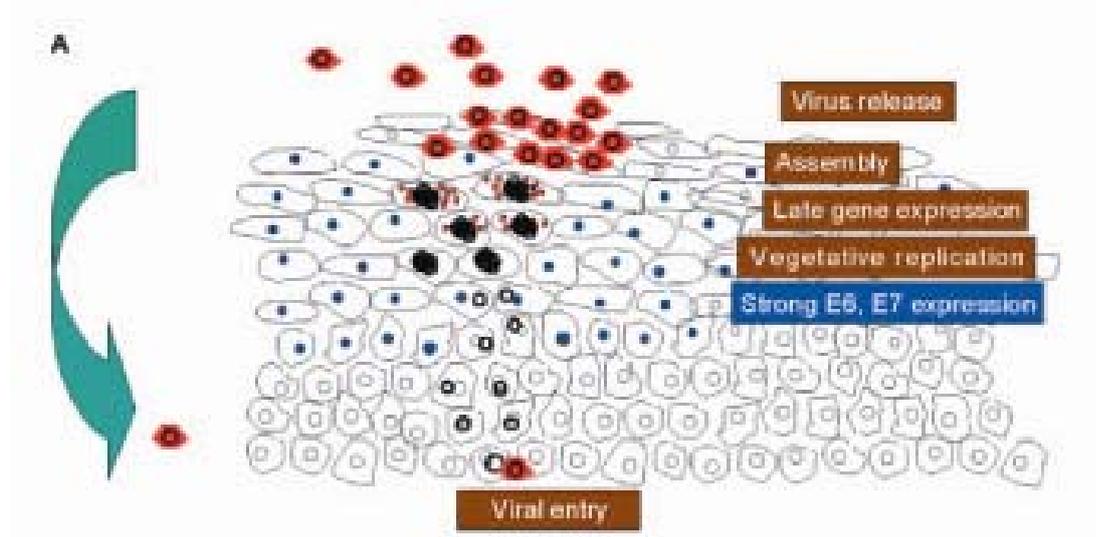


Figure 3: Virus replication (Snijders PJF et al., J Pathol, 2006)

It is now well established that a number of HPV genes can manipulate cell cycle control to promote viral persistence and replication. It leads to a deregulated viral gene expression and integration of the viral DNA into the host genome. Due to loss of function of the intracellular surveillance mechanism, viral oncogenes are expressed in the basal stem cells and initiate chromosomal instability (125).

In the squamous epithelium an ongoing growth of dysplastic cells with partially retained differentiation can be seen. The morphological changes include hyperchromasia due to severe changes of the overall DNA content (polyploidy or aneuploidy), a high nucleus/cytoplasm ratio, increased number of mitosis, atypical mitosis even in the upper layer of the epithelium. The maturation of the epithelium gets lost and with it the viral reproduction.

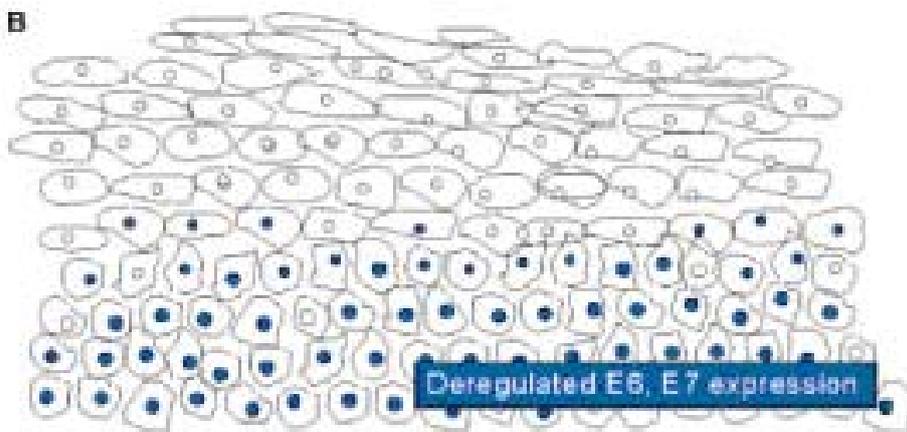


Figure 4: Virus integration (Snijders PJF et al., J Pathol, 2006).

I. 5. The development of HPV induced intraepithelial lesions

It is well known that high-risk HPV infections, although necessary, are not sufficient to induce cervical cancer alone. Cervical cancer requires several additive conditions and events to accumulate once an infection has become evident (133).

HPV infections are very common in young women but frequently resolve spontaneously. The life-time risk to ever contact HPV is estimated to be 80% and more than 80% of the HPV infections are likely to be transient without developing high grade CIN (73). Why certain HPV infections tend to persist and give rise to lesions, whereas many others do not, is still questionable but the interindividual immune response could be a possible explanation.

It is assumed that it takes on average 12-15 years before a persistent HPV infection may ultimately lead in a multi-step way to overt cervical carcinoma. After the infection with HPV development of CIN1 and CIN 2 lesions can occur within 1-2 years followed by spontaneous regression in many cases. Due to this, these lesions should be considered as a cytopathological effect of a productive virus infection.

In CIN1 and most CIN 2 lesions, the active viral replication and virion production are strongly coupled to the differentiation programme of the infected epithelium. HPVs rely entirely on the host cell DNA replication machinery for viral DNA synthesis (26).

By contrast, some CIN2 and mostly all CIN3 lesions exhibit a dramatic topographical change in viral gene expression, which includes an increase in E6/E7 expression in proliferating dysplastic cells (42). In addition, high-grade CIN lesions and cervical carcinomas show an integration of the viral genome into the host genome.

The additional (epi)genetic alterations that subsequently accumulate in high-grade CIN lesions result in overt malignancy via immortality and growth conditions that gradually become less sensitive to growth-modulating influences mediated by cytokines and cell-cell and cell-matrix adhesions (125).

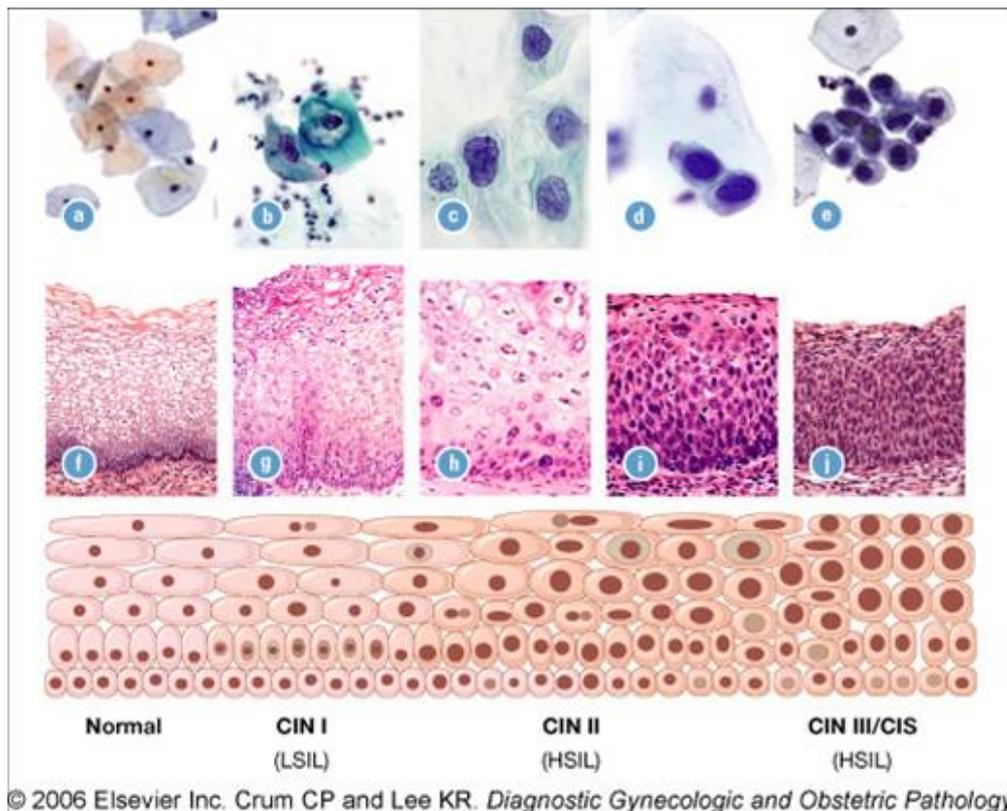


Figure 6: Cytological and histological pathomorphology of HPV.

I.6. Carcinoma of the uterine cervix

Carcinoma of the uterine cervix is the second most common cancer in women worldwide, with approximately 500 000 new cases diagnosed and 230 000 deaths each year. Almost 80% of new cases occur in the developing world where it is the leading cause of cancer-related death among women (97). In the former European Union (EU), cervical cancer was estimated to comprise about 3% of cancers in women; ranking eighth in importance and it

was the tenth most common cause of cancer-related deaths in women in 1998 (43). The recent expansion of the EU in 2006 will certainly cause significant changes in cervical cancer rates, because there is substantial excess in female mortality from the disease in most central and eastern European accession countries (65). For instance in Hungary, the age-standardized (world) annual incidence of cervical cancer was 15.7 and the mortality rate 6.7 women per 100.000, whereas corresponding data from Germany revealed an incidence rate of 10.8 and mortality rate of 3.8 per 100.000 population in 2002 (GLOBOCAN 2002, IARC).

After the introduction of regular, population-based cervical cytological screening in most developed countries, incidence and mortality rates of invasive cervical carcinomas fell impressively by up to 80 percent from 1950 to 1970 (48, 89). This has been mainly attributed to early detection and treatment of precancerous lesions.

However, in countries where cytology-based screening programmes are well developed, better screening tests than cytology may result in more efficient programmes, reduction of costs and reduction of the number of required screening events per women. Nevertheless, screening programmes are complex public health strategies of which the screening test being used is only one component. As a consequence, the advantages derived from the use of better tests would only result in noticeable reductions in mortality from cervical cancer if the screening programme as a whole is functional and efficient over extended periods of time.

I. 7. Cytology-based Cervical Screening

To date, there is strong evidence that cytology-based screening programs using the Papanicolaou (Pap) test have been effective in reducing the incidence of and mortality from the disease in developed countries. There is general agreement that high quality cytology is a

highly specific screening test with estimates of an average of 97% (range 86-100%). In contrast, sensitivity of a single smear may be between 30-87% (mean of 51%), although the sensitivity for high-grade disease alone is between 70 and 80% (88). Put another way, although the specificity of the Pap test is generally very high, the sensitivity to detect cervical intraepithelial neoplasia (CIN) or invasive cancer is low with poor sample processing and interpretation errors being the major problems. Blood, mucus and drying artefacts contribute to the difficulty of identifying abnormal cells in the traditional Pap smear, and up to 10% of slides can prove unsuitable for interpretation, necessitating repeat visits and sampling (40). Many of these problems can be overcome partially by improving methods of cell collection and presentation, but there remains a clear need to improve the traditional Pap test.

Newer technologies developed with the intention of improving cytological assessment include liquid-based cytology (LBC; ThinPrep, Autocyte), and computer assisted screening (PAPNET; AutoPap; IMAGER). Several sub-optimal studies (split-sample or historical) have been performed to determine sensitivity, specificity and predictive values of these new methods; however lack of an adequate reference standard in most of the studies hampers proper assessment and comparison of test characteristics (50). Nevertheless, the available evidence indicates that using liquid-based cytology, sensitivity is modestly higher for detecting any degree of cervical intraepithelial neoplasia (CIN), whereas specificity is modestly lower than with conventional Pap smears (88). This supports the conclusion that liquid based cytology is an acceptable alternative to conventional cervical cytology smears, which is reflected by the Food and Drug Administration of the US (FDA) approval of two liquid-based Pap systems for routine use.

Even though several independent reviews on comparing liquid-based versus conventional cervical cytology came to the conclusion that there is no significant difference between the two methods (36, 92), there are three major advantages of liquid based cytology

over conventional Pap smears: i) numerous investigators agree that liquid based cytology markedly improves specimen adequacy (39, 109, 13), ii) the residual material can be used for ancillary testing (e.g. for HPV DNA), and iii) recent studies have shown an improvement in sensitivity and specificity for biopsy proven adenocarcinoma in situ (AIS) and adenocarcinoma (8, 4).

Benefits of cytology-based screening

Taken as a whole, there is good evidence that regular screening of appropriate women for cervical pre-cancer and cancer with the Pap test reduces incidence of and mortality from cervical cancer.

Harms of cytology-based screening

Regular screening with the Pap test leads to unnecessary diagnostic procedures (e.g. colposcopy) and treatment for low-grade squamous intraepithelial lesions (LSIL) with uncertain long-term consequences on fertility and pregnancy. These harms are the greatest for younger women, who have a higher prevalence of LSIL that often regress without treatment.

Consequently, there is a need for alternative protocols e.g. using human papillomavirus (HPV) tests, or surrogate biomarkers of neoplastic transformation as well as test-combinations to enhance screening accuracy.

I. 8. HPV tests in cervical cancer screening programmes

It is now well established that the vast majority of cervical carcinomas and its precursors worldwide are caused by persistent infections with certain high-risk types of human papillomaviruses (HR-HPV) (115, 19, 133). Under optimal testing conditions, HR-HPV DNA can be identified in nearly all specimens of invasive cervical cancer (99.7%), in at least 70 % of CIN1, 80% of CIN2 and 96% of CIN3 precursor lesions. In terms of public health, these data

indicate that the existence of HPV-negative cervical cancer cases is negligible and does not require any interventional targeting by screening.

However, most women with HPV do not get cervical cancer either, owing to the fact that most HPV infections are transient in nature, especially in younger age groups (51, 83). Research is ongoing to determine acceptable protocols for HPV testing for three main screening- or management-related purposes: 1) in primary screening of asymptomatic women with negative cytology results for intraepithelial neoplasia or malignancy, 2) in triage of equivocal and abnormal Pap smears (\geq ASCUS), and 3) in patient management (treatment and follow-up) (138).

At present there are two HPV testing systems in widespread clinical use. Current hybrid capture technology (HC2 test) detects the presence of 13 types of oncogenic HPVs and 5 low-risk types using respective probe cocktails. Results are group-specific and do not allow distinction between different HPV genotypes. For clinical purposes, only the high-risk probe cocktail is used, with a reported sensitivity for detecting high- grade cervical intraepithelial neoplasias (CIN 2-3) between 84% and 100% (27, 139, 3). PCR-based methods mainly use consensus or multiplex primers that amplify a broad spectrum of HPV types. This methodology allows identification of individual HPV genotype/s.

I. 9. Clinical significance of HPV genotyping

It is now well established that only women with long-lasting latent HPV infections are at high risk for developing HSIL (52) and the persistence of at least one oncogenic HPV type is necessary for the emergency of cervical (pre-)cancer (61). Furthermore, there is now evidence that the different HPV types pose different risks for cervical cancer (85), which is

increased, in decreasing order of magnitude, in the presence of infection with HPV 16, 18, and 45 (28). Infection with HPV16 also poses an increased risk for cervical pre-cancer in women with equivocal or mild cytological abnormalities (24). HPV18 is preferentially involved in the development of cervical adenocarcinomas (21). Consequently, specific clinical measures should be taken with respect to certain HPV types in primary cervical diseases as well as in post-treatment disease control (15).

The type-specific detection of the persistence of cancer-associated HPV genotypes, and HPV types with yet unknown oncogenic potential can only be performed by genotyping of two consecutive probes within a reasonable period of time – about 12 months - because of the possibility of a new infection with another high-risk type during the follow up period. This can be performed by using different established PCR techniques (PGMY09/11-Amplicor LBA, GP5+/6+-EIA, SPF10-LIPA, PPF1/CP5-Sequencing) (30, 100). Amplified HPV DNA is identified by either microplate hybridization for the detection of PCR amplicons (GP5+/6+-EIA) or a reverse hybridization line blot assay (PGMY09/11-LBA, SPF10-LIPA) which provides information on the specific type(s) detected (99).

II. Material and methods

II. 1. IN GENERAL

The project was fully conducted at the Institute of Pathology Bonn-Duisdorf using equipments, reagents, know-how and manpower of the institution. Scientific supervision and consultation was provided by specialists at the Institute of Pathology, Medical School of the University of Pécs, with whom the Institute of Pathology in Bonn has a scientific collaboration for many years. Expert advice was obtained from specialists at the International Medical School of the University Münster.

a) Patients and samples

- 1) Office-based routine screening population of about 30 000 women (Bonn-region, West Germany)
- 2) Triage population of about 6000 women for HPV-related issues
- 3) Case series of different size and disease spectrum for methodical studies

b) Procedures

1) Cytology

For liquid-based cytology (ThinPrep), exfoliated cervical epithelial cells were sent to our institute in fixative solution in PreserveCyt vial for thin-layer preparations, which were made using the ThinPrep 2000 processor (ThinPrep; Cytoc Corp., USA). Smears were analysed by well-trained cytotechnologists who have a restricted workload of screening 10 smears per hour in accordance with quality assurance guidelines for cytological examinations issued by the German Medical Association. Cytological diagnoses and specimen adequacy were classified according to the modified Munich II Cytological classification (standard in

Germany) (127) and converted into the Bethesda 2001 terminology as follows: Pap I (unsuspicious) and Pap II (inflammatory) lesions were considered negative for intraepithelial lesion or malignancy (no-ASC); Pap IIw [(w = wiederholen/repeat): not an official class in the Munich II classification but very often used in daily practice] includes ASC-US and/or non-classic HPV-signs; Pap IIID lesions with mild dysplasia/CINI were considered low grade squamous intraepithelial lesions (LSIL) and Pap IIID with moderate dysplasia, PapIVa (severe dysplasia) were classified as high-grade squamous intraepithelial lesions (HSIL); Pap IVb was interpreted as HSIL/microinvasive carcinoma, whereas Pap V was considered as a correlate of invasive carcinoma. All cases with borderline or uncertain diagnosis (PapIIw, III/ roughly equating ASC-US, ASC-H) and those with squamous intraepithelial lesions (\geq PapIIID/LSIL-HSIL) were re-reviewed by a cytopathologist for affirmation.

Table 1: Correlation between the modified Munich (II) cytological classification and the Bethesda system 2001

Munich II classification	Bethesda 2001 correlate
PapI-II	Within normal limits/inflammatory
PapIIw *	Includes ASC-US and/or non classical HPV-signs
PapIII**	ASC-H and AGUS – cannot exclude high-grade disease or cancer
PapIIID	LSIL/HSIL
PapIVa	HSIL
PapIVb	HSIL/ microinvasive carcinoma
PapV	Invasive carcinoma

* PapIIw (w = wiederholen/repeat): not an official class in the Munich II classification but very often used in daily practice of most cytological laboratories.

** PapIII: slight uncertainty in the diagnosis, including ASC-H and AGUS cases but cannot exclude high-grade disease or cancer.

2) Histology

Gross and histological processing of surgical specimens were performed according to standardised surgical pathology protocols, histological diagnoses were made using the WHO classification.

3) PCR-based HPV DNA detection and genotyping by sequencing

Detailed protocols for the assays have been published previously (129). In short: HPV DNA detection was directly performed on residual material in the ThinPrep® collection vial with PCR-based assays using the improved MY09/MY11 consensus primers and the GP5+/6+ general primers for amplification of HPV DNA. Reaction parameters included initial denaturing at 95°C for 5 minutes, 40 cycles each at 95°C for 30 seconds, 37°C or 58°C for annealing the primer pairs GP5+/6+ or MY09/MY11, respectively. A final extension step was done at 72 °C for 5 minutes. The presence of human genomic DNA was verified by PCR amplification of the human globine gene. This reaction served as a positive control. PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers instructions. The sequence of one strand of the purified PCR fragments was determined with the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA) using 3-5 pmol of GP5+ or MY09 as the sequencing primers. Sequencing reactions were analysed on an ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, CA); results were compared with documented virus sequences available in the GenBank database using the BLAST program (Blast, Pittsboro, NC). Selection criteria for direct sequencing were: i) good quality PCR product, ii) cytology result with high prevalence of mild cytologic abnormalities and/or ii) demand of the women, since costs of HPV testing are not covered by general health insurance in Germany. Classification of HPV types was based on the epidemiological risk assessment of HPVs in the development of cervical cancer published by Munoz and co-workers (85).

4) DNA image cytometry

DNA ploidy measurements were carried out from the same liquid-based cytological material that was used for cytology and HPV-testing as published by our group previously (16). In short: after marking the location of dysplastic cells with a diamond writer on the back of the Pap-stained slides, the initial ThinPrep monolayer was restained according to the Feulgen method. The DNA content of at least 100 dysplastic epithelial cells and 30 normal-appearing intermediate cells for reference was measured interactively on individually focused nuclei using a cytometer system (Ahrens ICM-Cytometriesystem, Germany). Data were interpreted using the analysis software of the system. DNA histograms were classified as follows (14): i) diploid (stem line DNA peak between 1.80c and 2.20c), ii) polyploid (at least two stem lines between 1.8c-2.2c and 3.6-4.4c, or around 8c and 16c) and iii) aneuploid (stem line aneuploidy or single cell aneuploidy i.e. DNA-content > 9c).

5) In situ hybridisation

- To characterise cytomorphological and molecular basic features of HPV infection, the INFORM HPV III Family 16 Probe (B) was used in the BenchMark XT Staining Platform (Ventana Medical Systems) according to recommendations of the manufacturer. Briefly: First heat induced pretreatment in CC2 (citrate based buffer, pH 6.0, 28 min) and enzyme digestion (ISH-Protease III=alkaline protease, 4 min). Following the prehybridisation step, tissues were denatured and hybridised with the high-risk HPV probe-cocktail [HPV-genotypes-(DNP-labeled)]. The probe cocktail has demonstrated affinity to the following HPV genotypes: 16, 18, 31, 33, 35, 39, 51, 52, 56, 58 and 66. The amplification/detection steps were as follows: incubation first with a primary rabbit anti-DNP antibody, then an indirect biotin/streptavidin reaction (time and dilutions according to the Ventana machine protocol). Signal detection was performed with an alkaline phosphatase and NBT/BCIP-chromogen, which yielded a

blue staining. Nuclear fast red served as the counterstain giving a light pink colour to the negative nuclei. Punctuate and diffuse ISH signals were considered as indicators for the presence of both integrated and episomal HPVs, respectively. As positive controls, slides from CaSki-HPV-16 (200-600 copies/cell) and Hela-HPV18 (10-50 copies/cell) cell lines and “Alu-intactness of DNA control probe” (Ventana) were used. A HPV negative tissue slide as a negative control was used also with each probe.

- To characterise chromosomal imbalance in HPV infected cells, the fluorescence in situ hybridisation (FISH) technique was used with pericentromeric probes specific for chromosome 3 and 17 as previously published by our group (70). Briefly: Cells selected by gating larger than 5c and 9c DNA content were analysed for chromosome 3 and 17 numerical changes by FISH following the measurement of the DNA content. For this purpose, coverglasses were detached in phosphate buffered saline (PBS) and the slides were post-fixed in a 3:1 mixture of methanol and glacial acid (10 min, -20°C). Following air drying, cell-containing areas were exposed to pepsin digestion (15 minutes, 0.2mg/ml in HCl). Slides were washed twice in PBS (5 min each). The proteolysis was stopped in 3.6% formaldehyde/PBS (5 min., 4°C); the slides were washed in PBS (5min); dehydrated through ascending ethanol series and air-dried. Direct fluorescence-labelled chromosome 3 (SpectrumOrange) and Chromosome 7 (SpectrumGreen) alpha-satellite DNA probes were applied to the slides as recommended by the manufacturer (Vysis Inc., Dovers Grove, IL). The slides were sealed with rubber cement and dried at 37°C for 5 minutes. After denaturising at 73°C/10min, the slides were left over night at 37°C for hybridisation. Following the post-hybridisation washing (2x SSC/73°C/2min; 2xSSC/20°C/2 min; short PBS), slides were air-dried and covered by an antifade medium containing DAPI /Vectashield (Vector Corp., Burlingame, CA). Cells were analysed for red and green fluorescence hybridisation signals, images were captured by ISIS digital image analysis workstation

(Metasystems, Altlußheim, Germany attached to the microscope Aixoplan II, Zeiss, Jena, Germany).

6) Immunohistochemical detection of HPV L1 capsid protein

The reactions were performed using the Cytoactive® HPV-L1-high-risk antibody (Cytoimmun, Germany) as recommended by the manufacturer. Briefly: After deparaffinization antigen retrieval was performed in CC1 (Tris based buffer) for 30 minutes followed by blocking endogenous peroxidase with H₂O₂ (“iVIEW-Inhibitor”). The primary monoclonal antibody directed against the L1 capsid protein of all relevant HPV types was applied to the samples and incubated for 16 minutes. Immunostaining was performed with an I-VIEW DAB detection kit (Ventana), an indirect streptavidin-biotin-peroxidase system using DAB as chromogen. Sections were counterstained with haematoxylin.

7) HPV mRNA detection

Oncogene E6/E7 mRNA from HPV types 16, 18, 31, 33 and 45 was identified using the commercially available PreTect HPV-Proofer kit (NorChip AS Norway) based on real-time NASBA (nucleic acid sequence based amplification) technology according to recommendations of the manufacturer. Briefly, NASBA is based on isothermal mRNA amplification, accomplished by the simultaneous enzymatic activity of avian myeloblastosis virus (AMV) reverse transcriptase, T7 RNA polymerase, and RNase H. The NASBA amplification was carried out in a volume of 20 µl at 41°C for 2.5 h. A 5 µl volume of nucleic acids was included in the reaction. As performance control to avoid false negatives due to degradation of nucleic acid, we used a primerset and probe directed against the human U1 small nuclear ribonucleoprotein (snRNP)-specific A protein (U1A mRNA). The positive control was included in the kit, as negative control we used H₂O at each sample reaction.

II. 2. IN PARTICULAR

II. 2. 1. Studies for characterising cytomorphological and molecular basic features of HPV infection

a) Patients and specimens

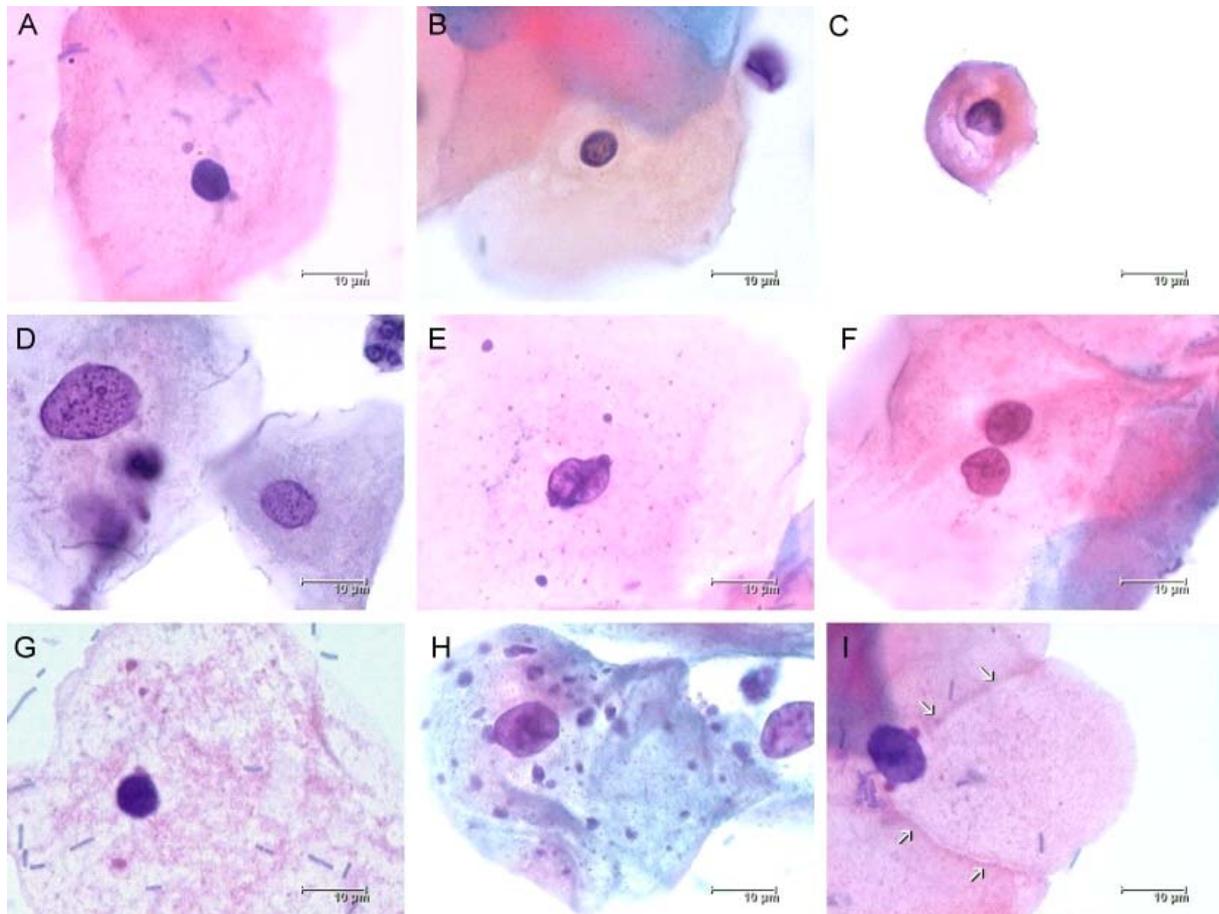
164 routine liquid-based cervical cytological specimens (ThinPrep monolayers) showing mild cytological abnormalities without classic signs of HPV infection, i.e. koilocytosis and dyskeratosis, or any degree of of squamous cell atypia, were selected retrospectively for this study. Specimens were obtained from different outpatient gynaecological practices from the federal state North Rhine-Westphalia, West Germany. The mean age of the patients was 39.5 years (range: 21-76 years). Results of cytology examination were reported using the second version of the Munich Classification, according to the German standards and translated into the Bethesda 2001 System as described in the general section (see Table 1).

Primary cytology was performed by trained cytotechnologists and confirmed by experienced cytopathologists who were also in charge of re-screening the selected smears for the study. For each monolayer a profile of mild cellular abnormalities was produced, with special emphasis on non-classic features suggesting the effect of HPV infection. These signs included: abortive koilocytosis, mild dyskeratosis, mild nuclear hyperchromasia, mild nuclear variations, bi/multinucleation, “measles cells”, parakeratosis, macrocytes, cytoplasmic folding and keratohyalin-like granules (Table 2 and Figure 7).

Table 2: Description of HPV-induced non-classic cytological alterations

Non-classic cytological signs	Cytomorphology
Abortive koilocytes	Superficial or intermediate cells showing some perinuclear cytoplasmic clearing but lacking the large, sharply demarcated perinuclear halo surrounded by a condensed cytoplasmic rim and nuclear atypia characteristic of a classic koilocyte
Mild dyskeratosis	Superficial squamous cells, singly or in sheets, with normal sized, regular or spindle shaped nuclei and orangeophilic cytoplasm
Parakeratosis	Small, heavily keratinized squamous cells with dense orangeophilic cytoplasm and small, pyknotic nuclei
Mild nuclear hyperchromasia	Mild nuclear enlargement with increased stainability without nuclear membrane irregularity. The chromatin is finely and uniformly granular.
Mild nuclear variations	Minor variation in nuclear size and form; presence of longitudinal nuclear grooves, wrinkled or spindle-shaped nuclei.
Bi/multinucleation	Double or multinucleated cells with regular chromatin pattern.
“Measles cells”	Condensation of the entire cytoplasmic matter into granules, the remaining cytoplasm appears empty; nuclei are sometimes missing.
Keratohyalin-like granules	Basophilic granules mostly around the nucleus, resembling to nuclear fragmentation
Macrocytes	Enlarged, swollen cells with faint or “empty” cytoplasm, i.e. cytoplasmic vacuolation
Cytoplasmic folding	Condensed filaments in the cytoplasm of superficial cells leaving the rest faintly stainable and resulting in a rough, multivacuolated appearance

Figure 7: Characteristic appearance of non-classic cytological HPV-signs in ThinPrep cervical cytology preparations



A) An *abortive koilocyte* showing some perinuclear cytoplasmic clearing around a small, pycnotic nucleus. **B)** *Mild dyskeratosis* in a large superficial squamous cell with normal sized, regular shaped nucleus and orangeophilic cytoplasm. **C)** *Parakeratosis* in a small heavily keratinized squamous cell with dense orangeophilic cytoplasm and small, pyknotic nucleus. **D)** *Mild nuclear hyperchromasia* and mild nuclear enlargement (cell on the left side) with fine, uniformly granular chromatin and regular nuclear membrane. **E)** *Mild nuclear variations* in a large superficial cell with a spindle shaped nucleus and a longitudinal nuclear groove; in the cytoplasm keratohyalin-like granules and “micronuclei”. **F)** A *binuclear* large superficial cell with regular chromatin pattern in the nuclei. **G)** “*Measles*” cell showing condensation of the entire cytoplasmic matter into granules and a pycnotic nucleus. **H)** *Keratohyalin-like granules*, spherical globuli around the nucleus resembling to nuclear fragmentation. **I)** *Cytoplasmic folding* in a large superficial cell with condensed cytoplasmic filaments (arrows) resulting in a multivacuolated appearance.

For statistical purposes and also because of cytomorphological relations, these signs were combined in: i) *minor nuclear abnormalities* (mild hyperchromasia, mild nuclear variations and bi/multinucleation), ii) *disorders of keratinisation* (mild dyskeratosis and parakeratosis), iii) *measles cells and abortive koilocytes*, and iv) *degenerative changes* (macrocytes, cytoplasmic folding and keratohyalin-like granules).

b) HPV detection and typing by PCR

HPV DNA testing was directly performed from residual material in the ThinPrep collection vial as part of the primary screening as described in the general section.

c) Statistics

Statistical analysis was performed to assess the diagnostic accuracy of each non classic cytological signs for identification of the presence of HPV infection by using the SPSS program for Windows. The absolute number of true-positives (TP), false-positives (FP), true-negatives (TN) and false-negatives (FN) were calculated from the original data. A case was considered TP if the respective non classic cytological parameter was present and it was positive for HPV DNA by PCR based assays. A TN case was defined if the monolayer did not contain the respective cytological variable and was negative for HPV DNA by PCR, as well. A case was considered as FN if the respective cytological sign was absent, but HPV DNA was detectable. A sample was defined as FP if the respective cytological parameter was present in the absence of HPV DNA.

The following test performance parameters were calculated at the threshold of HPV DNA positivity by PCR: sensitivity [$TP/(TP+FN)$]; specificity [$TN/(TN + FP)$]; positive predictive value [$PPV = TP/(TP + FP)$]; negative predictive value [$NPV = TN/(TN + FN)$].

The Pearson pair-wise correlation coefficient was used to estimate relationships between different cytological parameter. Association between HPV DNA and cytological variables

was studied by χ^2 statistics. Relation of cytomorphological signs between HPV positive and HPV negative groups was tested by the non-parametric Mann-Whitney *U* test.

For all statistics, cytological and HPV parameters were classified as being present (positive) or absent (negative). Significance was defined at $P < 0.05$; P was two-tailed.

II. 2. 2. HPV genotype distribution and spectrum of cervical diseases induced by low-risk and undefined-risk HPVs: Implications for patient management

a) Screening population and procedures

The study population was selected from a total of 5964 women who were investigated via HPV genotyping as an ancillary test to liquid-based cytology (ThinPrep®, Cytic, Boston, MA) during the 5-year interval between January 1st 2000 and December 31st 2005. Women were included in the study if they showed infection with only a single type of low-risk, undetermined-risk or novel HPV genotype and had no previous history of cervical dysplasia/carcinoma (\geq CIN2) or infection with HR-HPV. After excluding all ineligible women from the study, 293 patients remained who completely complied with the strict selection criteria. Patients who had more than one specimen tested were considered only once in the evaluation of the association of HPV types with epithelial lesions, and the highest grade of cytological abnormality was attached to the associated HPV type. We had a mean follow-up of 23 months (range: 6-84 months) in 243 patients. The mean age of women was 32.8 ± 7.9 (SD) years (range: 17-81 years).

b) Cytology

During the 5 years of the study, a total of 300 521 Pap tests (conventional and ThinPrep®) were evaluated at our institute. Cytological diagnoses and specimen adequacy were classified according to the modified Munich (II) Cytological Classification and

converted into categories of the Bethesda 2001 system as described in the general section.

In addition to standard cytological screening for the presence of atypical squamous epithelial cells of any grade, classic and particularly non-classic HPV-induced cytological alterations were also carefully assessed in all cases, as described previously.

c) Histology

All cases with cytological diagnoses of HSIL were proven histologically according to standard CIN classification. Histologies in lower cytological grades were not consistently available.

d) PCR-based HPV DNA detection and genotyping by sequence analysis

During the 5 years of this study, a total of 8503 HPV DNA tests were performed after informed consent of the women. Detailed protocols for the assays have been described in the general chapter. Classification of HPV types was based on the epidemiological risk assessment of human papillomaviruses in the development of cervical cancer (87) and according to their phylogenetical origin (37); respectively.

e) Statistical analysis

Statistical analyses were performed using the SPSS program (Version 12.0). Descriptive statistics for continuous measures are given as the mean with standard deviation (SD) and range, frequencies and prevalences are given in row numbers and percentage.

II. 2. 3. Validity of combined cytology and human papillomavirus (HPV) genotyping with adjuvant DNA-cytometry in routine cervical screening

a) Screening population and procedures

The current analysis is restricted to a screening population of 31031 female inhabitants in the Bonn-region in Western Germany who participated in their annual cervical screening in gynaecological practices between January 1, 2002 and December 31, 2002. Cytology and/or biopsy material was sent to our institute for routine reporting and ancillary testing as described in the general section. The median age of women was 36 years (range: 17-81 years).

b) Cytology

A total of 49275 Pap tests (conventional and ThinPrep) were evaluated at our institute in 2002. Cytological diagnoses and specimen adequacy were classified according to the modified Munich (II) Cytological Classification and converted into the Bethesda 2001 terminology as described in the general section. Positive cytology results were defined as PapIIw or worse (\geq PapIIw; $\sim \geq$ ASC-US).

c) Histology

Tissue material for histology was taken from 123 women during the reported period. The primary endpoint of screening was the histological verification of high-grade intraepithelial neoplasia using the SIL/CIN classification (Bethesda 2001). A positive disease classification was made on histological evidence of CIN 2, CIN 3, carcinoma in situ, microinvasive or invasive carcinoma (\geq CIN2). Women with no worse than CIN1 (\leq CIN1) on reviewed histology were classed as negative for high-grade intraepithelial neoplasia or cancer.

Colposcopy and repeat cytology was recommended for women with cytological diagnosis of PapIIID and worse (\geq LSIL), whereas biopsy was indicated from PapIVa (HSIL) upwards. Colposcopy directed biopsy was also taken from women who had equivocal cytology diagnoses (Pap IIw, Pap III) on repeated smears within 6 months and from all those exhibiting clinical symptoms and or visible cervical changes on cervicography. Consequently, histological confirmation in cytology negative women and the majority of those with mild or equivocal cytology was not available for the present analysis. Random sampling from screen-negative women was not performed.

d) PCR-based HPV DNA detection and genotyping by sequence analysis

A total of 1454 HPV DNA testing was performed during the reported year after informed consent of the women and written application from the smear-takers had been obtained. Detailed protocols for the assays have been described in the general section.

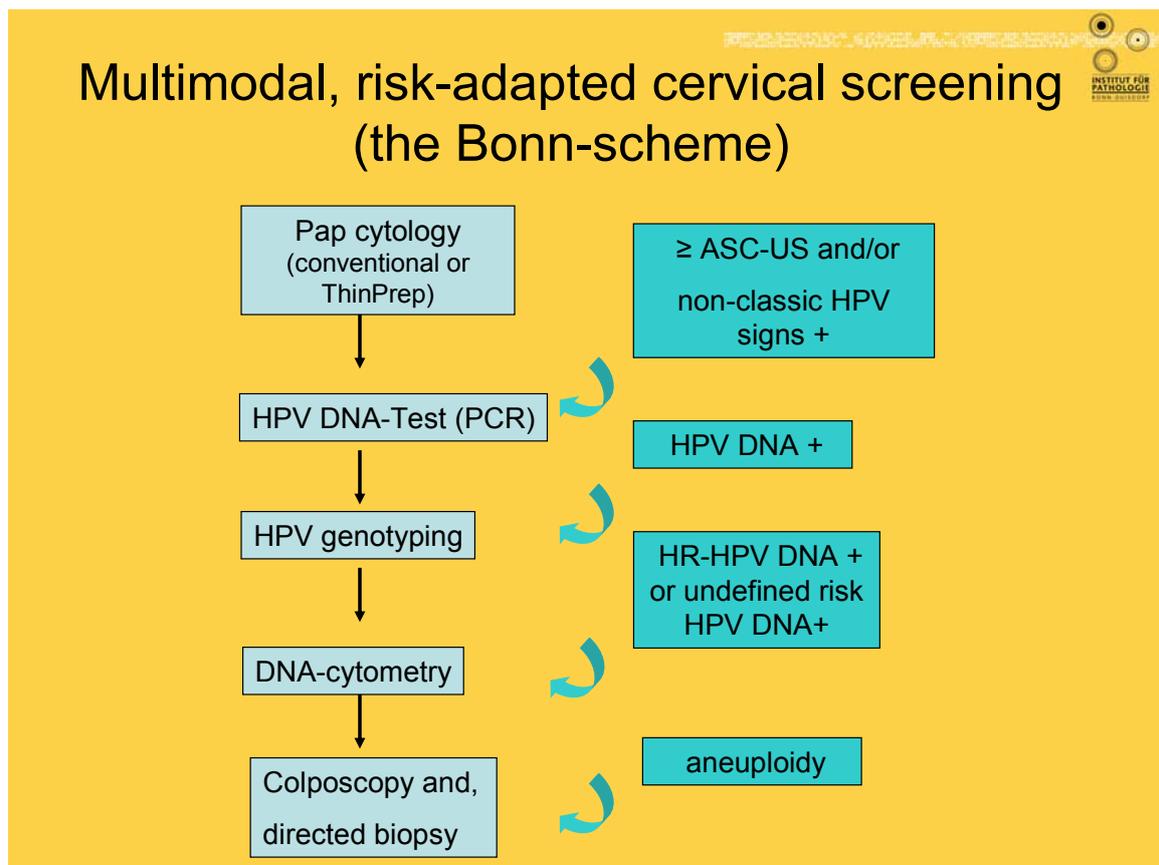
e) DNA image cytometry

DNA ploidy measurements were carried out from the same liquid-fixed cytological material that was used for cytology and HPV-testing in 81 women showing cytological abnormalities and/or infection with HR-HPV as described in the general section. For statistical analysis diploid and polyploid cases were grouped together as “normal/benign”, aneuploid cases were considered as lesions being at risk for progression into HSIL or worse.

f) Screening algorithm and patients management

The multimodal screening protocol included liquid-based cytology (ThinPrep®), PCR-based HPV detection and adjuvant DNA image cytometry. Screening algorithm and management of women with positive finding at any stage are demonstrated in Figure 8.

Figure 8: Multimodal risk-adapted cervical screening and management protocol



For the ideal case, the major steps included: 1) conventional Pap-test, or preferably by the ThinPrep technique with special focus on assessing HPV-related cytomorphological changes (classic and non-classic HPV signs) (13), 2) cytology results of \geq PapIIw are managed by using reflex PCR-based HPV DNA testing, 3) in HPV DNA + cases, genotyping by sequencing, 4) in high-risk HPV+ cases, DNA cytometry for assessing gross chromosomal instability as progression marker (16), 5) only DNA aneuploid cases should be referred to immediate colposcopy and directed biopsy, 6) treatment is reserved for biopsy-confirmed \geq CIN3 cases 7) after ablation, Pap-smears are to be scheduled at 6-months with a repeated HPV DNA test.

g) Statistics

The validity of the screening program by cytology was assessed by estimating the pickup rate of histologically confirmed high-grade disease as a surrogate marker of sensitivity. Specificity was derived by assessing the absence of histologically confirmed disease in cases reported cytologically as “abnormal” at the threshold of Pap IIw (ASC-US) and PapIII. This is in accordance with current general laboratory practice in screening a static population with stable disease prevalence. Test performance characteristics were calculated using 2x2 tables for each test modality individually and their combinations, if possible, in a group of 123 histologically verified cases taking histology diagnosis as diagnostic gold standard. Therefore, results of the different tests are to be interpreted as relative measures to each other and not as absolute values. Comparison of test results in the different groups was performed using the chi-square test and the Fisher’s exact test when frequencies were <5 .

III. Results

III.1. Cytomorphological and molecular basic features of HPV infection

III.1.1. Diagnostic value of minor non-classic HPV-related cytological alterations

Of the 164 women with Pap II and Pap IIw liquid-based cytology Pap test results, 76 (46.3%) had positive HPV result by PCR-based assays. Among the HPV positive cases, 41 were not sequenced either due to small amounts of PCR products or failing consent of the women, and were simply defined as “positive, not further specified” or “double infection, not further specified”. Eighteen of 35 sequenced cases (11%) were infected with high-risk HPV, including 6 cases with probably high-risk types and 1 sample harbouring double infection with high-risk types, respectively. Eleven samples (6.7%) were positive for low-risk HPV DNA and 6 cases (3.7%) for HPV types of undetermined risk, whereas eight samples (4.9%) showed double HPV infection (Table 3).

Table 3: Prevalence of HPV types* in positive cases (N=76).

High-risk types	Probably high-risk types	Low-risk types	Undet. ^o risk types	Positive; (nfs)	Double infection (nfs)
16 (N=7)	53 (N=4)	6 (N=1)	67		
33 (N=1)	66 (N=2)	42 (N=1)	(N=2)		
51 (N=1)		54 (N=2)	83		
58 (N=2)		61 (N=2)	(N=3)		
16+31(N=1)		70 (N=3)	84		
		72 (N=1)	(N=1)		
		54+70 (N=1)			
Total: 12	6	11	6	35	6

*Classification of HPV types is based on the epidemiological risk assessment of human papilloma viruses in the development of cervical cancer (85). ^o Undetermined; nfs = HPV positive, not further specified

In the HPV positive group, a mean of 6.68 cytomorphological signs was found in each monolayer in contrast to the HPV negative group, with a mean of 3.8 signs per ThinPrep, respectively. This indicates a significant difference between the two groups. Univariate statistical analysis by using the chi-square test confirmed highly significant associations for the diagnosis of IIw (69 Pap IIw/76 HPV+ vs. 74 Pap II/88 HPV-) and for almost each non-classic cytological signs with detection of overall HPV positivity, except cytoplasmic folding and keratohyalin-like granules. Presence of mild nuclear hyperchromasia, mild dyskeratosis, measles cells, abortive koilocytes and bi/multinucleation revealed the most significant association with HPV infection (Table 4). Prevalence and diagnostic accuracy of each cytological feature for detecting HPV infection are presented in Table 5.

Table 4: Univariate associations between diagnosis, mild cytological abnormalities and HPV infection in decreasing degree of significance

	Univariate χ^2 (log-rank test)	P value
Diagnosis (Pap II vs. Pap IIw)	91.89	0.000
Mild nuclear hyperchromasia	35.802	0.000
Mild dyskeratosis	30.014	0.000
Measles cells	28.702	0.000
Abortive koilocytes	20.344	0.000
Bi/multinucleation	19.994	0.000
Parakeratosis	16.303	0.000
Mild nuclear variations	15.890	0.000
Macrocytes	8.333	0.004
Cytoplasmic folding	2.077	n.s.
Micronuclei	1.399	n.s.

Abbreviations: n.s.= non-significant, P= probability* All parameters were classified as being present (1) or absent (0); degree of freedom was 1.

Table 5: Diagnostic accuracy of each 10 mild cytological abnormalities for cytomorphological detection of HPV infection

	Prevalence	Sensitivity	Specificity	PPV	NPV
	%	%	%	%	%
Mild nuclear hyperchromasia	38	87	59	65	59
Mild dyskeratosis	56	80	63	65	79
Measles cells	56	79	63	65	77
Abortive koilocytes	54	73	62	62	73
Bi/multinucleation	74	91	40	57	83
Parakeratosis	18	32	93	79	62
Mild nuclear variations	38	55	76	66	66
Makrocytes	61	73	49	56	68
Cytoplasmic folding	57	64	48	51	60
Keratohyalin-like granules	42	47	62	51	58

PPV= positive predictive value, NPV= negative predictive value

The highest sensitivity was achieved by bi/multinucleation (90.78%), followed by mild nuclear hyperchromasia (86.84%) and mild dyskeratosis (79.70%). Highest sensitivities were estimated for parakeratosis (93.02%), mild nuclear variations (75.86%) and measles cells/ dyskeratosis (63.21 and 63.20%, respectively).

Correlation analysis revealed highly significant co-expression of the following variants at the threshold of two-tailed $P < 0.05$: abortive koilocytes and measles cells (Pearson's $r = 0.71$), mild nuclear hyperchromasia and mild nuclear variations (Pearson's $r = 0.61$), macrocytes and cytoplasmic folding (Pearson's $r = 0.55$) (table not shown).

Based on these correlations and biological considerations, we combined the cytomorphological features into i) *mild nuclear changes* consisting of mild hyperchromasia, mild nuclear variations and bi/multinucleation, ii) *disorders of keratinisation* consisting of mild dyskeratosis and parakeratosis, iii) *measles cells and abortive koilocytes*, and iv) *degenerative changes* comprising macrocytes, cytoplasmic folding and keratohyalin-like granules.

By using these categories, we attained a maximum sensitivity and negative predictive value (100% each, respectively) for the parameter “mild nuclear changes”. It is of note, that parallel to this, specificity was very low (31%), resulting in 37% (61/164) false positive cases. Diagnostic accuracy of the 4 combined cytology parameters for detecting HPV infection is demonstrated in Table 6.

Table 6: Diagnostic accuracy of combined mild cytological abnormalities for cytomorphological detection of HPV infection

	Prevalence	Sensitivity	Specificity	PPV	NPV
	%	%	%	%	%
Mild nuclear changes	84	100	31	55	100
Disorders of keratinisation	60	84	61	65	82
Abortive koilocytes and measles cells	62	83	55	61	79
Degenerative changes	78	84	27	50	66

PPV= positive predictive value, NPV= negative predictive value

Due to the low number of cases in the different HPV risk groups, no statistical correlation could be computed between different HPV types and a specific constellation of cytomorphological features. However, cytomorphological discrimination between low-risk HPV positive and high-risk HPV positive cases does not seem to be possible (Table 7).

Table 7: Prevalence of combined mild cytomorphological signs in cases with high-risk HPV and low-risk HPV infection.

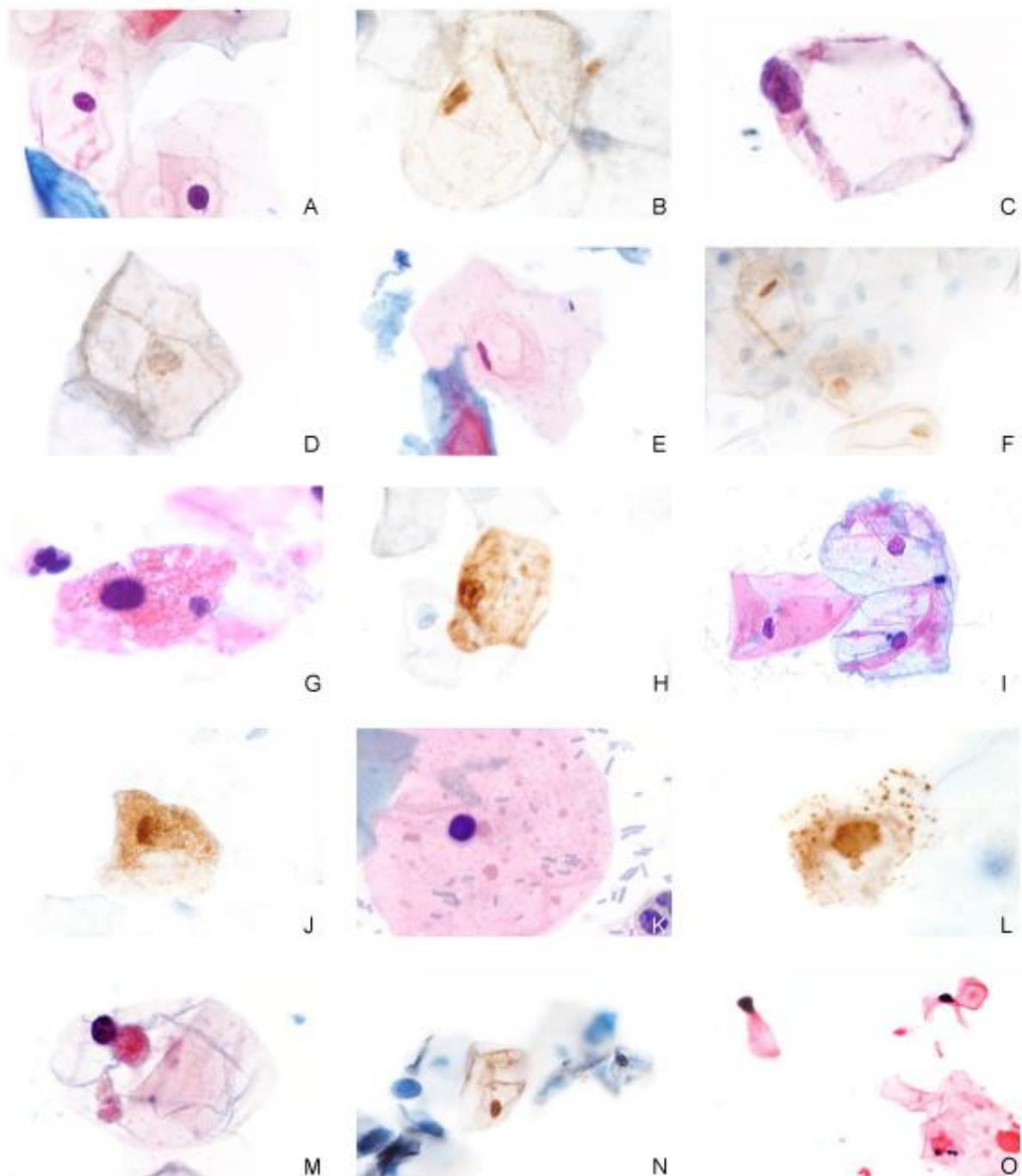
	High-risk HPV (N=18)	Low-risk HPV (N=11)
Mild nuclear changes	18	11
Disorders of keratinisation	16	8
Abortive koilocytes and measles cells	15	10
Degenerative changes	12	10

III.1.2. Molecular basic features of HPV infection

a) Immunohistochemical expression of the HPV L1 capsid protein and HPV DNA in situ hybridisation (CISH)

The cytomorphological correlation of the “non-classical” HPV signs and the HPV L1 expression (IHC) or HPV DNA evidence (CISH) is shown in Figure 9.

Figure 9: HPV L1 expression (IHC) and HPV DNA in situ hybridisation (CISH)



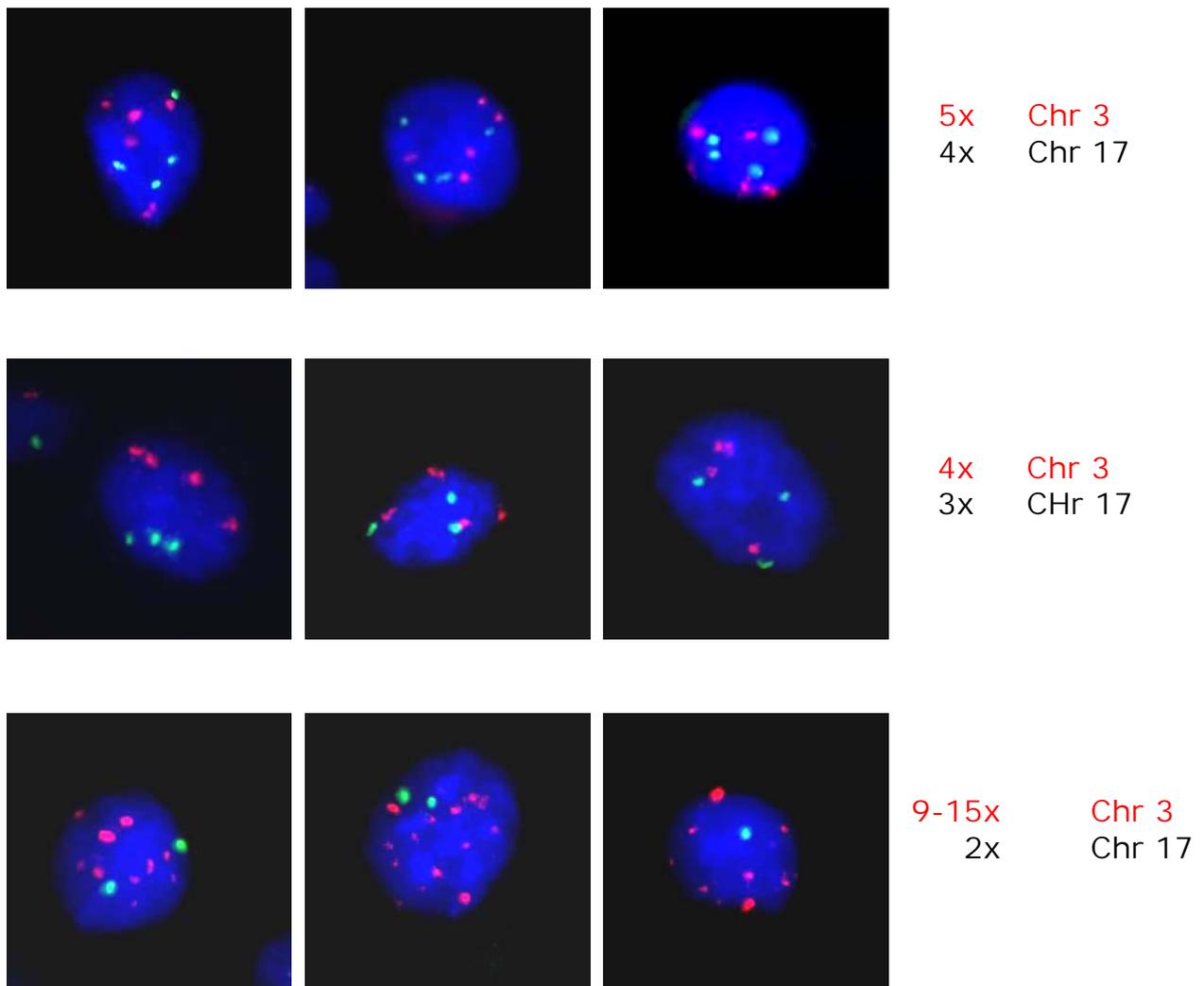
Legend Figure 9:

A) an abortive koilocyte [lower right position] showing some perinuclear cytoplasmic clearing around a small, pycnotic nucleus and cytoplasmic folding (Pap-staining); **B)** immunohistochemical (IHC) detection of HPV-L1 capsid protein (Cytoactiv®) both in the elongated angled nucleus and along the cytoplasmic folding; **C)** a ballooned macrocyte with a peripherally displaced nucleus (Pap-staining); **D)** HPV-L1-detection (IHC) both in the nucleus and along the cytoplasmic folding; **E)** spindle-shaped nucleus in a degenerated superficial cell, abortive koilocyte (Pap-staining); **F)** HPV-L1-detection (IHC) both in the nucleus and in the cytoplasm; **G)** a so called “measles” cell showing condensation of the entire cytoplasmic matter into keratohyalin-like granules and a slightly enlarged nucleus; **H)** HPV-L1-detection (IHC) both in the nucleus and in the cytoplasm of a measles-cell; **I)** strong cytoplasmic folding, amphophilia and spindle-shaped nucleus [lower left position] (Pap-staining); **J)** HPV-L1 detection (IHC) both in spindle-shaped nucleus and in cytoplasmic granules; **K)** a superficial cell with pseudo-hyalin granules and a hyperchromatic nucleus; **L)** HPV-L1 detection (IHC) both in the nucleus and in cytoplasmic granules; **M)** eosinophilic condensed cytoplasm at paranuclear location and cytoplasmic folding (Pap-staining); **N)** HPV-L1 detection (IHC) both in the nucleus and along cytoplasmic foldings; **O)** HPV-DNA detection in degenerated cells using chromogenic in situ Hybridisierung (CISH): positive staining in the nuclei of both „kite cells“ and dot-like reaction in the eosinophilic condensed cytoplasm within the cell in the lower right position.

b) HPV caused chromosomal imbalance with fluorescence in situ hybridisation (FISH)

All cells from the larger than 5c and 9c DNA content gate were repositioned in the cytometer after performing dual color FISH with DNA probes specific to chromosome 3 and 17. Aneusomy of both of the analyzed chromosomes was significantly more frequent in 5c cells and further increased in 9c cells in contrast to control cells from the near-to-normal DNA content range ($p < 0.005$). Samples with high proportions of polysomy showed low levels of aneusomy and vice versa. In three of the analyzed cases aberrant FISH pattern indicating aneusomy for chromosome 3 was found in more than 705 of the 9c cells. A similar accumulation of aneusomy 17 was also observed in 9c cells (Figure 9).

Figure 9: Recurrent chromosomal imbalance in polyploid cells of HSIL (Chr=Chromosome)



c) Diagnostic and prognostic value of the detection of E6/E7 mRNA transcripts in cervical cytologies up to PapIVa (HSIL/ in situ carcinoma)

Sixty-six women were included in this study arm (mean age: 34.6 years; range: 21-66 years). In thirty-three cases, histopathological diagnoses were also available after surgical treatment of the cervical lesions. Cross-tabulation of cyto- and histopathological diagnoses is demonstrated in Table (7). The chi-square statistics proved highly significant association between results of the two diagnostic tests (Pearson's χ^2 : 43.997; $P=0.001$).

Table 8: Distribution of and association between cyto- and histopathological diagnoses in 33 of 66 women investigated for HPV mRNA expression in their cervical diseases

HISTOLOGY					
CYTOLOGY	no histo	no CIN	CIN II	CIN III	Total
Pap II	3	0	0	0	3
PapIIw/ASC-US	18	2	0	3	23
Pap 3D/LSIL	11	2	3	3	19
Pap 3D/HSIL	0	2	0	9	11
Pap IVa/HSIL	1	0	2	7	10
Total	33	6	5	22	66

Highly significant association was computed between HPV genotypes and the mRNA types expressed in the samples (Pearson's Chi-square: 147,283, P = 0.000) as demonstrated in Table 8.

Table 9: Association between HPV genotypes and HPV mRNA expression

HPV-DNA	HPV mRNA							total
	16	16 + 45	18	31	33	45	neg.	
16	26	1	-	-	-	-	10	37
16+18+42	-	-	-	-	-	-	1	1
16+31	2	-	-	1	-	1	-	4
18	-	-	2	-	-	-	1	3
31	-	-	-	1	-	-	6	7
31+51	-	-	-	-	-	-	1	1
33	-	-	-	-	2	-	2	4
45	-	-	-	-	-	2	1	3
neg.	-	-	-	-	-	-	4	4
weak pos.	-	-	-	-	-	-	2	2
total	28	1	2	2	2	3	28	66

Four cases were both negative for HPV-DNA and mRNA. All HPV-mRNA positive cases were also positive for HPV-DNA, whereas 24 HPV-DNA positive cases did not express transcribed mRNA. Diagnostic validity parameters for the HPV mRNA test (NorChip) for detecting HPV DNA were calculated using 2x2 tables as shown in Table 9.

Table 10: Diagnostic validity of the HPV mRNA test (NorChip)

	DNA positive	DNA negative	total
mRNA positive	38	0	38
mRNA negative	24	4	28
total	62	5	66

Sensitivity: 61.29% (48-73; 95% CI),

Specificity: 100%,

Positive predictive value (PPV): 100%

Negative predictive value (NPV): 14.28% (4-32; 95% CI).

Thirty-four patients had cytological progression and 32 patients showed cytological regression or persistence of their cervical diseases within 12 months follow up. Based on these associations, the prognostic validity for the HPV mRNA test (NorChip) was calculated, as shown in Table 10.

Table 11: The prognostic power of the HPV mRNA (NorChip) test

	Disease progression	Disease regression	
mRNA positive	28	10	38
mRNA negative	6	22	28
	34	32	66

Sensitivity: 82.35 % (0.69-0.95; 95% CI)

Specificity: 68.75% (0.52-0.84; 95% CI)

Positive predictive value (PPV): 73.68 (0.59-0.87; 95% CI)

Negative predictive value (NPV): 78.57% (0.63-0.93; 95% CI)

III.2. HPV genotype distribution and the spectrum of cervical lesions induced by low-risk and undefined-risk HPVs

Overall, 30 different HPV types were detected in the 293 patients investigated in this study arm: 11 low-risk types (LR-HPVs) and 19 undetermined-risk types (UR-HPVs); the latter group included 3 novel HPV genotypes. LR-HPVs contained HPV 6, 11, 40, 42, 43, 54, 61, 70, 72, 81, and CP6108; UR-HPVs contained all other types detected that are not assigned as low-risk, high-risk or probable high-risk types yet in the epidemiological classification of HPVs published by Munoz and coworkers (85). Respective data are demonstrated in Tables (11) and (12). There was no difference in the mean age of the patients in the different groups with LR- or UR-HPV infections (32.97 and 32.89 years, respectively).

Table 12: The prevalence of low-risk HPV types according to their corresponding cytological diagnoses

LR-HPV types	No. of cases (n)	no-ASC (n)	ASC-US (n)	LSIL (n)	HSIL (n)
6	38	3	21	12	2
11	5	2	0	3	0
40	1	0	1	0	0
42	45	6	28	9	2
43	3	1	1	1	0
54	11	2	7	2	0
61	38	4	26	5	3
70	22	1	13	7	1
72	1	0	1	0	0
81	12	1	8	3	0
CP 6108	1	0	1	0	0
Total	176	20	107	43	8

Table 13: The prevalence of unknown-risk HPV types according to their corresponding cytological diagnoses

UR-HPV types	No. of cases (n)	no-ASC (n)	ASC-US (n)	LSIL (n)	HSIL (n)
30	3	0	1	0	2
32	5	0	4	1	0
34	1	0	0	0	1
62	11	3	6	2	0
67	16	0	8	8	0
69	1	0	1	0	0
71	1	0	1	0	0
74	3	0	3	0	0
83	14	1	9	4	0
84	18	1	12	5	0
86	3	0	3	0	0
87	2	0	2	0	0
90	19	1	16	1	1
91	11	0	6	3	2
CP8304	4	1	3	0	0
I A09	1	0	1	0	0
JC 9710	1	0	0	1	0
MM 4	1	0	0	1	0
W13B	1	0	0	0	1
Total	117	7	76	26	7

Abbr.: no-ASC = normal, or inflammatory cytology (Pap I-II); ASC-US = atypical squamous cells of undetermined significance (PapIIw, non-classic HPV signs)

LR-HPV = low-risk HPVs; UR-HPVs = unknown-risk HPVs

The most frequent cytological diagnosis was ASC-US (n = 183; 63%); 107 of the cases (59%) were associated with LR-HPVs and 76 cases (41%) with UR-HPVs. The second largest cytological group was LSIL (n = 69; 23%); about two-thirds of the cases (43/69) were associated with LR-HPVs and one-third (26/69) with UR-HPVs. Twenty-seven cases (9%) were negative for intraepithelial lesion or malignancy (no-ASC) indicative of reactive, inflammatory or atrophic changes. About three-quarters (20/27) of the cytologically “negative” cases were associated with LR-HPVs and one-quarter (7/27) with UR-HPVs.

None of the patients had cervical carcinoma; 15 women (5%) had HSIL in cytology – of these, 2 were histologically diagnosed as carcinoma *in situ*, 8 as CIN3 and 7 as CIN2 lesions. Seven HSILs were associated with UR-HPVs (2 x HPV 30, 1 x HPV34, 1x HPV 90, 2 x HPV 91 and 1 x W13B) and 8 with LR-HPVs (2 x HPV6, 2 x HPV 42, 3x HPV 61 and 1 x HPV 70)

The most prevalent HPV types in the study population were: HPV 42 (n= 45; 15%), HPV 6 and HPV 61 (both n=38; 13%), HPV 70 (n=21; 7%), HPV 90 (n= 19; 6.5%) and HPV 84 (n=18; 6.1%).

There was no difference in the mean age of the patients in the different groups with LR- or UR-HPV infections (32.97 and 32.89 years, respectively).

After re-classifying our data according to the phylogenetical grouping of known HPVs (37), 12 of 19 UR-HPVs could be assigned either as LR types (HPV 32, 62, 83, 84, 86, 87, 91, 74) or probable high-risk types (HPV 69, 30, 67, 34) as demonstrated in Tables 13 and 14.

Table 14: Phylogenetical grouping, corresponding epidemiological risk-classification and cytological findings in monoinfections with low-risk HPVs from the alpha genus

Genus/type	HPV type (species)	No. of cases (n)	no-ASC (n)	ASC-US (n)	LSIL (n)	HSIL (n)
known low-risk HPV types						
(and probable low-risk types based on genetical relationship or no proven association with cancer)						
α1	32	5	0	4	1	0
	42	45	6	28	9	2
α3	61	38	4	26	5	3
	72	1	0	1	0	0
	81	12	1	8	3	0
	62	11	3	6	2	0
	83	14	1	9	4	0
	84	18	1	12	5	0
	86	3	0	3	0	0
	87	2	0	2	0	0
α7	70*	21	1	13	7	1
α8	40	1	0	1	0	0
	43	3	1	1	1	0
	91	11	0	6	3	2
α10	6	38	3	21	12	2
	11	5	2	0	3	0
	74	3	0	3	0	0
α13	54	11	2	7	2	0
?	CP 6108	1	0	1	0	0
?	CP 8304**	4	1	3	0	0

Types written in black were classified epidemiologically by Munoz et al. (85). Types written in red have not been classified epidemiologically according to risk for cervical cancer.

Table 15: Phylogenetical grouping, epidemiological risk-classification and cytological findings in mono-infections with probably high-risk, unknown-risk and novel anogenital HPVs from the alpha genus

Genus/type	HPV type (species)	No. of cases (n)	no-ASC (n)	ASC-US (n)	LSIL (n)	HSIL (n)
probable high-risk HPV types						
(based on genetical relationship or rare association with cancer)						
$\alpha 5$	69	1	0	1	0	0
$\alpha 6$	30	3	0	1	0	2
$\alpha 9$	67	16	0	8	8	0
$\alpha 11$	34	1	0	0	0	1
?	MM 4***	1	0	0	1	0
unknown-risk HPV types						
$\alpha 15$	71	1	0	1	0	0
	90	19	1	16	1	1
novel HPV types						
?	I A09	1	0	1	0	0
?	JC 9710	1	0	0	1	0
?	W13B	1	0	0	0	1

Types written in black were classified epidemiologically by Munoz et al. (85), types written in red and blue have not been classified according to risk for cervical cancer, yet and types written in green are novel HPV genotypes.

Concerning cytology, all negative smears (no-ASC) and ASC-US cases showed at least one but generally more minor non-classical cytological alterations indicative of HPV effect. These included in decreasing order of frequency: mild nuclear changes, disorders of keratinisation, abortive koilocytes and “measles” cells as well as degenerative changes, as published recently by our group (16) and demonstrated in Figure 7.

The majority of LSIL and HSIL cases showed a combination of both classic (koilocytosis and dyskeratosis) and non-classic HPV-signs.

No cytological progression of lesions was observed in 243 patients in a mean follow-up period of 23 months (range: 6-84 months).

III.3. Efficiency of a risk-adapted multimodal screening and management strategy (the Bonn-protocol) for cervical cancer prevention

1. Global results of cytology, histology, HPV testing and DNA ploidy for the whole screening population in the year 2002

A total of 49275 Pap smears (conventional and ThinPrep®) from 31031 women [mean age: 39.3 years (range: 17-81 years)] were analysed. Prevalence and distribution of different cytological and histological diagnoses are demonstrated in Tables 15 and 16.

Table 16: Global prevalence and distribution of cytological diagnoses for 31031 women screened for cervical cancer and pre-cancer in 2002

<i>Pap test results</i>	Number (%)
I-II	47503 (96.4%)
IIw	1265 (2.7%)
III	22 (0.04%)
IIID	430 (0.8%)
IV	48 (0.009)
V	7
Total	49275 (100%)

Prevalence of „within normal limits“ and abnormal cytology in the screening population	
Pap I-II	95.8% (29740/31031)
Pap ≥ IIw	4.2% (1291/31031)

Table 17: Histological diagnoses in 123 women from the screening population, who were treated surgically in 2002

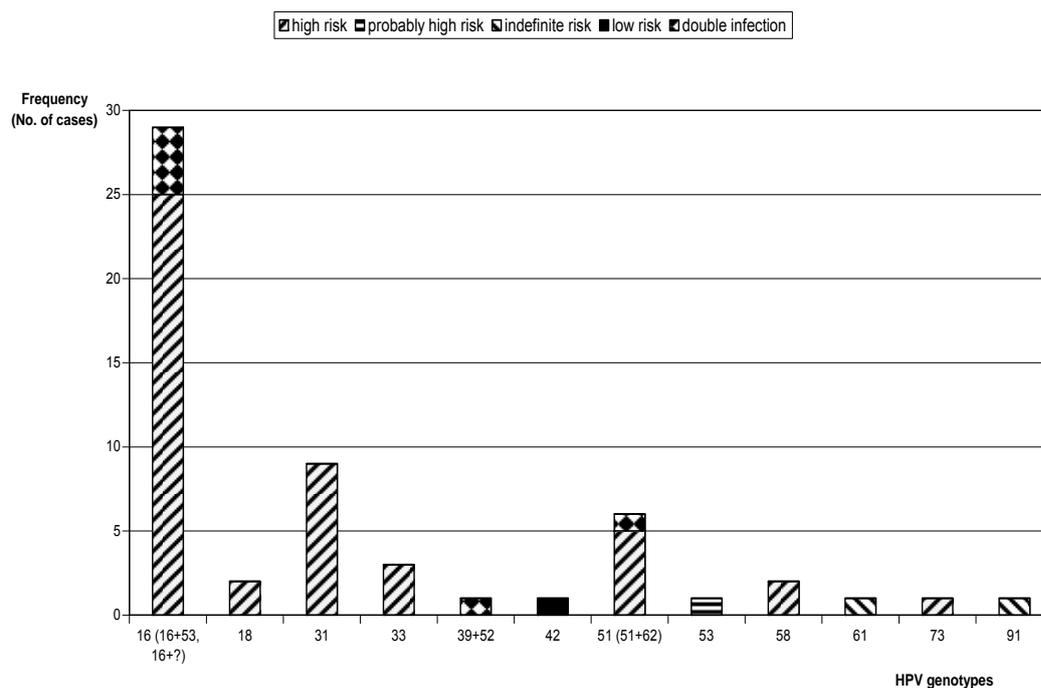
Histology results	
Negative for HPV or dysplasia	3 (2.4%)
Condyloma acuminatum	5 (4.0%)
CIN1	15 (12.2%)
CIN2	19 (15.4%)
CIN3	33 (26.8%)
Carcinoma in situ	35 (28.5%)
Carcinoma	13
Total	123 (100%)

Prevalence of histologically confirmed cervical disease in the screening population	
≥CIN2	0.32 %(100/31031)
Carcinoma	0.04% (13/31031)

The prevalence of histologically verified moderate and higher grade cervical neoplasias (\geq CIN2) in the total screening population was 0.32 %; the prevalence of invasive carcinoma *per se* was 0.04%. There was a highly significant correlation between cytological and histological diagnoses (Pearson's $R = 25.6$, $P = 0.000$). The overall sensitivity of our cytology screening program for detecting \geq CIN2 lesions was 81.3%, program specificity was in 96.5% at the cytology threshold of \geq PapIIw (\approx ASCUS).

Of 560 reflex HPV-DNA tests, 61.8% were positive with a prevalence of 36.4% in PapIIw, 57.1% in PapIII, 92% in PapIIID and 100% in PapIV cytology classes. HPV DNA sequencing detected infections with a single HPV type in 56.5%, double or multiple infections in 16.7% of cases, whereas genotyping could not be carried out in 26.8% of the cases due to small virus load or insufficient PCR-product. All \geq CIN2 cases were HPV positive, however the presence of HR-HPV DNA was not significantly associated with \geq CIN2 in histology. HPV prevalence and genotype distribution is demonstrated in Figure 10.

Figure 10: Prevalence and distribution of HPV genotypes in 57 of 123 histologically verified cases



Of 81 DNA histograms, 18 cases (22.2%) were diploid, 6 (7.4%) were polyploid and 57 (70.3%) were aneuploid. All 10 cases with PapIVa-b and V cytology showed aneuploid DNA profile (100%), whereas the Pap group IIID included 44/63 (69.8%) aneuploid, 15/63 diploid and 4/63 polyploid cases. In the PapIIw class (n=3), all three DNA profiles occurred once each. An aneuploid DNA profile was significantly associated with the presence of \geq CIN2 lesion in histology (chi square =6.86, P=0.008)

Figure 11: A diploid DNA histogram.

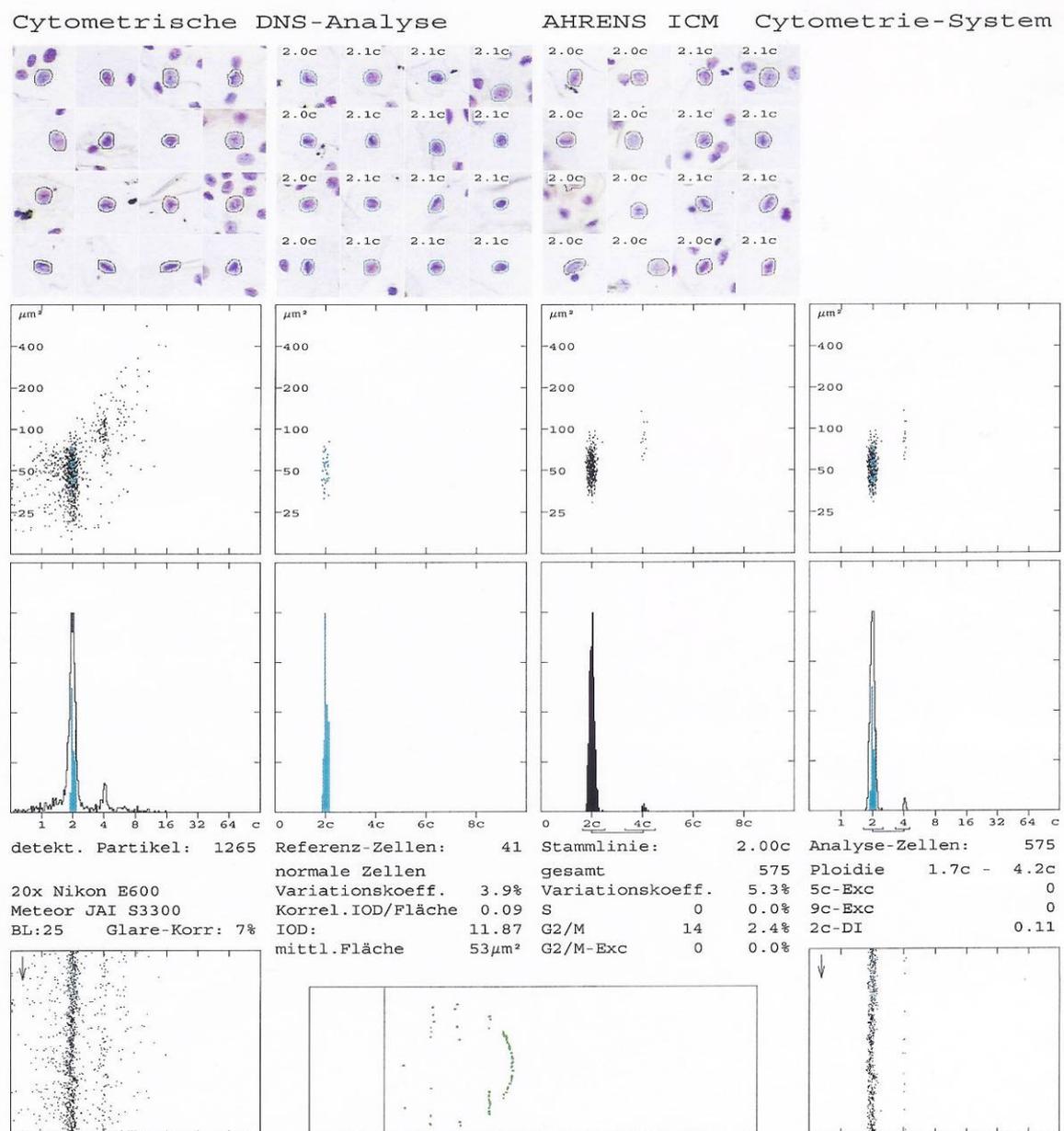


Figure 12: A polyploid DNA histogram.

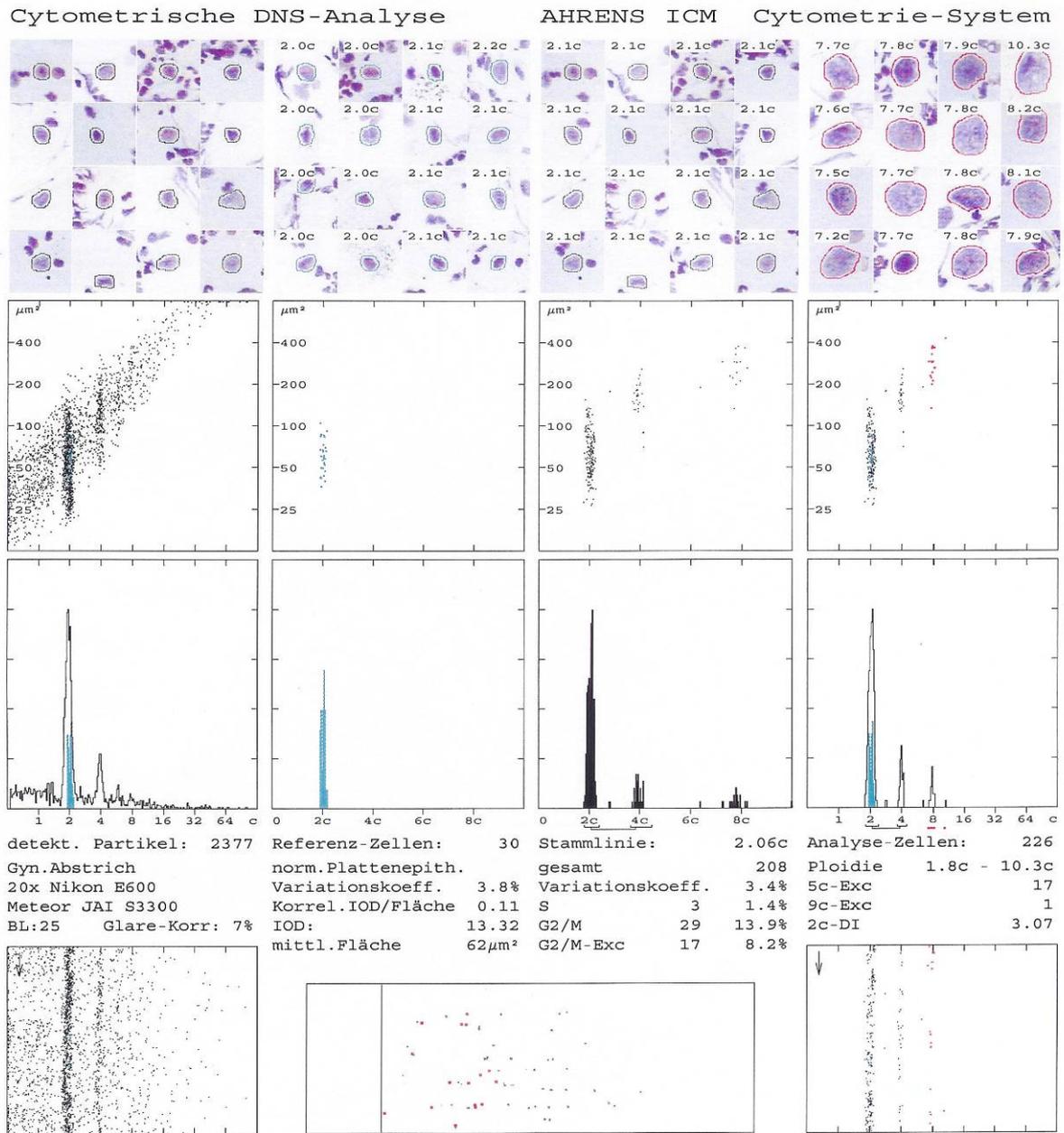
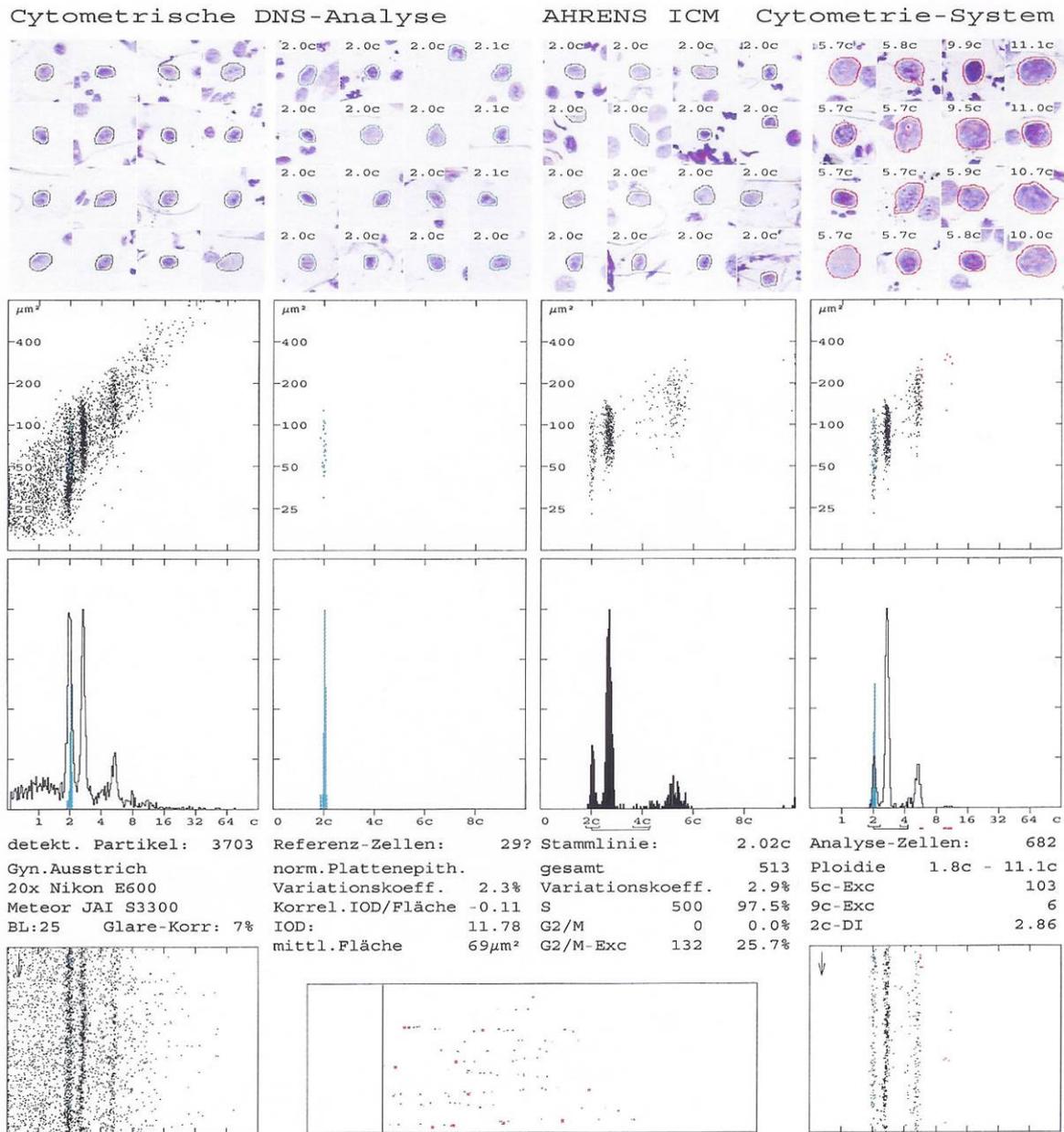


Figure 13: An anaploid DNA histogram.



As single techniques, HPV genotyping had the highest positive predictive value (PPV) for \geq CIN2 (85.2%), followed by DNA cytometry and cytology (83.7% and 81.3%, respectively) as demonstrated in Table 18.

Table 18: Positive predictive value (PPV) of single tests for \geq CIN2 lesions

Histology	Cytology*	HPV genotyping [#]	DNA cytometry [§]
\geq CIN 2	81.3%	85.2%	83.7%
\geq CIN 3	65.9%	66.7%	63.3%
\geq pT1a	10.6%	1.9%	2.0%
Carcinoma	9.8%	-	2.0%
Total number of tests	123	57	54

* positive cytology = Pap IIw-V

[#] threshold for HPV genotyping: HR+ *versus* NOT HR +

[§] threshold for DNA cytometry: aneuploidy *versus* diploidy and/or polyploidy

2. Comparison of relative test performances and correlations between cytology, HPV testing, DNA ploidy and histology

These results were achieved by the analysis of 123 cases with histologically verified diagnosis of \geq CIN2. HPV testing by PCR was performed in 63 of 123 cases, all results were positive. HPV genotyping could be carried out in 57/63 cases, which revealed infection with high-risk HPV (HR-HPV) types in 53 of 57 cases (93%), low-risk HPV types in 2 and undefined risk HPV types in 2 samples. The presence of HR-HPV DNA was not significantly associated with \geq CIN2 in histology. Eight of 57 sequenced cases (14%) were $<$ CIN2, 7 of them were CIN1 (5 aneuploid, 1 diploid, 1 without DNA profile), and one case was negative in histology.

An aneuploid DNA profile was significantly associated with the presence of \geq CIN2 lesion in histology (chi square =6.86, P=0.008). Nine of 54 analysed cases (16.6%) were false positives, 7 of them were CIN1 (5 with HR-HPV positivity, 1 with low-risk HPV infection, two without HPV testing) and 1 case was a condyloma acuminatum.

The PPVs of combined strategies for different cervical lesions are shown in Table 19.

Table 19: Positive predictive value (PPV) of test combinations for detecting histologically verified cervical diseases

Histology	Cytology + HPV genotyping	Cytology + DNA cytometry	Cytology + HPV genotyping + DNA cytometry
HPV signs and \geqCIN 1	100%	100%	100%
\geq CIN 1	98.2%	98%	100%
\geq CIN 2	82.0%	82%	88.2%
\geq CIN 3	69.1%	62%	67.6%
\geq pT1a	1.8%	2.0%	-
Carcinoma	-	2.0%	-
Total number of tests	57	54	38

The highest PPV for \geq CIN2 (88.2%) was achieved by the combination of all three methods.

Cumulative relative test performance characteristics are demonstrated in Table 20. Efficacy measures of cytology at \geq Pap IIw threshold are restricted to PPV and detection rate (81.3% for each). Test sensitivity was identical for HPV genotyping, DNA cytometry and the combination of cytology and HPV-genotyping (app. 98%, each). Even the less sensitive method, which was the combination of all the three test modalities, has achieved 91% in sensitivity. The highest specificity had DNA cytometry (25%), followed by the three-test combination (20%). The highest PPV was achieved by the three-test combination (88%), whereas the highest NPV was yielded by DNA cytometry. Pre-test probability of a positive test result could be multiplied by a factor of maximum 1.3 by an aneuploid DNA profile.

False positive rate was the lowest with DNA cytometry (75%) and the highest using HPV genotyping (100%). Detection rate of \geq CIN2 was the highest using the combination of cytology and HPV genotyping. The most efficient test for detecting \geq CIN2 was the combination of cytology and HPV genotyping (82.5%). Even the least efficient test method, DNA cytometry alone, could correctly detect 81% of \geq CIN2.

Table 20: Cumulative relative test performance characteristics of single tests and combinations in 123 women with PapIIw-V cytology (threshold \sim ASCUS) who had histological verification of their cervical disease (threshold \geq CIN2)

Relative test performance characteristics									
	n	Sensitivity %	Specificity %	PPV %	NPV %	[§] LR+	⁺ False+ rate %	[#] Detection rate %	Efficiency %
for \geq CIN2	100								
Cytology (\geq PapIIw)*	123	n.d.	n.d.	81.3	n.d.	n.d.	n.d.	81.3	n.d.
HPV-HR+	57	97.9	11.1	85.2	50.0	1.10	100.0	80.7	80.7
DNA-aneuploidy	54	97.6	25.0	83.7	75.0	1.30	75.0	75.9	81.5
\geq PapIIw and HPV-HR+	57	97.8	10.0	83.6	50.0	1.08	90.0	80.7	82.5
\geq PapIIw and DNA-aneuploidy	54	95.3	18.0	82.0	50.0	1.16	81.8	75.9	79.6
\geq PapIIw and HPV-HR + and DNA-aneuploidy	38	90.9	20.0	88.2	25.0	1.14	80.0	78.9	81.6

n.d. = not defined; *all cases were positive at cytology threshold \geq PapIIw, thus no 2x2 tables could be constructed.

Setting the threshold of cytology at \geq Pap IIID/HSIL, sensitivity was 87%, specificity 50%, PPV 89.7%, NPV 50%, LR+ 1.74, LR- 0.26, false+ rate 43.5%, detection rate 70.7% and efficiency 81.3%.

[§]The positive likelihood ratio (LR+) was calculated as sensitivity/(1-specificity)

⁺False positive rate was the ratio of false positive results with the index test among definite or suspected non-diseased women.

[#] Detection rate was calculated by dividing the number of true positive cases for the index test by the total number analysed

3. Accuracy of single tests and combinations in the multimodal screening setting

Of 38 women receiving the complete screening protocol, 31 had \geq CIN2 and 7 had CIN1 in histology. All cases would have been detected by cytology alone (1 PapIII, 30 PapIIID, 7 Pap IVa). Regarding disease detection, 7 cases were false positive (all CIN1, 6 HR-HPV+, 1 LR-HPV+, 6 aneuploid, 1 diploid). The PPV for \geq CIN2 by cytology alone was 88.2% (30/34) the detection rate was 79% (30/38).

All 38 cases were positive for HPV-DNA by PCR analysis. On sequencing, 36 cases contained HR-HPV DNA (16x HPV 16, 1x HPV 18, 3x HPV 33, 8x HPV 31, 5x HPV 51, 2x HPV 58, and 1x HPV 73 types), 1 sample had low-risk HPV DNA (type 42) and another one had an unknown risk type (HPVU 91). Regarding disease detection by a HR-HPV+ test, there were 6 false positive cases (all CIN1, 5 aneuploid, and 1 diploid). The PPV for \geq CIN2 by HPV genotyping alone was 83.0% (30/36) the detection rate was 79% (30/38).

Thirty-six of 38 cases had aneuploid DNA content; two cases were diploid (one CIN1 and one CIN2). Using DNA as a single test, 6 false positive cases (all CIN1, 5 HR-HPV+, 1 LR-HPV+, and one with PapIIID/LSIL cytology) and one false negative case (CIN2, HR-HPV+) would have been detected. The PPV for \geq CIN2 by DNA cytometry alone was 83.0% (30/36) the detection rate was 79% (30/38).

The combination of all three modalities resulted in the highest PPV (88.2%) and the second highest efficiency (81.6%) with a false positive rate of 80%. Sensitivity was the lowest (90.9%) and specificity the second highest (20%) among the other modalities investigated. Test efficiency did not differ significantly between the different screening strategies (Table 19).

IV. Discussion

Cervical cancer screening is acknowledged as currently the most effective approach for cervical cancer control. Despite never being examined in a randomised controlled clinical trial, a large body of consistent observational data supports the fact that regular cytology-based cervical screening programmes with the Papanicolaou (Pap) test have been effective in reducing the incidence of and mortality from cervical cancer (64). However, there is also good evidence for the potential harms of the Pap test. These lie on the one hand in the detection and (over)treatment of many lesions that would never progress to cervical cancer, which cause unknown long-term consequences on fertility and pregnancy especially in younger women. On the other hand, there is a high rate of undetected high-grade pre-cancerous lesions and even carcinomas suggesting problems with the sensitivity of the test. These data indicate that a plateau of what can be achieved by conventional cytology is now being reached.

In the meantime, the fundamental role of human papillomaviruses (HPV) in the aetiology of cervical cancer has been firmly established. Accordingly, infection with oncogenic HPV types is now being considered as the primary cause of almost all cervical carcinomas with persistent infection with certain high-risk HPV types representing the first step in cervical carcinogenesis (133, 18, 85).

Therefore, it is timely to consider the role of HPV testing within the cervical screening programme. Furthermore, it is also timely to consider rational and biologically relevant combinations of different test modalities, including the detection of certain molecular biomarkers, to enhance the net effectiveness of screening and enable prognostication of prevalent cervical lesions.

In the present work, we were aiming at getting better insight into the natural history of cervical carcinogenesis and directly transferring novel scientific results and enhanced research methodology in routine diagnostic pathology.

IV. 1. Cytomorphological and molecular basic features of cervical HPV infection

Human papillomavirus testing is becoming an important tool in cervical cancer screening. The objectivity of the HPV test enhances the performance of cervical cytology and helps to clarify the meaning of an equivocal Pap smear (68). The ASCUS-LSIL Triage Study (ALTS) has clearly demonstrated that HPV testing, by the Hybrid Capture II assay, can be safely used in the triage of Pap tests showing atypical squamous cells of undetermined significance (ASCUS), with approximately half of women who are HPV negative not requiring colposcopy (1).

In our present study of 164 liquid based cytology specimens with mild cytological abnormalities roughly equating to the ASC-US category in the Bethesda 2001 system, 88 cases (54 %) were found HPV negative by highly sensitive GP5+/6+ and MY09/MY11 PCR assays. All HPV negative cases were classified as Pap II corresponding to reactive cellular changes associated with inflammation, cellular repair, intrauterine contraceptive device, or atrophy both in the Munich II and Bethesda 2001 systems. Additionally, the Munich Pap II class also encompasses HPV infection without significant nuclear changes, similarly to the borderline nuclear change category in the UK British Society for Clinical Cytology (BSCC) terminology (120). In the Bethesda classification, signs of HPV infection are considered as low-grade squamous intraepithelial lesion (LSIL).

By using a panel of 10 “non-classic” cytomorphological features suggesting the effect of HPV, we found 3.8 signs per ThinPrep on the average in the HPV negative samples, in

contrast to 6.68 signs per ThinPrep observed in the HPV positive group. This is in line with findings of Schneider and co-workers (118) with corresponding values of 2.0 and 4.8 of 9 signs per sample, respectively. The non-classic signs we found in the HPV negative samples were mainly related to degenerative changes, such as cytoplasmic folding and presence of macrocytes, as well as bi/multinucleation also seen in reactive conditions. The prevalence of mild nuclear changes, including mild hyperchromasia and nuclear variations was significantly lower than in the group of HPV positive cases. The rather low specificity of only 31 % for the nuclear HPV-sign can be partly due to the fact that, even by PCR methodology, HPV can be detected only above a certain viral load (126). However, HPV infections under the detection threshold of PCR might produce cytopathological changes visible by light microscopy. This assumption is supported by the fact, that in some HPV-PCR negative cases we could detect HPV either by FISH or by immunocytochemical demonstration of the capsid protein as demonstrated in the present dissertation.

Intriguingly, the negative predictive value of mild nuclear changes was 100%, i.e. the absence of this feature in a mildly abnormal Pap smear predicted a negative HPV status with a certainty of 100% in the present setting (Table 6). The mean age of HPV negative women was 43.44 years; only 1 patient was younger than 30 years. This is concordant with results of the ALTS study revealing increasingly common HPV negative AS-CUS cases in women older than 30 years. The epithelial changes detected in this age group are likely to be due to declining oestrogen levels and general changes in epithelia throughout the body occurring with aging (122).

HPV positive women with mildly abnormal Pap test represent a clinical dilemma. Published prevalence rates vary significantly, mainly depending on age and the HPV test used (45).

In the present study, overall HPV positivity was detected in 76 of 164 cases (46.3%) with minimally abnormal Pap smears using sensitive PCR methods. In the ALTS study, 50.7 % of women with ASCUS cytology were found positive for the high risk panel of HPV types with the hybrid capture II (HC II) assay (1). Schneider and co-workers (118) have found overall HPV positivity in 9.5% of asymptomatic women with cervical smears showing classical and non-classical cytological signs of HPV infection using in situ filter hybridisation for HPV 6/11, HPV 16 and HPV 18, respectively. In another study of 335 lavage specimens with original diagnoses as “within normal limits” or “inflammatory”, Cramer and co-workers (33) upgraded 9 cases as AS-CUS upon re-examination, and found overall HPV positivity in 20.6% of the cases by using the HC assay. The significant difference in study results is mainly due to different sensitivity of the molecular tests used (45).

The mean age of women with HPV positive smears in our study was 25.27 years and 69/76 (90.7%) were classified as Pap IIw indicating equivocal diagnoses at first screening. The significantly higher prevalence of younger women with HPV infection compared to women 30 years of age and older is well documented in HPV natural history studies (77). This corresponds with viral transience and lower incidence at older ages (113).

Regarding cytomorphology, none of the 10 non-classic signs evaluated in the present study was diagnostic for HPV infection. All characteristics were found both in HPV negative and HPV positive samples, however with significantly higher prevalence in the latter. Eight of 10 features were significantly associated with the presence of HPV-DNA; the strongest association was computed for mild nuclear hyperchromasia, followed by mild dyskeratosis and the presence of measles cells in the ThinPreps (Table 5). Upon combining the cytomorphological signs into four relevant groups, all HPV positive women were correctly classified by the combined feature of mild nuclear changes (sensitivity 100%), however with a simultaneous specificity of 31% (Table). The most balanced combined HPV-associated

features were keratinisation disorders (84% sensitivity and 61% specificity), closely followed by abortive koilocytes and measles cell with 83% sensitivity and 55% specificity. Although degenerative changes were also encountered in the majority of HPV positive cases (sensitivity 84%), low specificity as well as positive and negative predictive values make this sign unusable for detecting HPV. This is in line with our previous results of nearly 100% sensitivity and specificity of the combination of classical and non-classical HPV signs for PCR-based detection of HPV infection in women with squamous intraepithelial lesions (SIL) and high-grade intraepithelial lesions (HSIL), respectively (128, 16, 17).

In a solely morphological study, Checcini and co-workers (25) described weakly independent association of mild koilocytosis with colposcopic and histological evidence of HPV infection, whereas overall sensitivity (46%) and specificity (87%) of “nonclassic” cytological signs were regarded not to be sufficient for cytological detection of HPV. Cramer et al. (33) also found significant associations of partial koilocytosis, spindled nuclei and multinucleation with overall HPV positivity and with high risk HPV types, whereas for the low-risk HPV types, only partial koilocytosis was independently predictive of the presence of HPV DNA.

In our present study, we had patients’ consent for analysing type specific HPV infection in 35 of 76 positive cases. We could identify 15 different HPV types as single infections and 8 double infections. Twelve of the patients harboured infection with oncogenic HPV types, 6 with probably oncogenic types and 11 with non-oncogenic types (Table 3). Based on cytomorphology alone, no difference could be made between type specific HPV infections (Table 7), it is in contrast to results of Cramer et al. (33).

The presence of HPV in women with equivocal Pap smears has prognostic significance. Because only persistent infections with at least one oncogenic HPV type pose a risk of neoplastic progression (113), only type specific molecular testing of HPV can

reassuringly identify which women with borderline cytology are at real risk and require colposcopy. However, safe selection of HPV-negative women who are at extremely low risk for CIN3+ and need only reassurance and screening in extended intervals (85) could significantly contribute to cost effectiveness of risk adapted cervical cancer screening.

Our present results provide evidence that effective pre-selection of HPV-negative women with minimally abnormal Pap tests could be achieved by critical evaluation of non-classic HPV-associated cytomorphological signs, especially those with mild nuclear changes, at primary screening. These women, 16% in the present study, should not be triaged by molecular HPV testing and further management should be in accordance with nationally accepted guidelines for HPV negative and cytology negative women.

Ancillary techniques to detect the physical and functional state of HPV in cervical epithelial lesions

The majority of existing or proposed HPV screening protocols are DNA-based tests providing results on the presence or absence of HPV in general or with regard to specific genotypes in cytological samples. Type-specific persistence of oncogenic HPV is considered to be the true precursor of neoplastic progression (61) and the expression of the E6/E7 oncogenes is necessary for malignant transformation and maintenance of the neoplastic state (135, 54). Therefore, detection of the E6/E7 mRNA of the respective HPV genotypes might serve as a better prognostic test than mere DNA detection for the development of HSIL and invasive carcinoma (128).

In our study, we used the PreTec HPV-Proofer assay (NorChip) to detect E6 and E7 mRNA expression from HPV types 16,18,31,33 and 45 - which are the most prevalent high-

risk HPV types in Europe and North America (28) - in 66 cervical cytologies up to HSIL/in situ carcinoma. The results were compared to those of consensus and type-specific PCRs, cytology and histology. Ninety-six percent of the cases (62/66) were positive for HPV-DNA, confirming the importance of HPV in cervical carcinogenesis from the lowest end of epithelial changes. All HPV-mRNA positive cases were also positive for HPV-DNA, whereas 24 HPV-DNA positive cases did not express the respective mRNA. The latter cases may represent HPV infections either with non-integrated (episomal) viral DNA or HPV infections with low transcriptional activity producing mRNA below the detection threshold of the test. Fifty-one percent of patients had cytological progression of their cervical disease and 49% had cytological regression or persistence during 12 months of follow up. The prognostic power of the NorChip test for detecting disease progression versus regression was: 82% sensitivity, 69% specificity, 74% PPV and 79% NPV (Table 11). These results show that HPV mRNA detection by the NorChip test has very high prognostic value for the course of cervical lesions and the assay offers an improvement for the triage of women with ASCUS/LSIL Pap cytologies. We carry on with our studies to collect more data and longer follow-up to confirm our preliminary results, which are concordant with findings of others (81).

In this context, we also investigated the immunohistochemical expression of the HPV L1 capsid protein in a representative sample of women from our screening population using the Cytoactiv ® test. HPV L1 capsid antigen is considered to be a major target of cellular immune response, which plays a significant role in the natural regression/elimination of the majority of HPV-induced squamous intraepithelial lesions. This biological property of the L1 antigen is also implemented in the vaccination against HPV. The virus-like particles in the HPV vaccine have an outer L1 protein coat, but they have no genetic material inside. This structure enables the vaccine to induce a strong protective immune response.

Experimental data indicate that the expression of L1 capsid protein is significantly reduced in cervical lesions with integrated high-risk HPVs, particularly with HPV 16. The development of viral capsid antigen L1 depends upon transcriptional factors which only can be expressed during maturation process from basal to superficial epithelial cells (69). In HSIL both the natural structure and the maturation of the epithelium are disturbed leaving dysplastic basal epithelial cells as the predominant cell type with reduced L1 capsid protein expression. As a consequence, a reduction or loss of L1 capsid antigen production might result in a reduction of cellular immune response (49) enabling HSIL to persist and progress. Thus, the detection of L1 capsid protein has been proposed as a novel prognostic marker for cervical dysplasia.

In our ongoing study, we correlate the immunohistochemical expression of L1 capsid protein with HPV genotypes, DNA ploidy, cytology and histology in 209 women with LSIL (n=145) and HSIL (n=59). Our preliminary results show that the L1 antigen can be detected in about 50% of the cases investigated, practically irrespective of the cytological grade of SIL. Forty percent (69/145) of LSILs and 50% of HSILs (29/59) were positive for L1 expression. This indicates that HPV oncogene integration might not be the only explanation for reduced L1 expression in SIL. Further analysis of our data with additional follow-up results may help to find out a possible association between loss of L1 capsid antigen expression and SIL progression/recurrence.

In another approach, we investigated the physical status of HPV (integrated versus episomal) and HPV-induced genomic changes at the chromosomal level, i.e. copy numbers of chromosomes 3 and 17, by fluorescence in situ hybridisation.

Using chromogenic in situ hybridisation (CISH) with a high-risk HPV-specific probe cocktail (HPV 16, 18, 31, 33, 35, 39, 51, 52, 56, 58 and 66) in the Benchmark XT Staining Platform, we could demonstrate the presence of both integrated (punctate CISH signal) and episomal

(diffuse signal) virus DNA in a significant number of cases with non-classic minor cytological aberrations and in ASCUS. Most of these cases also expressed the L1 capsid protein detected by the Cytoactiv® test. Further analysis of our data is in process.

With regard to chromosomal imbalance in HPV infected cells, we found an accumulation of abnormal chromosome 3/17 ratio in hyperdiploid cells with >5c and >9c DNA content in HSIL samples detected by fluorescence in situ hybridisation (FISH) using pericentromeric probes for the respective chromosomes after image analytic measurement of DNA content and repositioning of the highly aneuploid cells. We interpreted the recurrent FISH signal imbalance as a strong argument for a common origin of the respective cells. This feature suggests that highly aneuploid cells of HSIL accumulate clonal genetic aberrations that are not detectable in peridiploid epithelial cells in early progressive lesions. Further studies are ongoing to analyse associations between classical and novel markers with special emphasis to predictive testing of early CIN behaviour.

IV. 2. HPV genotype distribution and the spectrum of HPV-induced cervical lesions

According to current knowledge, cervical cancer develops from pre-cancerous cervical lesions (CINs) over the course of many years, following a well defined path with identifiable intermediate stages (125). In many respects this process mirrors the natural course of other HPV-associated benign diseases. Changes caused by HR-HPV infections and those associated with common LR-HPV types are well characterised because of their outstanding clinical significance and high prevalence. There are, however, a growing number of new or rare HPV types with yet unknown oncogenic potential and poorly characterized phenotypic appearance of infected cells.

In our present strictly selected collective, none of the 293 cases with single non-high-risk HPV infection only was diagnosed as a carcinoma and 15 (5%) HSILs were classified by cytological assessment. This, and the finding of no disease progression during follow-up, supports the well-known low oncogenic potential of LR-HPVs and affirms that the 19 unknown-risk types detected in the present study (see Table 12 and Table 13) are probably alike. However, in our screening population, 2 verrucous carcinomas and one transitional cell cervical carcinoma were detected after persistent infections with HPV 6, 11 and 42, respectively [unpublished data]. These women were ineligible for the present study.

In current cytological classification systems, HPV infections are diagnosed on the basis of classic cytopathic effects of HPV such as koilocytosis, dyskeratosis, and “raisinoid-shape” nuclear atypia. While these cytological or histological changes are, when strictly used, specific, they are only moderately sensitive for diagnosis of HPV (118). These are the morphological correlates of productive viral infections. However, many HPV infections remain suppressed for a long time without showing these prominent cytological alterations, or striking colposcopic evidence of disease, but are detectable with HPV DNA by sensitive PCR-based methods. Reported rates of HPV-positive “normal” specimens encompass 25% (103), 29% (80), 36% (98) and 44% (135), respectively. In our present study, 27 cases (9%) were classified as “normal” (no-ASC) in the strict sense of showing no signs of cytological atypia. However, using our recently published expanded cytological classification of HPV-related alterations (13) minor non-classic HPV-signs, especially mild nuclear changes, could be seen in all these specimens. Similar observations were made by others as well (118, 25, 33). These results suggest that the rate of “normal” cytology with a positive HPV test would be radically reduced by careful assessment of non-classic HPV-effects.

Intriguingly, it was not possible in any of these studies to discriminate between HR-HPV and non-HR-HPV positive cases by cytomorphology. This is of particular note,

especially in women with minimally abnormal Pap tests, because patients with HR-HPV positive “normal” smears are at increased risk for developing CIN3 (108, 134), whereas the probability of progression to HSIL in women who screen positive for LR-HPVs is extremely low (90). Thus, HPV typing may have clinical importance for risk adapted individual patient management.

The most common diagnosis in our present study was ASCUS (63%) referring to cells with nuclear atypia of which 59% were associated with LR-HPV types and 41 % with UR-HPV types. Because of the general presence of one or more non-classic HPV-related signs in the ASCUS smears and the epidemiological evidence that infection with HR-HPV types - undistinguishable morphologically from non-HR-HPVs - is associated with cervical neoplasia, mild cytological HPV-related changes should be viewed with greater concern and possibly added to the ASCUS definition. This is further supported by the prospective follow-up in the ASCUS/LSIL Triage Study that suggested a similar risk for subsequent CIN grade 2 or 3, approximately 12% over 2 years, among women with HPV-positive ASCUS and LSIL indicating that these two cytological findings are clinically equivalent (31).

Of the 69 LSIL cases (23%) in the present study, 39% were associated with UR-HPV types and 61% with LR-HPV types. It was not possible to predict, based on cytological appearance, which HPV type was responsible for the infection, nor was it possible to distinguish between a “dysplasia” (neoplastic transformation) and a “simple HPV infection” of a productive type. The few HSIL cases, (15; 5%) were associated in almost equal frequency with LR-HPVs (n = 8) and UR-HPVs (n = 7) in the present setting.

Though indistinguishable morphologically, the risks of viral persistence and neoplastic progression to CIN3/carcinoma differ markedly with HPV type, with genetically related types appearing to act most alike (87). Accordingly, phylogenetic grouping may predict the natural

history and carcinogenicity of individual HPV types (114) even if they occur rarely and thus cannot be evaluated epidemiologically.

In the present study, 8 of 19 unknown-risk HPVs could be classified as LR-types (HPV 32, 62, 83, 84, 86, 87, 91, 74) based on their common genetic origin (see Table 14). Additionally, our present findings on the relative high prevalence of HPV 90 (n = 19; 6.5%), which belongs to species $\alpha 15$ together with HPV 71, and its association with 16 ASC-US, 1 normal, 1 LSIL and 1 HSIL cases suggest a low oncogenic potential of this type as well. From the remaining types, CP8304 has also been detected in smears with normal cytologies but not in cancer (79, 98, 11). Our present data also support the classification of CP8304 as a low-risk type. Risk assignment of HPV 70 is controversial; it belongs to the high-risk $\alpha 7$ species, but was characterised as low-risk epidemiologically by Munoz et al. (85). In our present study, HPV 70 was found in 21 women (1 normal cytology, 13 ASC-US, 7 LSIL and 1 HSIL). The majority of these infections has been persisting for over years indicating that the immune system cannot readily eliminate this HPV type and the development of associated higher grade pathologic changes is rare and slow. The species IA 09, JC 9710 and W 13B, found in a small number of patients only, represent novel genotypes with an as yet undetermined oncogenic risk.

Intriguingly, four viruses from the UR-types (HPV 69, 30, 67, 34) could be assigned as probable high-risk species based on their genetic relationship with other HR-HPVs. Of them, HPV 67 occurred in 16 women (8 ASC-US and 8 LSIL). This type belongs phylogenetically to species $\alpha 9$, which comprises the most carcinogenic HPV types including HPV16. Also MM44, which was detected only in 1 LSIL in our present setting, has been found by other investigators in a few cervical carcinomas in women from different geographical areas indicating its high oncogenic potential (79). HPV 30 was detected in one laryngeal carcinoma by Kahn and co-workers (59) and it was found in two women with

HSIL/carcinoma in situ in our present study. Thus, the detection of probable high-risk genotypes in LSIL and lower cytologies can serve as a prognostic indicator as to the chance of progression to HSIL and cancer, and such patients would possibly benefit from more intensive management than women with other HPV types.

The prophylactic and therapeutic targeting of HR-HPV infections, particularly HPV 16 and 18, is the subject of much current research. However, it is important to note that together HPV 16 and 18 currently account for about 70% of cervical cancer cases (18). This leaves ~30% caused by at least 20 other genotypes and the theoretical concern that additional HPV genotypes could expand to fill the gap left after effective vaccination against only HPV 16 and 18. Therefore, further research is needed to understand the pathobiology of papillomavirus infection, in which each HPV type should be considered as a separate sexually transmitted infection posing different risk for cervical cancer.

Our present findings highlight the importance of type-specific HPV testing and the use of expanded cytological classification of HPV signs from the lowest end of HPV-related cellular changes and upwards. Non-classic signs of HPV-effect should be viewed with greater concern and possibly added to the ASC-US definition. The efficiency of cytology-based cervical cancer screening programs could be increased by including objective individual risk factors for women with normal or equivalent cytology, or with cervical diseases at the lower end of squamous intraepithelial lesions, such as the detection and risk assignment of HPV-type(s) according to epidemiological classifications or phylogenetical origin.

IV. 3. Efficiency of risk-adapted multimodal screening and management strategy for cervical cancer prevention

Based on good evidence, regular cytological screening of appropriate women for cervical cancer reduces the incidence and mortality of the disease (64). However, the Pap smear as a screening test unavoidably produces a certain percentage of false positive and false negative results, both around 4% (29). In our present analysis, the Pap test performed well with relative sensitivity and specificity in the upper range of its published performance (88). This may reflect improved test accuracy by the increasing use of the ThinPrep method (102) and the expertise of the single pathologist who supervised all equivocal smears. Furthermore, the systematic assessment of non-classic cytomorphological signs of HPV effect for pre-selecting cases for molecular HPV testing (13) may also have enhanced sensitivity of the Pap test for detecting cervical cancer precursors. However, there is still a significant rate of false positive cytology results, 3.5% in our present study, which leads to unnecessary additional workups and over-treatment of benign conditions in many women. Taking the published test performance and the recommended practice of annual screening in account, the life-long risk of a false positive result is 36-84% in Germany (10). With the implication of a risk-adapted multimodal protocol, we were aiming at improving the accuracy of routine cervical screening with special focus on possible reduction and prognostic assessment of false positive results.

Extensive data from large screening studies show that HPV DNA testing is substantially more sensitive (around 95%) for detecting prevalent CIN3 than either conventional or liquid-based cytology (around 70%) (68). In our present study, HPV genotyping had a relative sensitivity of 97.9%, 10.9% higher than cytology at PapIIID/HSIL threshold, for detecting histologically proven \geq CIN2 in women with abnormal cytology. The relative test specificity was astonishingly low due to the high rate of false positives (100%).

Combining HPV genotyping with cytology, sensitivity, specificity and the false positive rate slightly decreased parallel with a minor increase in test efficiency (Table 20). This performance is fairly typical compared with other published studies (46).

However, despite their oncogenic potential, most infections with HR-HPV typically resolve within 1-2 years and only a minority, those with a long-term persistent infection with a single HR-HPV variant, progress to CIN3 with subsequent high risk of becoming invasive if untreated (91, 61). Therefore, we imply two consecutive PCR-based HPV tests with the ability for genotyping in our screening protocol with the second assay being performed after 12-14 months after the first high-risk positive genotype test. Using this approach it is possible to distinguish between persistent infection and a new infection with another high-risk HPV type during the follow up period in most of the cases.

Nevertheless, even the highly sensitive test combination of cytology and HR-HPV genotyping cannot assess the biological potential of prevalent cervical intraepithelial neoplasia towards progression or regression. Experimental results suggest that increasingly deregulated expression of the E6-E7 oncogenes of HR-HPVs in epithelial stem cells first results in chromosomal instability and induces DNA aneuploidy followed either by subsequent integration of the HR-HPV genome into the affected cell clone (78) or alternative oncogene activation mechanisms (110). Clinical studies support this concept in that cervical lesions with an aneuploid DNA profile are more likely to persist or progress than those with diploid or polyploid DNA content (12).

Previous studies, including ours, have reported that DNA ploidy measurement by image cytometry on cervical smears positive for HR-HPV help to detect women at high risk for developing high-grade cervical lesions (67, 16). On analysing the 6 false positive cases by HPV-HR genotyping of 38 women receiving the full methodical spectrum of multimodal screening in the present analysis, all were diagnosed as CIN 1 in histology; 5 of them had aneuploid DNA

content, and one displayed a diploid profile in DNA cytometry. We presume that co-positive results for HR-HPV and DNA aneuploidy in low-grade CIN probably indicate that an oncogenic HPV virus has already enhanced genetic instability and rendered cells susceptible to malignant transformation and consequent progression if left untreated. On the contrary, the one diploid CIN1 lesion with HR-HPV infection will probably regress. Any other test combinations could not have made this prognosis even with superior test accuracy.

Of the 31 histologically confirmed \geq CIN2 lesions, all were correctly detected by cytology alone, 30 by type specific detection of HR-HPV DNA (1 case contained HPV DNA of undefined risk type) and 30 by aneuploid DNA profile. The combination of cytology, HPV genotyping and DNA cytometry resulted in an increase of positive predictive value (PPV) up to 88.2% for moderate to high-grade cervical dysplasias and carcinomas (\geq CIN2) compared to single tests or double combinations. This approach had the additional benefit of being able to predict the possible outcome of histologically proven CIN1 lesions detected as false positives by single tests.

We conclude that our multimodal cervical screening (Figure 8) might permit identification of those women with low grade squamous intraepithelial lesions likely to progress at earlier and curable stage of disease and lengthen screening interval in those with transient minor lesions caused by productive HPV infection. Using a combination of methods, although more expensive per screening, might be cost effective if the increased sensitivity permits lengthening of the screening interval.

V. Literature

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VII. LIST OF PUBLICATIONS

Original articles [impact factor: IF]:

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Review

1. Bollmann R, Bankfalvi A, **Varnai AD**, Bollmann M: Risiko-adaptierte multimodale gynäkozytologische Krebsvorsorge – Der Pap-Test der Zukunft. *Pathologe*, 2007 (in press)

Book chapter

1. Bollmann R, **Varnai AD**, Bankfalvi A, Bollmann M: Alternative approaches to cervical cancer prevention: risk-adapted multimodal laboratory cervical screening. In: *New Research on Cervical Cancer*, Eds: George Z. Rolland, Nova Science Publishers, Inc., 2007 .

Congress Posters & Presentations:

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