The potential of RNA as a target for national screening of pre-cancer

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Abstract

Whole national screening of pre-cancer is done only in some few countries, dominated by The Netherlands, Denmark, UK, Norway and Finland. These national screenings are done combining national cancer registry, national public health and national medical bodies or hospitals. Until some few years ago national screening was only done using morphological or visual methods or technology. Today a number of molecular methods have been implemented to serve these national screening programs. Based on all the discussions within this review, it is clear that the main driving engine and the cause of cervical pre-cancer and the main cause of invasive cervical cancer is the expression of E6 and E7 oncoproteins from HPV 16, 18, 31, 33 and 45. However, the main challenge is the role of morphology or imaging-based diagnosis in the original definition of pre-cancer disease. This definition is not based on the cause of cervical pre-cancer but based on a complex, subjective, morphological observations. The difference between these two definitions are discussed in this review. The unique discovery done while validating the first standardized detection technology used against mRNA, confirmed that the presence of both abnormal E6 and/or E7 mRNA and protein is the cause of cervical pre-cancer or severe neoplasia and the main cause of invasive cervical cancer. This confirmation was evident even though all these studies were disturbed by the above defined biases from morphology or imaging-based diagnosis. The use of the screening target that cause stable and high expression of the most carcinogenic compounds ever discovered, must cause a more accurate screening program. A number of studies have proved that the detection of E6/E7 mRNA followed-up by indirect or direct treatment in a well-organized national screening program, would reduce the incidence of cervical cancer. This review discusses the main studies involved in the scientific, clinical evaluation and how this unique technology could be used as a new medical gold standard for national screening of cervical pre-cancer.

Introduction

The diagnosis or screening of cervical pre-cancer and invasive cervical cancer has for the last 80 years relied on the histology, colposcopy and the Pap methods including cytological identification of abnormal cells.[1-7] In addition the colposcopy has also being involved making the gynecologists and pathologists working together.[4-6] Before treatment or interventions in the cervix based on cytology or colposcopy, histology has to be used as the gold standard in order to confirm the presence of histological CIN2+.[8,9] Therefore, an abnormal pap smear has only being an indicator that a disease process is present and further tests are required to make the complete diagnosis.[2] However, the main problem with these morphological tests have been its subjective nature, loss of repeatability, lack of fellow standards, lack of internal controls and the high number of false negatives and positives.[10] In the accuracy evaluation of cervical cytology few studies of initial screening were unaffected by workup bias, but the few that were provided estimates of the specificity of Pap smear screening of 97 to 100 percent and sensitivity of 29 to 56 percent, indicating sensitivity estimates much lower than those generally believed to be true. The evidence regarding the accuracy of the newer technologies on cervical cytology screening is insufficient for several reasons. Most effect on sensitivity are based on surrogate reference standard (cytology) and the assess of the effects of thin-layer cytology or computer rescreening on specificity.[10] Another main problem with these morphological tests is the inability to include them in complete performance evaluation.[10] The third main problem with cytology is the lack of clinical sensitivity towards invasive cervical cancer. Even in the countries where a more complete national screening program is done, cytology is able to miss around 50% of the cancer positive samples.[11-13] Another significant problem with cytology is the relative high number of women that still become positive (4-7%) without improving the discovery of invasive cervical cancer (ICC) in a national screening program. However, the major problem for all women may be the need for follow-up on the majority of cytological diagnosis for months or years without any diagnostic conclusion.

The Cancer Registry of Norway (CRN) is responsible for several registers of pre-malignant lesions and cancers as well as the screening program for cervical cancer. The register of pre-malignant cervical lesion treatment, and the Cytology Register have been linked by the use of the Norwegian person identification number (PIN). This PIN is a unique 11 digital-number given to each Norwegian citizen, and is used in all official register. The database of CRN uses a reporting system that is based on pathology, autopsy and cytology reports, clinical records and death certificates. One record is the result of accumulated reports on one person for each disease. The registration of Invasive Cervical Cancer (ICC) is practically 100% complete in Norway. Virtually all PAP smears are registered into the Cytology Register. The use of a unique personal identification number given to all Norwegian residents in all these registers made it possible to compare all cases with a CIN 2/3/ACIS (N=8586) with ICC (N=777) in the period 2000-2002. All Pap smears since

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1992 were used to characterize detection mode and screening history. The report from this follow-up was conducted by Nygård and collages.[11,12,14] Fifty-five percentage of the women diagnosed with CIN2/3/ACIS had an adequate screening history. Of women diagnosed with ICC, 45% in stage I and 10.5% in stage IV had an adequate history. Women with ICC stage II-IV, 40.9% had no Pap smear within 24 months of diagnosis while 5.7% had a normal Pap smear. For women with CIN2 or CIN3, more than 30% had an abnormal Pap smear. Again, the Pap smear sensitivity varied greatly, with estimates from 11-99 for detecting CIN2 or worse lesions, with meta-analyses reporting a mean sensitivity of 50%.[15,16] Data suggest that the Pap test is unable to discriminate between histologic diseased and non-diseased individuals with concurrently high sensitivity and specificity. It is very clear that a Pap smear in an asymptomatic woman is a screening procedure, not a preventive act. The important for pap is the regular use often enough to establish high enough accuracy. This may also create the main bias behind the so-called cytological screening success. However, the cost of this lack of objective clinical accuracy is around 35 000 women (CRN) every year strongly exposed to unnecessary psychological stress. Increasing number of cytological examinations would naturally give a higher number of possible examinations; including colposcopy examinations and histology of biopsies. It is also very likely that most of the cytological success may be related to a much higher random number of biopsies followed by histological examination. It may also happen that that a higher frequency of cytological examinations causing a higher number of colposcopy examination including Pap diagnosis. This higher number of morphological or image analysis cause a higher frequent disturbances or lesions of the transformation zone. Lesions in the cervix may cause a higher number of HPV infections in the basal cell layer and thereby cause more production of E6 and E7 oncoproteins following presence of an abnormal E6/E7 mRNA and cervical pre-cancer.

Within the area of cervical cancer prevention, a wide range of molecular methods and HPV biomarkers have been introduced during the last 30 or so years. Common for most of these biomarkers (*e.g.* HPV-DNA tests) is a low positive predictive value for histological CIN2+ in screening (typically below 10%) making it unlikely to treat women solely on this diagnosis.[17-20] However, due to very high clinical sensitivity based on possible biased presence of both carcinogenic and natural HPV infection, these HPV DNA based methods are selected and FDA approved, despite the high number of false positives and the lack of targeting true pre-cancer. A recent very large study in US on 256 648 women confirmed this bias made HPV DNA test (HC2) (Qiagen, Hilden, Germany), missing 19% of the invasive cancer cases in the first run or at baseline.[21] More than 80% of women within a country may have been infected by natural HPV infections and only a fraction of these women should have been included in the statistical risk of developing several pre-cancer. Despite this fact, 15% of the cytological normal cases from the above large study, were identified to have high-risk Human Papillomavirus (hrHPV) infection using the HC2. Recently, in China, a similar study showed that more than 19% of the women in the big cities were positive by high-risk HPV by the gold standard HPV DNA test; HC2.[22] All FDA approved HPV tests have been validated against this HC2 test. Recently, a national screening related study in Norway has presented related results, showing that COBAS 4800 HPV DNA test missed 40% of the HPV 16 infections in histological normal/CIN1 cases.[23]

The medical opinion still demand that all studies should involve morphological methods. Therefore, all studies involving objective molecular methods have to undergo verification bias from morphology. Comparing morphological and very subjective diagnosis with accurate molecular test targeting the cause of cervical pre-cancer, had to fail. Another challenge was the selection of HPV DNA tests targeting many HPV DNA types not able to separate between transient and transforming infection. This caused another verification bias creating a lot of positive results from HPV DNA infections that did not cause transforming infection of true cervical pre-cancer. A third challenge include the use of gold-standard histology done on tissue that was not representative for the whole risk area of the cervix. This also produced another bias when possible accurate molecular test targeting the cause of disease was evaluated. The bias or noise from morphological methods are included or have to be included, in most studies that involve validation of new molecular methods. Therefore, it has been nearly impossible to perform studies and present data, using objective and very accurate molecular methods, without verification bias. The cause of cervical pre-cancer or severe neoplasia/dysplasia is identified based on accurate analytical performance, but cannot and should not always confirm subjective morphological diagnosis. This may be the main reason for confusions involved when opinion leaders worldwide discuss screening strategies in order to improve primary screening of cervical cancer. The use of the suggested gold standard method presented in this review used in a primary screening setting, would give a direct same day follow-up treatment based on accurate identification of the cause of pre-cancer. The methods discussed in this review are used for direct detection of cervical pre-cancer. The methods are based on the direct identification of E6/E7 abnormal modified mRNA (include human RNA sequences) from the carcinogenic methods directly on extracted mRNA from collected cervical cells. A standardized method that is able to fulfill this demand are PreTect HPV mRNA products (PreTect AS, Klokkarstua, Norway).[23-44] The combined name used in this review is E6/E7 mRNA technology.

The true invasive potential for CIN3 is in fact smaller than 50% according to 30 years follow-up of CIN3, studies done by McCredie and colleagues.[45,46] Histology is the gold standard morphological method to detect CIN3 but not persistent CIN3. On the other hand, mRNA tests are based on an oncological biomarker profile indicating the presence of pre-cancerous lesions by a high positive predictive value,[37,47-49] even though it cannot directly confirm persistent pre-cancer (Figures 1 and 2). Likely, the female immune system will remove more than 50% of these severe neoplasia or severe oncogene expression before it become an invasive cancer (Figure 1). Therefore, methods directly targeting oncogene activity and not only a risk, should have a clinical sensitivity between 50-70% against CIN2+ and not higher. The high positive predictive value demonstrated by the E6 mRNA technology makes it possible for clinicians to use this method exclusively when deciding which women should be treated to avoid progression towards invasive cervical cancer.[41,50,51] E6/E7 mRNA technology identifies the presence of the well-known oncogenic activity involved in the cervical cancer carcinogenesis. HPV DNA based PCR methods are only targeting small fragments of the HPV DNA and they do not distinguish between transforming and transient infections (Figures 1 and 2). The HPV DNA based PCR methods may not distinguish between the driver and the passenger HPVs with regards to the viral type. Therefore, Matsukura and Sugase concluded that only the seven HPV types (Type 16, 18, 31, 33, 35, 52 and 67) may be the true carcinogenic types.[52] HPV 31, 33, 35, 52 and 67 may be highly prevalent in female populations, diagnosed to be cytological normal or histological CIN2+. However, when women with invasive cer-

vical cancer are evaluated for HPV types by PCR in countries with high prevalence of *e.g.* HPV 35,52 or 66 in the normal population, the prevalence of the same types are dramatically reduced or gone in women with true invasive cancer.[53,54] It means that even among these seven carcinogenic HPV types there may be transforming infections that is not involved in driving carcinogenic process all the whole way from CIN2+ cases into invasive cervical cancer (Figure 1).[55]

Short introduction to morphological methods for national screening of cervical cancer

Cervical smears or epithelial cells from the transformation zone are normally collected by a gynecologist or a physician using different kinds of brush or swabs. However, nurse or normal health personal also collects more and more samples and self-sampling equipment has been introduced to the market (CRN). Most of the swabs used for this collection have not been developed for this purpose. However, several of the brush types have been developed for better collection of cells from the cervix area.[56,57] Evaluation of cervical cytology accuracy the initial few screening studies were unaffected by workup bias, but few provided estimates of the specificity and sensitivity of Pap smear screening as 97 to 100 and 29 to 56 percent respectively, indicating much lower sensitivity estimates

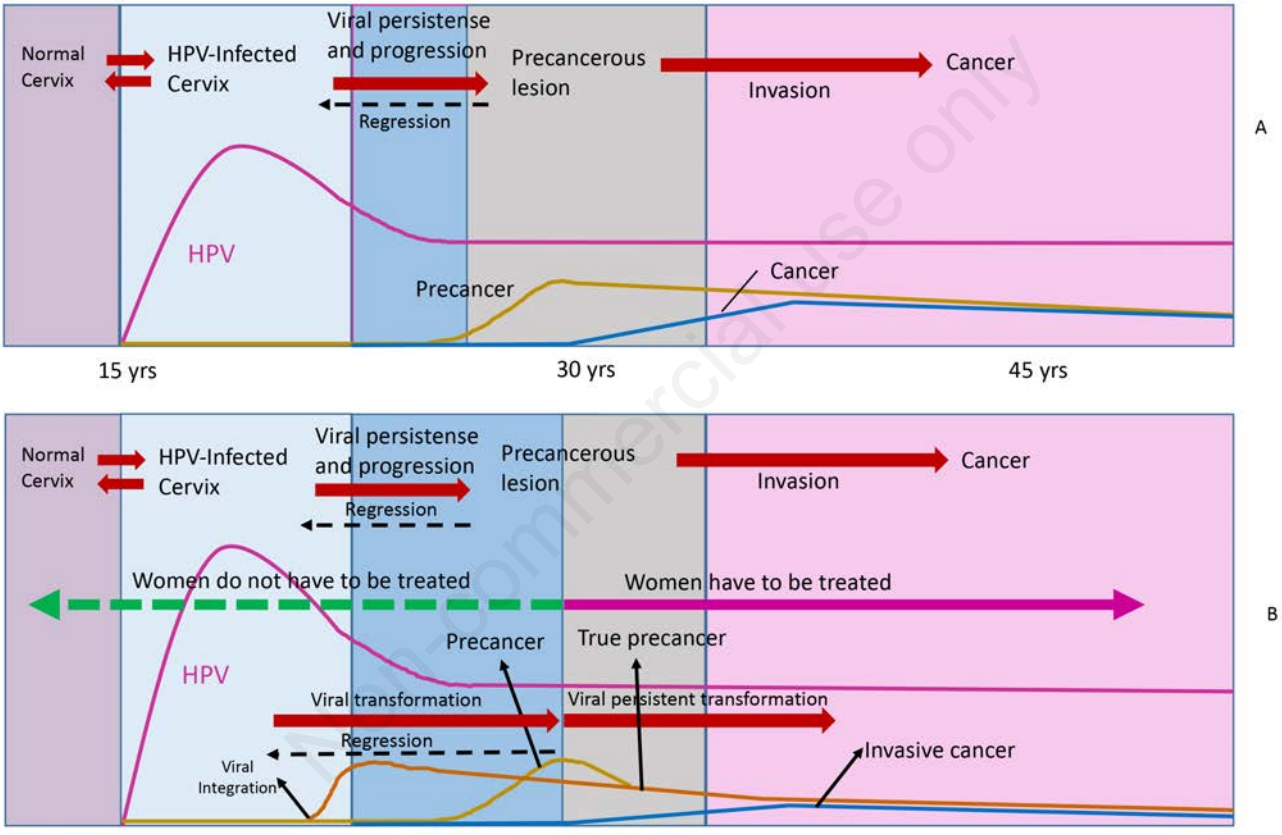

Figure 1. A) Natural history of HPV infection, transformation and invasive cervical cancer. As being presented by Schiffman and Castle in 2005 (55) the natural history of HPV infection and invasive cervical cancer (With permission from the NEJM): The peak prevalence of transient infections with carcinogenic types of HPV occurs among women during their teens and 20s, after initiation of sexual activity. The peak prevalence of cervical precancerous conditions occurs approximately 10 years later and the peak prevalence of invasive cancer at 40 to 50 years of age. The peak of the curves is not drawn to scale. In this review article we have extended the upper Figure into the lower part of the B) to teach more about abnormal HPV activities. The use of E6/E7 mRNA technology has presented another natural history of cervical true pre-cancer. After initial infection by HPV through a lesion in the transformation zone, HPV may accidently become integrated into the infected basal cells even when women are in their early twenties (The Figure do not illustrate the necessary time for integration). This will in turn cause a stable production of full-length E6 and E7 causing viral transformation into neoplastic cells, true dysplastic cells, precancerous cells or CIN2+ like cells. Women having this stable oncogene production may be defined as having true pre-cancer. The immune system is still able to remove this kind of transformed cells causing a regression of the oncogenic phenotype. However, at this time it is also possible to perform a conization that will remove all the transformed cells. However, if the E6/E7 mRNA technology repeatedly identify the presence of same type E6/E7 mRNA due to lack of treatment or lack of immune defense, persistent viral transformation will secure continuous oncogene activity. This will in turn secure continuous accumulation of mutations and instable genomic and rnomic transformation by the activity of full-length E6 and E7 proteins. In the end, without treatment or functional immune defense, the transformed cells will break through of the basal cell layer into invasive cervical cancer. The main challenge is that the history of HPV DNA infection told by the PCR methods, do not tell anything about this transforming history. Another challenge is that the bias from morphological methods makes it very complicated to evaluate this transforming history.

than those generally believed to be true.

After a collection of the cells have been transferred to a microscope glass, conventional techniques or liquid based techniques are used. The cell smear that has been prepared is examined through a microscope using what we call a morphological examination or cytology. The cell smear may also be examined using automatic image analyses. The evaluation is typically following the recommendations from Bethesda,[58] with more or less defined matrix or cell morphology given the diagnosis: ASCUS-L, LSIL, ASCUS-H, AGUS, MSIL, HSIL and Cancer. Normally, there are many more kinds of defined diagnosis that may be used. ASCUS and LSIL normally have to be repeated until a normal diagnosis or ASCUS-H, AGUS, MSIL or HSIL are discovered.

When HSIL or ASCUS-H is discovered by a cytologist, the gynecologist normally takes 4 biopsies from the transformation zone following guidelines from a colposcopy. The biopsies are sent to the pathological department fixed by formaldehyde or some other fixative. Thin sections of the tissue are places on an object glass and stained before the pathologist is performing histological diagnosis: CIN0, CIN1, CIN2, CIN3, Cancer In-situ and Invasive Cancer. Following examination, many more kinds of defined diagnosis may normally be given by pathologists. Anything similar to CIN2 or more severe are than sent to the gynecology oncologists for treatment including LEEP or LETZ conization.

Short introduction to the molecular methods for national screening of cervical cancer

There are many molecular methods and in particular many commercial molecular methods available for detection of HPV DNA, p16INK4a or similar markers. There are also some few commercial molecular methods that identify E6/E7 proteins or mRNA. Some in-situ methods are also available. The main HPV assays may be divided into target amplification methods including consensus or type-specific primers, HPV mRNA amplification including E6/E7 targeting primer-sets and probes or signal amplification methods.[59] The main commercial HPV assays are: The hybridization full genome method HC2 including CareHPV test (High-risk types not type-differentiated; Qiagen, Gaithersburg), The PCR L1 based method Amplicor HPV test (High-risk types and not type-differentiating; Roche, Branchburg), The hybridization L1 based Cervista HPV HR test (High-risk types not type differentiating; Hologic, Madison), the reverse line-blot hybridization on PCR products L1 based CLART test (Differentiate 13 or more high-risk types; Genomica, Coslada), the reverse line-blot hybridization on PCR products L1 based INNO-LiPa HPV Genotyping (Differentiate 13 or more high-risk types; Innogenetics, Gant), the reverse line-blot hybridization on PCR products L1 based Linear Array HPV Genotyping test (Differentiate 13 or more high-risk types; Roche, Branchburg), the reverse line-blot hybridization on PCR products L1 based Digene HPV Gentyping RH test (Differentiate 13 or more high-risk types; Digene, Hilden), microarray on PCR products E1 based Infiniti HPV-HR QUAD test (Differentiate 13 or more high-risk types; Autogenomics, Carlsbad), microarray on PCR products E1 based PapilloCheck (Differentiate 13 or more high-risk types; Greiner Bio-one, Frickenhausen), hybridization Cervista HPV16/17 also targets E6 and E7 test (Hologic, Madison), real-time PCR COBAS 4800 HPV test (specific genotyping information for HPV types 16 and 18, while concurrently detecting 12 other high-risk HPV genotypes as a pooled results; Roche, Pleasanton), real-time PCR Real Time High Risk (HR) HPV test (Differentiate 14 high-risk types with current distinction of HPV-16 and HPV-18 from 12 other HPV genotypes; Abbott, Des Plaines), transcription mediated amplifica-

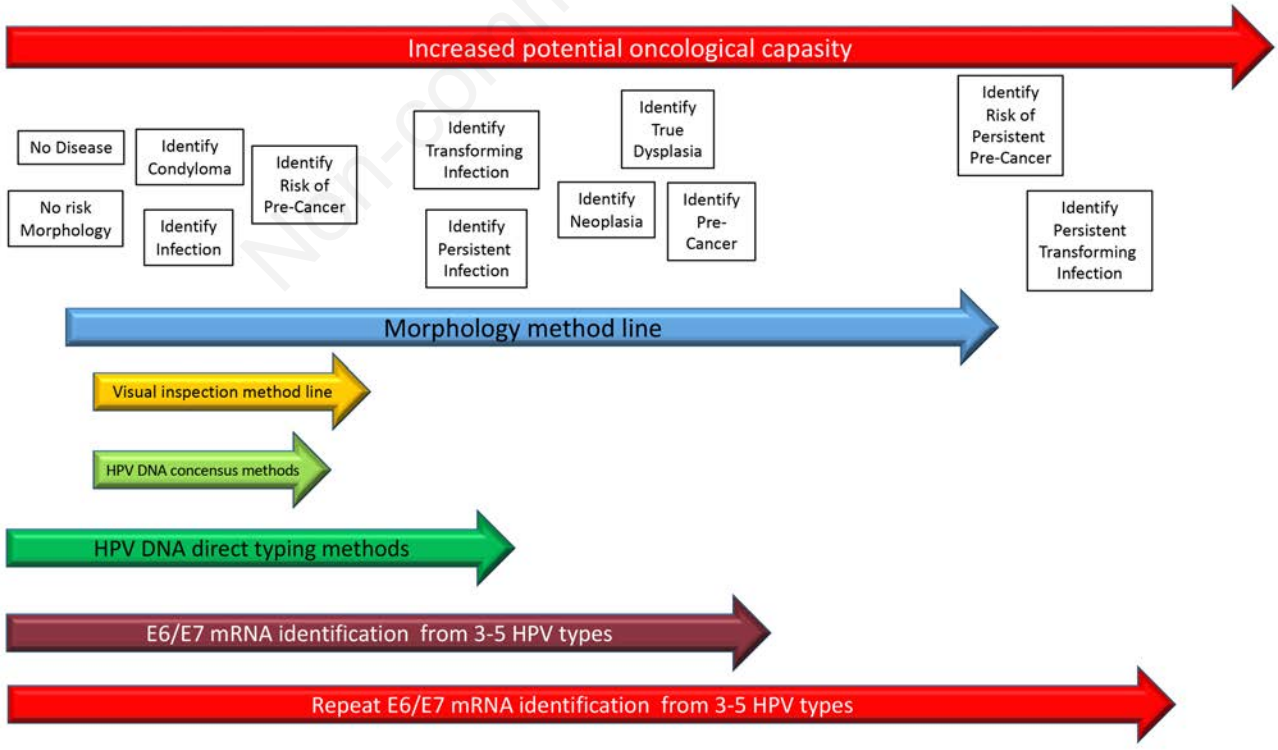

Figure 2. Description of cervical carcinogenesis with HPV infection or persistent oncogene expression.

tion APTIMA HPV (AHPV) ASSAY targeting E6/E7 mRNA (14 high-risk types as a group, not type differentiating; Hologic, San Diego, Madison),[60] transcription mediated amplification AHPV-GT assay targeting E6/E7 mRNA (type-specific detection of HPV type 16 and for combined detection of HPV types 18 and 45; Hologic, San Diego, Madison),[60] real-time Nucleic Acid Sequence Based Amplification (NASBA) E6/E7 mRNA based PreTect HPV-Proofer assay detecting E6/E7 mRNA (type-specific differentiation of HPV 16, 18, 31, 33 and 45 including human mRNA control detection; PreTect AS, Klokkarstua), real-time Nucleic Acid Sequence Based Amplification (NASBA) E6/E7 mRNA based PreTect SEE assay (type-specific differentiation of HPV 16, 18 and 45 including human mRNA control detection; PreTect AS, Klokkarstua).[59]

The main challenge with nearly all the commercial HPV DNA detection methods is the dependency of the consensus primer. These consensus primer-sets have a lower analytical sensitivity compared to a direct typing real-time PCR method (Figure 3). Due to the low analytical sensitivity of these consensus primer-sets, the analytical sensitivity of these PCR methods may not be higher than HC2 or other similar hybridization methods. This may cause a problem when large numbers of histological or cytological normal samples are analyzed. Another challenge for the consensus primer's used in PCR is the lack of analytical specificity. Therefore, consensus PCR done in samples with two or more infections may completely miss one or two of the types due to the combination of low analytical sensitivity and specificity. Even a rather high number of HPV 16 (14-40%) or invasive cervical cancer cases (19%) may be missed due to this challenge.[21,23,61] The AHPV-GT RNA/DNA detection method has the same challenge due to dependency of consensus primer-set before using the type-specific probes.[62] Another challenge

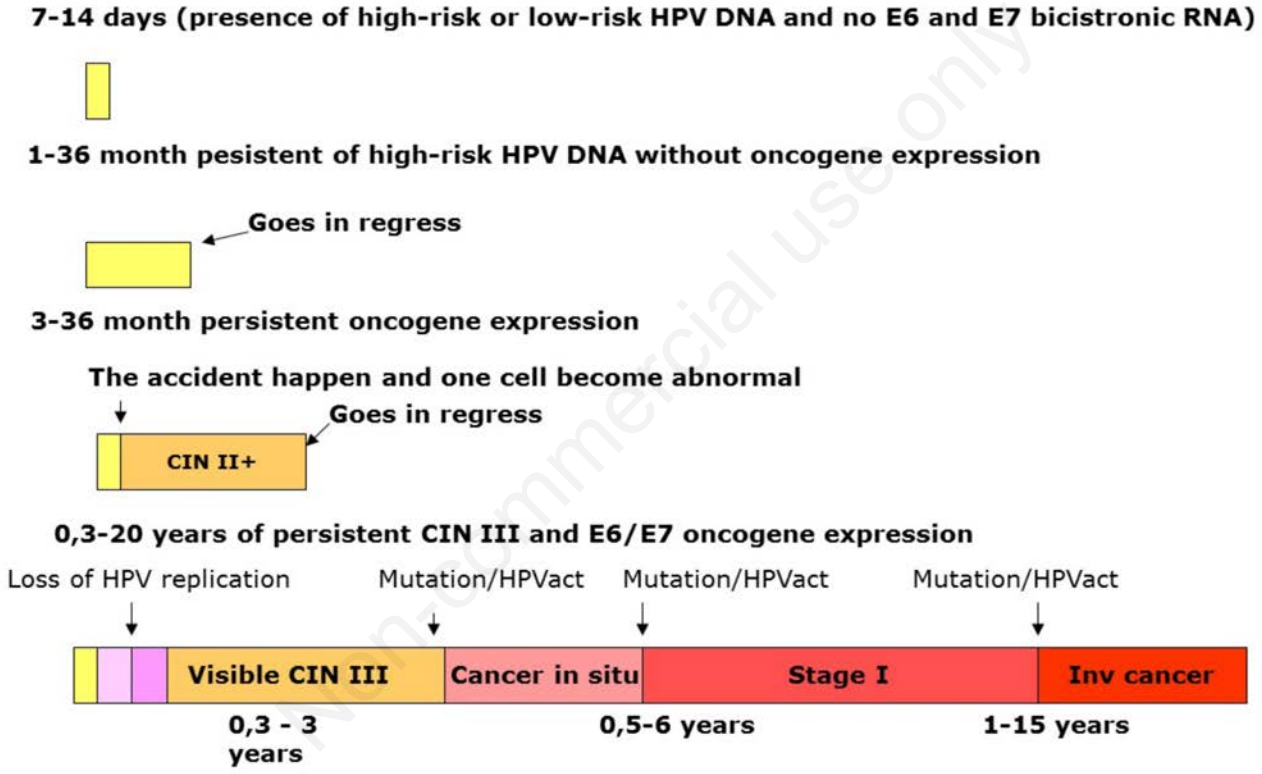

Figure 3. Illustration of the potential oncological capacity behind cervical pre-cancer national screening methods. If cytology or histology in the morphology method line is just observing normal epithelial cells, there is probably no oncological activity. However, many times both histology and cytology may not analyze representative cell samples or may not at all be sensitive enough. Cytology may miss 50% of samples with cancer cells. The morphological diagnose condyloma may represent an infection but may not have anything to do with cervical pre-cancer or risk of developing pre-cancer. Morphological methods may not always be able to define a case without a disease. Cancer in-situ detected by morphological methods is very likely to present a persistence until invasive cervical cancer. The visual inspection with acetic acid or colposcopy may identify a risk of a cervical pre-cancer development or a normal infection. However, this kind of visual inspection methods may not be able to judge whether there is a present pre-cancer or only a presence of an infection or just an abnormality not related to cancer. The visual inspection method line may be strongly hampered by a rather high number of false positives. The HPV DNA consensus methods may detect and amplify any DNA from 12 HPV types or more and cannot separate between transient and transforming infections. HPV DNA consensus PCR methods may miss infections in samples that are normal using the morphological line. This is probably due to the lack of analytical sensitivity. HPV DNA consensus PCR methods may detect infection that is not related to any disease. The HPV DNA consensus PCR methods cannot identify directly the presence of a pre-cancer and are only able to identify a risk of the development of a cervical pre-cancer. However, the type-specific direct PCR methods are able to identify persistent infections that is more likely to have cervical pre-cancer or develop into cervical per-cancer. The E6/E7 mRNA from 3-5 HPV types identified by real-time NASBA using PreTect HPV mRNA products may represent the presence of a true dysplasia or true pre-cancer. A repeated presence of E6/E7 mRNA may show that the immune system is not able to remove transformed of neoplastic cells in the cervix. If E6/E7 mRNA are present after months of repeat testing, the transformed neoplastic cells maypersists until invasive cervical cancer.

with the AHPV technology is the detection of HPV E6/E7 DNA in addition to HPV E6/E7 mRNA.[60] According to the Getman and colleagues that has developed this AHPV technology, the AHPV or the AHPV-GT will detect all HPV DNA copies in cervical samples containing 600,000 or one million Siha like cancer cells.[60] According to experienced cytologists (personal communication), normal cytological samples collected from women in a screening program contain between 1-10 million cells. Therefore, the AHPV-GT technology will detect HPV E6/E7 DNA in the same way as HPV E6/E7 mRNA in typical female screening population.

In the next generation of commercial methods envisage provide same day sampling, diagnosis and treatment in addition to more conventional physician-gynecologist-cytologist-labtest-oncologist road. This kind of same day services are normally called point-of-care diagnosis and treatment.

The rest of the document discuss the key characteristics of the E6/E7 mRNA technology and the role of oncogene expression from abnormal HPV genomes as the cause of cervical pre-cancer, the main cause of cervical cancer, and the basis for the assessment of E6/E7 mRNA and E6/E7 oncogenic proteins in the cervical mucosa. In addition, a high number of per-review articles and clinical data are available that supports or evaluate the use of PreTect HPV mRNA products in the diagnostics of pre-cancer lesions and cervical cancer.[23-44,47,51,63-86]

Key biological properties of the E6/E7 mRNA technology

Introduction and summary

The main HPV assays may be divided into target amplification methods including consensus or type-specific primers, HPV mRNA amplification including E6/E7 targeting primer-sets and probes or signal amplification methods.[40] The first standardized E6/E7 mRNA method detects the presence of E6 and E7 proteins following the expression of abnormal E6/E7 mRNA from the five carcinogenic HPV types. The E6/E7 mRNA method detects an abnormal presence of E6/E7 mRNA in the cervical mucosa samples that has been shown to be related to the loss of transcriptional regulation (discussed below).[23,24,37,38,40,42,44]

The stable significant expression of the E6/E7 proteins and mRNA can only happen when the infected epithelial cells lose control. This loss of regulation is expected to happen when the carcinogenic process starts. This loss or regulation has been documented to be related to cell instability, accumulation of E6/E7 full-length proteins, binding of E6 and E7 to cell cycles regulating proteins, integration of the carcinogenic HPV types and accumulation of mutations (Figures 1 and 2). Stable expression of full-length E6/E7 cannot happen without the continuous presence of full-length (and not spliced mRNA) E6/E7 transcripts.[87] Therefore, the presence of E6/E7 mRNA expression in the cervical area, in mucosa or in vaginal or vulva may always be the same as the presence of cell abnormalities (Figure 1). The main cause of this lack of regulation is the integration of carcinogenic HPV according to a number of international papers.[88-95] Loss of regulation causes deregulation of promoter 97 resulting in abnormal stable expression of E6/E7 mRNA.[96-109] This would automatically give high and stable production of the E7 and full-length E6 proteins (Figures 1-3). This production causes cell abnormalities or pre-cancer in the basal cell layer.[110-120] The abnormal epithelial cells with lack of regulation will after few hours, end up in the cervical mucosa. Each of these epithelial cells produce from 100-1000 copies of the E6/E7 mRNA.[121,122] Therefore, a transformed epithelial cell from the dominating carcinogenic HPV types, will likely produce E6 mRNA positive result. During the further carcinogenic process and even towards the advanced invasive cervical cancer and metastases, the E6 and E7 proteins continue to be the main player and cause.[91,123-126] The stable high or low transcription of E6/E7 full-length and modified mRNA, is probably the main reason for continuous expression of the E7 protein and the full-length E6 protein.[91]

E6 and E7 proteins from the carcinogenic HPV types are the most oncogenic macromolecules ever discovered.[127-131] This has been documented in more than 5000 international peer-review articles (Pubmed Search) since 1985 giving the following key properties of the E6 and E7 proteins:

1. The E6 protein from carcinogenic HPV (but not from none-carcinogenic HPV) produce three nuclear localization signals making it operational both in cytoplasm and in the nucleus
2. The expression of the E6 protein alone immortalize several human cells[132]
3. E6 activate vascular endothelial growth factor promoter and fibroblast growth factor –binding protein mediating angiogenesis[133]
4. E6 induce over-expression of 16ink4a and p27kip1 to enter S phase (only in carcinogenic HPV types)[134]
5. Both E6 and E7 cause via anaphase bridges telomerase activity and chromosomal instability[135]
6. E6 from the carcinogenic HPV types inhibits onco-suppressive functions of p53 thereby preventing cell cycles arrest or apoptosis induction. E6 can compete with p53, repress p53 and inhibit p53 translocation[136-138]
7. E6 degrade p53 together with ubiquitin ligase, E6-AP and together with E6-AP the E6 protein bind to several kinases of the SRC family[138]
8. E6 is involved in the deregulation of transcription and DNA replication[139]
9. Carcinogenic E6 protein binds to PDZ motifs causing regulation of cell adhesion, apicobasal polarity, and proliferation. This binding also causes unblocking of hDlg/APC tumor suppressor functions[140]
10. E6 binds to interferon regulatory factor, calciumbinding protein E6-BP. E6 inhibit insulin signaling, Jak-STAT pathway and p63 pathway[141]

Clinical experience indicates that any malignant growth is preceded by specific pre-cancer or pre-cancerous lesions (Figure 3).[142] Dysplasia became one of the key morphological criteria of the concept of pre-cancer.[143,144] The term dysplasia became widespread in both practical morphology and therapy. In the cervical epithelium, dysplasia is characterized by abnormal cell composition and architectonics.[144] The cells become heteromorphic demonstrated with wide variation in the size and shape. The nuclei become hyperchromatic and oversized relative to the normal nuclei. This phenomenon is called dyskaryosis. The number of mitotic figures increases and they are found in unusual sites of the epithelial layer. In cervical dysplasia, mitoses can be detected in any (including surface) layer of the multilayered epithelium as against basal cells only in the norm. However, atypical mitoses are not commonly observed. Dysplasia is also characterized by abnormal architectonics as a loss of the normal epithelial structure, polarity, and sometimes histotypic or organotypic pattern: the vertical anisomorphy of cells is lost in the multilayered squamous epithelium and the layer is replaced with basal cells instead of progressive differentiation of the basal elements into squamous cells (Koss's diagnostics Cytology). Strong evidence has shown that E6 and E7 proteins cause abnormal cellular changes on the molecular level[145,146] which may or may not correlate with morphological dysplasia or neoplasia criteria of the concept of pre-cancer. A study performed in Africa, which included histology

and cytology from all the women, showed that more than 50% of the histological negative women still had expression of E6/E7 mRNA.[37] However, all clinical studies always include a rather high number of HPV DNA or RNA positive cases without any morphological positive diagnosis. The papillomavirus type DNA may be present in a clinical sample with dysplasia, neoplasia or CIN2+ without being the cause of further on-going pre-cancer or persistent CIN2+ or invasive cancer.[52] This means that HPV 52, HPV 35 or HPV 31 may be present in a clinical sample claimed by morphological analyses to have pre-cancer but may never cause persist oncogenic expression until presence of invasive cervical cancer. The detection of HPV DNA by PCR in clinical samples with neoplasia or dysplasia morphology not able to progress into persistent CIN2+ or invasive cervical cancer, may cause too many false-positives within a typical nationwide screening setting or program.

Definition of and comparison between CIN and Presence of E6/E7 mRNA expression as detected using pathological methods or E6/E7 mRNA technology

Definition of *Cervical Intraepithelial Neoplasia (CIN):* All precancerous intraepithelial abnormalities of the uterine cervix that are capable of progression to invasive cancer, albeit with a low frequency for the better differentiated (low-grade) lesions and a higher frequency for poorly differentiated (high-grade) lesions.[147-151]

Definition of *Presence of E6/E7 mRNA expression in cervical mucosa:* Precancerous abnormalities are present in the uterine cervix that is capable of progression to invasive cancer.

Similarities between pathology and E6/E7 mRNA technology*:* i) Both can identify abnormal cervical cells; ii) Both can identify cervical pre-cancer; iii) Both are related to hyperchromatic and oversized nuclei relative to the normal nuclei; iv) Both identify mitosis.

Differences between pathology and E6/E7 mRNA technology: i) E6/E7 mRNA technology cannot identify the location of the oncogene expression in the epithelial cell layers; ii) More than one morphological lesion will often be present making diagnosis very subjective. The E6 and E7 is present or not present and the difference is not subjective;[40,44] iii) E6/E7 mRNA technology may be optimal for detection of molecular oncology in completely normal samples, while morphological methods may be dependent upon the collection of biopsies in order to detect any oncological activity;[37,40] iv) Morphological methods are dependent on subjective counting of number of cell abnormalities (Koss's Diagnostic Cytology). E6/E7 mRNA technology may only be used as a qualitative method;[40,152] v) 250 different kinds of morphological diagnoses have been observed by histological examination. Many different kinds of morphological diagnosis have also been observed by cytological examination. Molecular diagnosis is either positive or negative and there is now room for questions;[11,12,153] vi) E6/E7 mRNA technology has undergone complete performance evaluation showing robustness, sensitivity and specificity.[38,40] Cytology can undergo complete performance control but only against another morphological methods. The typical high number of ASCUS/LSIL cases including a number of undefined diagnosis definitions may cause problems when cytology is correlated with the type-specific PCR or the E6/E7 mRNA technology; vii) More than 70% of the following morphological diagnosis may be negative with E6 mRNA technology: ASCUS, hyperplasia, metaplasia, condyloma, HPV-changes, LSIL or CIN1 diagnosis;[42,44,152,154] viii) More than half of the cases with moderate cervical intraepithelial neoplasia are always negative with E6 mRNA Technology.[154,155] It means that it is difficult to understand how cells with neoplasia and dysplasia that do not supporting expression of E6/E7 mRNA is able to progress to invasive cervical cancer.

Function and importance of the different E6/E7 mRNA transcripts and their expression

Function and importance of the different E6/E7 mRNA transcripts

HPV E6 and E7 expression is primarily regulated at the transcriptional or post-transcriptional level. For example, for HPV 16, the E6 Open Reading Frame (ORF) encodes at least three distinct variants of the E6 protein, which all may have different roles in the viral life cycle. These transcripts are either unspliced (full-length E6-E7 transcript) or spliced transcripts: E6*I is spliced from nucleotide 226 to 409 and E6*II from nucleotide 226 to 526, all being transcribed from the promoter p97 located just upstream of the second ATG of the E6 ORF. The full-length E6 protein has been reported translated from the E6 full-length transcript. The E7 protein is likely encoded by the E6*I or E6*II and for some time it was thought that this splicing event was a means of obtaining high levels of E7 expression.[20] In fact, a report by Stacey et al. states that the HPV-16 E7 protein is also translated from full-length E6-E7 mRNA structures, demonstrating that splicing is not required for E7 synthesis.[21] Additionally, only the full length E6 protein, not the spliced E6 variants, is found to efficiently bind to and promote the degradation of p53[22] and it is further suggested that spliced transcripts of the HPV 18 E6 gene may encode an E6 modified protein that inhibits the full-length E6 mediated degradation of p53.[23-25] Moreover, unspliced E6 mRNA is found to be more closely associated with tumorigenicity as compared to the spliced transcripts and studies including cervical cancer samples show that the full-length transcript is always present, either alone or together with the spliced transcript.[27] The detection of HPV 16 E6*I/E6*II representing the way the RT-PCR can detect the full-length transcript may serve to identify transcription patterns indicative of cervical disease progression and help physicians to decide clinical management.[34] Taken together, these studies point to the full-length transcript as being the transcript important for the carcinogenic process.

The importance of E6/E7 mRNA expression

The role of E6/E7 mRNA expression in the natural lifecycle of human papillomavirus is very clear:

In a normal HPV infection at the lower layers of the cervical epithelium, the E6/E7 transcripts or full-length transcripts are transcribed from the p97 promoter. However, when the infected cells move up to the surface (terminal differentiation) of the cervical epithelium the p97 promoter will be more and more down regulated and finally turned off. Therefore, the transcripts that are transcribed from this promoter in cells with normal HPV infections cannot be detected in the upper layers of the stratified squamous epithelia.[141] With other words: This totally turn off the transcription of E6 and E7 mRNA in the upper layers of the stratified squamous epithelia (The upper part of the spinous layer, the granular layer and the cornified layer).[121] A normal papillomavirus infection regulate down the p97 promoter in order to increase the transcription from the p670 promoter preparing the ground for viral particle production. During the terminal differentiation an increased level of E1 and E2 are produced turning down the activity of the p97 switching to the p670. The p670 promoter produces transcripts that are spliced out in order to preferential expression L1/L2 transcripts and proteins.[58] The viral early promoter, which controls E6 and E7 expression, is thought to be constitutively active during differentiation (in the lower parts of epithelia) in order to maintain the cells in a "pseudo" S phase state necessary for high-level replication of

viral episome.[136] In cervical pre-cancer cases (without normal HPV infection) this regulation and the regulation of mRNA splicing is lost and all cells coming to the cornified layer will be transcribe from the p97 promotor giving full-length E6 transcripts in addition to different variants as well as full-length E6 and E7 proteins.

Therefore, the detecting of E6/E7 mRNA in the cervical mucosa tells us the following (Figure 1): i) Something is wrong with the normal HPV life cycle;[156-158] ii) There must be a lack of transcriptional control;[91,159] iii) This lack of transcriptional control is most likely related to integration of HPV by deletion of whole or parts of the E1 and E2 genes;[120,160] iv) The splicing of HPV mRNA is probably not regulated anymore giving a possible stable production of full-length E6 mRNA as well as full-length E6 proteins. This is in particular the case detected by the HPV 16 primer-sets and probes, walking over the area between the E6 and E7 genes, that can only detect full-length HPV 16 E6/E7 mRNA; v) Stable expression of E6/E7 mRNA giving stable expression of E7 and full-length E6 proteins is by a molecular oncological definition, the cause of cervical pre-cancer (First Author definition); vi) Stable expression of E6/E7 mRNA giving stable expression of E7 and full-length E6 proteins is also the main cause of invasive cervical cancer and metastasis; vii) The only possible cure of this irreversible lack of regulation and uncontrolled oncogene activity is the removal of the pre-cancer cells by treatment or by the natural immune system.

This should be a strong indication that E6/E7 cannot be detected in factual normal smear samples. In fact, we have at least two studies showing that the E6/E7 expression is totally absent in factual CIN1 cases diagnosed defined by a panel of experienced pathologists.

One option is the level of E6/E7 mRNA expression. In samples with a malignant progression the loss of regulation of E6/E7 protein or mRNA expression due to integration or instability is the real problem, proved in a high number of scientific studies. Loss of regulation may not give different defined levels of mRNA expression that correlate with different level of malignancy detected or different stages of carcinogenesis. We believe that integration or loss of regulation is resulting in any level of expression throughout the stratified squamous epithelial, and is visible on the surface of the epithelium following the differentiation. Even a very low level of mRNA expression will cause high production of full-length E6 and E7 proteins. We do not know about any method that always detects a level less than 20 mRNA copies per cell, when the method is positive in a routine screening setting. The E6/E7 mRNA technology has documented a PPV for CIN2+ higher than 50% in clinical studies even with strong bias from cytology or histology. This indicates that it is very likely that E6/E7 mRNA technology when positive is detecting underlying pre-cancer in the majority of cases. This also indicates that assays (typical HPV DNA assay's) detecting more than 70-80%, positivity rate (or sensitivity) towards CIN2+ cases will have an increased level of false positive or may not reflect the true oncological state of the lesion.

Evaluation of different transcripts from HPV 16 discovered in clinical samples

Design of E6/E7 mRNA technology

The E6/E7 mRNA technology detects the presence of E6 and E7 proteins following the expression of abnormal E6/E7 mRNA from the five carcinogenic HPV types. E6/E7 mRNA technology detects an abnormal presence of E6/E7 mRNA in the cervical mucosa samples that has been shown to be related to the loss of transcriptional regulation (discussed below). Normally human papillomaviruses do not express the E6/E7 mRNA in the three upper layers of the epithelium. It is only when something goes wrong with the human papillomavirus that it is able to express E6/E7 mRNA in the upper layer of epithelial cells. It is only these kinds of infections that is defined as transforming infection and will be persistent transforming infection if the human immune system is not able to remove the transformed cells.

Design of the primer-set and probes

The sequences and positions of the primers and molecular beacons within the E6/E7 sequences and targets are patented but it is still all business secrets. However, the discussion below presents evidence related to the selection of correct transcripts for optimal cervical cancer screening. As a positive reaction control for the different primer-sets, artificial oligos were designed based on the primer and probe sequences.

HPV 16 transcripts in normal cells, CIN2, CIN3 and SCC lesions

The NASBA method was used to analyze the HPV 16 transcript pattern in HPV 16 DNA positive samples with a normal cytological diagnosis (n=75) and histological CIN2 (n=7), CIN3 (n=21) or SCC diagnosis (n=116, Karlsen and Molden *et al.,* not published). The E6/E7 full-length transcript was detected in approximately 1/3 of the normal Pap smears while the E6*I/II, E1, E2 and E1^E4^E5 transcripts were detected in almost twice as many normal samples. The E1^E4^L1 transcript was detected in 41% of the normal samples. Full-length transcripts detected by the use of E6/E7 primer-set (PreTect HPV-Proofer) were detected in all CIN2 and CIN3 and SCCs included in this study, while transcripts amplified by the use of E1^E4^L1 primers were detected in 30-50% of the lesions. In the SCCs, the E6*I/II primer-set detected transcripts in 114/116 (98%). The E1 transcript(s) was detected in all SCCs, and it was more prevalent in CIN3 than in CIN2 and normal samples. In contrast, the E2 transcripts were detected in 95% of the normal smears positive for HPV 16 transcripts. The use of E2CE5 primer-set detected 3/116 (3%) SCC.

HPV 16 transcripts in relation to the physical state of HPV in SCC

Karlsen and Molden (not published) were also interested in the aspect of transcript patterns in relation to the physical state of HPV in SCC determined by *in situ* hybridization (ISH). The physical state of HPV identified as viral integration (I) (n=50) or as viral integration/episomal (I/E) (n=64) has been described previously.[161] In addition, two samples were not positive for HPV 16 DNA by ISH. The transcripts detected by the use of primer-set E6/E7 (E6/E7 mRNA technology), E6*I/II, and E1 are present in almost all the SCCs, integrated as well as integrated together with episomal states of the virus. The transcripts detected by the use of the E2 primer-set, was detected in 72% of the lesions where the virus was found to be integrated in the human genome, while it was detected in 97% of the lesions which contained both integrated and episomal forms of the virus. The E1^E4^L1 and E1^E4E5 transcripts were detected in roughly half the SCCs with only integrated viral DNA in contrast to nearly all SCC with episomal DNA as well and hence support the current understanding about disruption within the E2 gene region upon viral integration in SCCs.[162] We would not expect to detect these transcripts when integration has occurred in the E1-E2 gene region.[162] However, we cannot be sure that these samples are not containing small amounts of episomal forms of DNA as well, due potential lack of analytical sensitivity by the ISH method.

Cellular aspects of tumor progression

Precancerous and early cancerous lesions are of clear interest. Clinical experience indicates that any malignant growth is

preceded by specific changes. However, there is no common view of 'precancer'; some propose a narrow definition, while others tend to broaden it. Finally, in addition to the concept of cancer progressive development, the concept of its *de novo* development exists.[89] Dysplasia became one of the key morphological criteria of this concept. This term became widespread in both practical morphology and therapy. At the same time, the limits of the term are getting more and more fuzzy, which necessitates the definition of 'dysplasia'. Metaplasia is the replacement of one distinctive tissue with another one that differs morphologically and functionally. During metaplasia, the epithelium loses the organotypic form and function, while the histotypic type and function are preserved. These changes in tissue differentiation rely on pluripotent basal cells, which are sources of any epithelium development. Morphological evaluation of metaplastic changes should consider not only the histotypic and structural properties, but also the cytological properties of tissue elements. Dysplasia is an abnormal differentiation giving rise to cells with pathological properties. Metaplasia and dysplasia can develop independently; however, it is very important that dysplasia can develop on the background of metaplasia. The relationships between metaplasia, atypical hyperplasia, and dysplasia remain controversial, which leads to terminological confusion and complicates the interpretation of data. Dysplasia should be considered only as controlled and reversible precancerous abnormalities of epithelial differentiation resulting from the proliferation of cambial elements (undifferentiated pluripotent cells of the basal layer) to atypical cells with no polarity and affected histological structure without the membrane invasion.[27,138] In the cervical epithelium, dysplasia is characterized by abnormal cell composition and architectonics. The cells become heteromorphic and demonstrate wide variation in the size and shape. The nuclei become hyperchromatic and oversized relative to the normal nuclei. This phenomenon is called dyskaryosis. The number of mitotic figures increases and they are found in unusual sites of the epithelial layer. In cervical dysplasia, mitoses can be detected in any (including surface) layer of the multilayered epithelium as against basal cells only in the norm. However, atypical mitoses are not commonly observed. Dysplasia is also characterized by abnormal architectonics as a loss of the normal epithelial structure, polarity, and sometimes histotypic or organotypic pattern: the vertical anisomorphy of cells is lost in the multilayered squamous epithelium and the layer is replaced with basal cells instead of progressive differentiation of the basal elements into squamous cells. Dysplasia of the cervical multilayered squamous epithelium features limited numbers of proliferation foci with affected vertical anisomorphy of cells in the layer, basal cell hyperplasia, nuclear polymorphism and hyperchromatism, enlarged nuclei, higher nuclear/cytoplasmic ratio, hyper- and parakeratotic lesions, and high mitotic activity. At the same time, the pathological elements to different extents replace the epithelial layer usually not reaching the surface layers. Different stages (grades) of dysplasia are recognized according to the degree of epithelial proliferation and structural and cellular atypia, affecting the cell organization.[9,165] The most significant morphological features of dysplasia include nuclei polymorphism and abnormal mitoses. The limits between different grades of dysplasia and preinvasive cancer or sometimes invasive cancer are not always clearly defined by morphological analysis.[1,2,4,33,64,98,110] Adequate identification of dysplasia and its grade is of principal clinical significance and largely determines the risk of malignant transformation and treatment approach. The probability of malignant transformation of the regenerating, hyperplastic, or metaplastic epithelium is low. The risk of transformation increases in the case of dysplasia, and severe dysplasia demonstrating cellular changes similar to cancerous ones appears to correspond to the highest risk. The progress of the recent years in studying cervical carcinogenesis is primarily due to the elucidation of the role of papillomaviruses. The integration event invariably results in the expression of two viral proteins, E6 and E7.[91,163-165] These two proteins are capable of transforming cells individually and cooperate to immortalize primary human epithelial cells. *In vitro* tissue culture studies indicate that HPV E6 and E7 are oncogenes, and that their oncogenicity is due in part to their capacity to inactivate cellular tumor suppressor genes. The behavior of E6 and E7 *in vitro* and the genetic evidence from analysis of human cancers suggest that the E6 and E7 genes play a significant role in the development of cervical cancer.[166-172] Aneuploidy, the most frequent form of genomic instability in human carcinomas, develops as early as in nonmalignant cervical precursor lesions. In addition, cervical neoplasia is frequently associated with abnormal multipolar mitotic figures, suggesting disturbances of the cell-division process as a mechanism for chromosome segregation defects. Spindle poles are formed by centrosomes, and the high-risk HPV E6 and E7 oncoproteins can each induce abnormal centrosome numbers.[173-175] These two HPV oncoproteins, however, induce centrosome abnormalities through fundamentally different mechanisms and, presumably, with different functional consequences. High-risk HPV E7, which targets the pRB tumor suppressor pathway, can provoke abnormal centrosome duplication in phenotypically normal cells. On the contrary, cells expressing the HPV E6 oncoprotein, which inactivates p53, accumulate abnormal numbers of centrosomes in parallel with multinucleation and nuclear atypia. These two pathways are not mutually exclusive, since co-expression of HPV E6 and E7 has synergistic effects on centrosome abnormalities and chromosomal instability. Taken together, these findings support the general model in which chromosomal instability arises as a direct consequence of oncogenic insults and can develop at early stages of tumor progression. In summary E6-E7 gene products submerge control of the cell cycle and mitotic spindle pole formation through complex interactions with various cellular protein complexes and induce severe chromosomal instability. The E6 and E7 activity should therefore not cause normal differentiation, morphology, metaplasia, hyperplasia or atypical hyperplasia. The activity of other markers like Ki67, p14 or p16 may cause metaplasia, hyperplasia or atypical hyperplasia. However, the activity of E6/E7 should cause nuclear polymorphism, nuclear hyperchromatism, enlarged nucleic, cellular atypia, abnormal cell organization, abnormal epithelium organization, higher nuclear/cytplasmic ratio, hyper-and parakeratotic lesions, high mitotic activity and abnormal mitoses.[173-175] In fact, these kind of changes should very much be linked to the typical definition of severe dysplasia or severe dyskaryosis and should cover broad range of cervical pre-cancer behavior and characteristics. Cells having this kind of E6/E7 activity can only be removed by the immune system or by surgery. The very presence of E6/E7 mRNA in the mucosa or in cells collected at the upper epithelium layer is linked to integration of the carcinogenic HPV and loss of E6/E7 expression regulation including ongoing activity from promoter p97. It is logical that no presence of E6/E7 mRNA in the upper epithelium layer or in the mucosa would not cause any translation of the E6 and E7 proteins, making the presence of cellular abnormalities unlikely. It is quite obvious that both the E6/E7 full-length mRNA and the E6 and E7 proteins are involved in the cervical carcinogenesis and therefore are the main cause and engine behind invasive cervical cancer.[160,175-192]

The potential of E6/E7 mRNA technology for national screening of cervical cancer

Some possible definitions

There is no doubt that colposcopy, histology and cytology has been very valuable morphological examination methods in order to define cervical dysplasia, neoplasia, pre-cancer, cancer in-situ and invasive cancer. However, many studies have shown that subjective morphological examination even including automatic or manual image analyses are disturbed by the complexity of a cell smear or a tissue including thousands of different matrices, networks, structures caused by millions of very different cells. The results are that national cancer registry may operate with more than 250 kinds of different histological or cytological diagnosis given by very different pathologists before it is sorted into more Bethesda like defined diagnosis.[12-14,193]

HPV DNA based amplification and detection methods have increased the possibility to detect any potential pre-cancer like morphology, even though these methods is not able to separate between natural HPV infection and transforming/carcinogenic HPV infection. Natural HPV infections are not directly related to the development or the presence of cervical pre-cancer. Therefore, the HPV DNA methods detecting 14 or more HPV types containing mostly natural HPV infections, make many more women positive than the cytological method, making many more unnecessary women the idea that they may have cervical pre-cancer. It is obvious that even the detection of natural HPV 16 or 18 infection, is not the same as the detection of a cervical pre-cancer causing, is also producing false-positives. Only using cytology as the national screening test in Norway produced 17000 women every year with a not relevant risk of developing cervical cancer (The Norwegian Cancer Registry). The repeat use of a very sensitive HPV DNA method mostly detecting natural HPV infection, have to increase the number of women with not relevant positive diagnosis without any transforming/carcinogenic HPV infection.

An ideal gold standard method for the identification of cervical pre-cancer or cancer may be defined with the following number of properties: i) The method must identify the cause of the disease and not a risk of the disease. ii) The method must identify the true pre-cancer or neoplasia that progress to invasive cervical cancer if the immune system is not working. iii) The method must create a minimum number of false positives and negatives. iv) The method must give a simple yes or no conclusive diagnosis with highest possible analytical performance (in order to be used in national screening settings). v) The method should be able to be used in a national screening setting that causes the highest possible coverage rate. vi) The sample collection method used related to the method should be possible to be done by normal health personal or the woman herself. vii) The method must be accurate enough to cause the same day treatment.

How these properties are working related to different screening strategies are discussed below.

1. Morphological examinations are not able to identify the cause of the disease but maybe the results of a disease. Morphological methods may miss more than 50% of invasive cervical cancer. The HPV DNA methods cannot identify the cause but only the risk of developing the disease. Many enough false-positives detected by the HPV DNA increase the likelihood of detecting invasive cervical cancer. However, the first time HPV DNA methods is used it is not able to detect more than 90% of the women with invasive cervical cancer. The PreTect HPV mRNA product, PreTect HPV-Proofer identifies the cause of cervical pre-cancer and the main cause of invasive cervical cancer. The detection of similar or more numbers of invasive cervical cancer cases than a typical consensus PCR or Hybridization method targeting only five HPV types compared with 14 or more HPV-types, teach that PreTect HPV-Proofer identifies more specific cancer markers (Table 1). It also teaches that the standard DNA methods use a lot of biomarkers that is not related to the main cause of invasive cervical cancer. Table 1 also confirm that it is very likely that PreTect HPV-Proofer detect the real disease markers with very few false negatives making it very likely that the PreTect HPV-Proofer detect the cause of cervical pre-cancer.
2. Morphological examinations are not able to identify the true pre-cancer or neoplasia disease directly making it necessary to repeat the test many times within time intervals,[12-14,193] The HPV DNA methods can only identity the presence of an HPV type that may have a risk of developing severe neoplasia or true pre-cancer.[194-202] PreTect HPV-Proofer identify the cause of pre-cancer or severe neoplasia and progress to invasive cervical cancer, if the immune system is suppressed.[37,41,44]
3. Morphological examinations always create a high number of false positives and negatives. The HPV DNA methods always create a high number of false positives but with less false negatives. However, it has been shown by Castle's research group that some HPV 16, 18 or 45 infections are not detected with the consensus PCR methods.[203-205] The E6/E7 mRNA technology may detect a minimum number of false negative and positives. But, because it is difficult to perform a study without verification

Table 1. E6/E7 mRNA technology has similar sensitivity as DNA test against samples positive for Invasive cervical cancer.

Study	N.	PreTect HPV-Proofer, %	DNA-tests
Lie AK *et al.* (2005) DNA versus RNA based methods for HPV detecting cervical neoplasia. Gyn Oncol Vol 97, Issue 3, June 2005, 908-915	20	100	90% (hc2)
J. Moecle (2007) Evaluation of a E6/E7 – RNA – transcripts as a predictor of cervical neoplasia in a multicenter study of a high-risk population	35	97	94% (hc2)
Kraus *et al.* (2006) Presence of E6 and E7 mRNA from Human Papillomavirus Types 16, 18, 31, 33 and 45 in the majority of Cervical Carcinomas, J. Clin. Microbiol Vol 44, Issue 4, April 2006, 1310-1317.	204	89	93% (GP5+/6+)
Basu *et al.* Human papillomavirus Genotype Distribution in Cervical Cancer in India: Results from a Multi-center study. Asian Pacific J. Cancer, 10, 2009, 27-34.	278 DNA 276 RNA	83	83% (My09/11)

bias and with proper treatment of all PreTect HPV-Proofer positive it is difficult to confirm these minimum numbers.[37] It may be claimed that the E6/E7 mRNA technology may miss some few cancer's or samples with true pre-cancer cells since it is not detecting HPV 35, 52 or 58 However, It has been proved that morphological and DNA methods may also miss some cancer's and true pre-cancer cells.[203,206-209]

4. Morphological examinations including ASCUS, LSIL or CIN 1 can never produce a simple yes or no conclusion. Most of the morphological methods produce conclusions that just demands coming back for a new evaluation. Even several ASCUS diagnoses may not cause a simple yes or no conclusion. The analytical performance of morphological diagnosis cannot be fully defined. The HPV DNA methods may not come up with a yes or no diagnosis as long as they are not performing type-specific analyses. Including a HPV-DNA type-specific diagnosis it may still not tell the clinicians what to do with HPV 35, 52, 58, 66, 67, 56, etc.[210,211] When these HPV types are discovered in Europe it may only contain HPV DNA that do not support stable production of oncogenic E6 and E7. The HPV DNA type-specific diagnosis is not telling the clinicians if this infection is a transforming or transient infection.
5. The use of morphological methods demands large cytological laboratories with intra and inter control quality system connected to a very well-organized national screening system that is able to follow-up all women carefully and repeat the cytological examinations several times. The HPV DNA methods will also create many positive women that have to be followed-up for several times before treatment. The most problematic example is the use of HC2 in China creating a positivity rate of more than 19% in a normal population.[22]
6. Samples collected for morphological methods have to be done by a professional medical person. With the rather large number of self-collected samples methods it has been proved that PCR based DNA technology methods can be used.[212-215]
7. Most positive morphological diagnosis cannot be used for direct treatment. The same is the case for all the HPV DNA based results. However, the PreTect technology identify the cause of pre-cancer and have the potential to be used as test and treat.

Recent evaluation and meta-analyses of the accuracy of mRNA tests used in screening settings compared with the definition of diagnostic gold standard

Two studies have evaluated the use of PreTect HPV-Proofer and Nuclisens Easy Q HPV in different studies.[78,216] Verdoodt and colleagues concluded that the HPV assays for detecting of 5 hrHPV types may reduce the over-diagnosis of women who have minor cytologic abnormalities. However, given the lower sensitivity, women with negative mRNA test results cannot be considered free of CIN2+ and require further surveillance. Origoni and collagues (2015) concluded the following: Compared to hrHPV-DNA testing, which actually represents the most validated alternative to cytology in screening settings, mRNA tests present the valuable improvement of a better specificity, and consequently, higher positive predictive value (PPV) towards high-grade cervical lesions (CIN2+).[216] HPV E6/E7 mRNA testing may serve as a more specific discriminator between transient cervical dysplasia and potentially progressive lesions. According, testing for high-risk HPV E6/E7 mRNA might reduce the psychological burden associated with HPV-DNA testing.

It is very clear that the main engine behind the progression to cervical pre-cancer and invasive cervical cancer is stable expression of E6 and E7 full-length proteins followed by the stable presence of full-length E6/E7 mRNA. It is also very clear that histological defined CIN2+ or cytological HSIL is a typical definition of cervical tissue with severe dysplasia/neoplasia or pre-cancer. However, it is very well known that the female immune system is able to both remove cells with stable expression of E6/E7 proteins and to remove CIN2+ or HSIL like cells from the transformation zone. Very large follow-up studies have shown that 50% of cervical pre-cancer cells may not progress to invasive cervical cancer weather they are defined as CIN2+, HSIL or with E6/E7 expression.[46] Luckily, many women have an immune system that is able to remove these pre-cancer-like cells. The question is whether it is possible to claim that CIN2+ or HSIL like cells without detectable E6/E7 expression is pre-cancer like cells in the same way as cells with E6 and E7 expression without detectable CIN2+ or HSIL like cells. It may be claimed that women with E6/E7 expression from carcinogenic HPV types without detectable CIN2+ or HSIL like cells may have more carcinogenic pre-cancer cells than in women with CIN2+ or HSIL without detectable E6/E7 from the carcinogenic HPV types. The CIN2+ or HSIL diagnosis is a subjective morphological diagnosis that is not covered by any analytical performance evaluation and is very dependent upon how skilled and well experienced the histologists are. In addition, the histological examination is dependent upon a more complex collection of representative biopsies by a medical person and only one or 10 pre-cancer cells may not be enough for making a diagnosis. The molecular test is only dependent upon a simple collection of cervical samples using a Cervex brush. The histological examination does not include an objective control of sample (detection of human mRNA) in the same way as included in the PreTect HPV- mRNA products. Key statistical properties of E6/E7 mRNA technology

E6/E7 mRNA technology detects the presence or absence of E6/E7 mRNA and the active molecular oncogenes (E6 and E7) followed by the expression of E6/E7 mRNA from for the HPV-types 16, 18, 31, 33, and 45 or only HPV-types 16,18 and 45.[23,40] The key statistical properties of E6/E7 mRNA technology are as follows: i) High positive predictive value towards CIN2+ (30-50%);[37,43,44,78] High sensitivity towards cervical cancer (comparable to HPV-DNA detection methods, typically ≥90%);[41] High specificity in a screening population (comparable or slightly better than cytology).[37,41-43,155]

This chapter gives an overview of these key statistical properties of the E6/E7 mRNA technology and discusses, in addition, the same characteristics of cytology and HPV-DNA testing technologies. The calculations are based on the clinical and scientific documentation, experience and knowledge behind cytology, HPV-DNA and E6/E7 mRNA technologies.[37,40]

The key statistical properties have to be estimated using three different scenarios in order to evaluate the predictive accuracy.

i) When measuring PPV it is important to focus on pre-cancerous lesions which may be identified or verified by independent technologies such as histology.[216,217] Although many direct prognostic markers have been identified to date, most of these did not make it into actual clinical decision algorithms.[218] The biomarker has to match the current clinical diagnostic gold standard without any verification bias.
ii) Since only a minority of histologically confirmed CIN2+ progress to invasive cervical cancer,[46] real sensitivity must be measured against invasive cervical cancer. This was also concluded about in the recent study done in USA against

more than 250 000 women having undergone Pap or HPV DNA testing one year before a colposcopy directed biopsy was evaluated.[21] The conclusion was: Since most CIN3 did not progress to cancer, it is of more concern that approximately 19% of women with biopsy-documented cancer in the study tested negative for HPV.

iii) Specificity indicates the volume of false positives. Since invasive cervical cancer and also pre-cancerous lesions are rare in a population,[11,14] correct screening program specificity can only be measured in a screening population supposed to be normal.

Negative predictive value (NPV) is a statistical property that is independent of technology due to the low number of annual invasive cervical cancer cases.

Positive predictive value towards CIN2+

In diagnostics, it is important to have a high positive predictive value. In cervical cancer prevention, the different technologies available have quite dissimilar PPV's for detecting a histologically confirmed CIN2+ condition. The discussion below is based on overview of results from the literature. To be able to estimate the actual incident level of CIN2+ in a population, the sensitivity of cytology towards CIN2+ is important. However, there are very few studies which are properly designed for measuring this sensitivity. Meta-analysis indicates the sensitivity of cytology at about 50%.[15,16]

PPV of cytology

Cervical cytology is based on a range of different diagnosis, of which the most common ones are normal, ASCUS, LSIL, HSIL, and invasive cervical cancer (ICC). Therefore, the PPV of cytology will, of course, differ depending on the cut-offs used. In the United States about 6%, or about 3.5 million of 55 million yearly Pap smears, are classified as abnormal.[16] The majority of these women are referred to colposcopy and biopsy. We have not found documentation on how many women that are treated by conization each year in the United States that are based on identified CIN2+ histology. However, based on numbers from Norway,[11,12,14,153] we estimated that less than 1% of the women screened are treated by conization annually. This indicates an overall PPV of less than 17% for cytology (*PVV* = 1% / 6%)

PPV of HPV-DNA testing technologies

HPV-DNA testing technology is acknowledged to have higher sensitivity than cytology, but at the same time finds many more women as positives. Scientific studies indicate that the positivity rate of HPV-DNA testing technology is about 3 times higher than the rate of abnormal cytology in a screening population.[219] Thus, since the rate of abnormal cytology is about 6% in USA it can be expected that about 18% of the women undergoing screening annually will be positive with an HPV-DNA testing technology. The higher sensitivity of HPV-DNA testing technology is estimated to 90-95%,[219] resulting in considerably more women being found as histologically confirmed CIN2+ compared to cytology; roughly estimated to 1.9% of the women screened (*i.e.* 95% of 2%). Many opinion leaders advocate the use of cytology to limit the number of HPV-positive women referred to colposcopy and for collection of biopsies.[220] This implies that the estimated sensitivity increase of HPV-DNA testing technologies will be counteracted by the subsequent cytology analysis. These observations indicate an overall PPV of 5% [*PPV* = (1.9% × 50%) / 18%]for HPV-DNA testing technologies.

Recently, an HPV-mRNA assay has been introduced (APTIMA HPV, Hologic, San Diego, Madison). Published studies indicate that the properties of this assay are quite similar to the HPV-DNA testing technologies already present in the market, with a possible slight reduction in the positivity rate. Since the positivity rate is similar to the HPV-DNA testing technologies, cytology is still needed in order to reduce the number of referrals to colposcopy and biopsy. This implies that this HPV-RNA assay may have similar or possibly a slightly increased PPV compared to HPV-DNA testing technologies. However, there is no evidence that the PPV of this HPV-RNA detection technology will approach the PPV of cytology.

PPV of E6/E7 mRNA technology

Studies show that E6/E7 mRNA technology has a higher sensitivity than cytology and finds a number of positive women that is about 25% less than those found to be abnormal by cytology,[37] *i.e.* it can be estimated that E6/E7 mRNA technology will be positive in about 4.5% of the women undergoing screening annually in the United States. Based on the sensitivity of cytology in the United States, which is estimated at 50%, and the expected treatment rate of 1% (see paragraph on 'PVV of cytology'), we expect that about 2% of the women have an incident underlying CIN2+ abnormality. Since the number of E6/E7 mRNA technology positive women are comparable to the number of women referred to colposcopy and biopsy today, no cytology triage is needed. Thus, the increased sensitivity is not altered by a cytology triage step as is the case for HPV-DNA testing technologies. Studies show that E6/E7 mRNA technology has a sensitivity of 70-80% towards CIN2+. This indicates that about 1.4-1.6% of these women will be found by E6/E7 mRNA technology, indicating a PPV of 31% (PVV = 1.4% / 4.5%). However, existing primary screening studies indicate an even higher PPV of about 50%. One explanation may be that the expected sensitivity of on-time cytology is even less than 50%, probably approaching 30%. It is only repeating cytology than may have a clinical sensitivity high enough to be defended used in national screening services.[12]

Sensitivity towards cervical cancer in screening algorithms

The aims of all cervical cancer screening programs are to reduce the incident of invasive cervical cancer and at the same time reduce the number of deaths caused by invasive cervical cancer. Since the disease is invasive cervical cancer, all preventive diagnostics, should first of all be evaluated towards confirmed invasive cervical cancer cases.

Traditionally the cervical cancer prevention programs have transformed the main aims into the pseudo endpoint of detecting pre-cancerous lesions defined as histologically confirmed CIN2+. However, previous and recent studies have shown that only very few CIN2 cases will ever develop into invasive cervical cancer and that only 30% of the CIN3 cases will ever develop into invasive cervical cancer.[45,46] Since almost all CIN2+ cases are treated there is today a huge overtreatment in connection with existing screening programs. This overtreatment does not contribute to the above-mentioned main aims of the cervical cancer prevention programs.

Since only a minority of the histologically confirmed CIN2+ cases progress to invasive cervical cancer, it is obvious that at any time a significant proportion of these cases are regressing to a normal condition. If all CIN2+ cases would eventually develop into invasive cervical cancer, diagnostic sensitivity could be measured towards this pseudo endpoint. Since this obviously is not the case, it is not correct to measure diagnostic sensitivity towards CIN2+. Therefore, diagnostic sensitivity must be measured towards the real disease which is invasive cervical cancer. It is the sensitivity towards invasive cervical cancer which is the basis for estimating the effect of applying different diagnostic technologies in the cervical cancer prevention programs. We

are aware that most professionals measure diagnostic sensitivity towards the pseudo endpoint of histologically confirmed CIN2+. For diagnostic tests with a sensitivity of 90-95% towards CIN2+ (*e.g.* HPV-DNA testing technologies) this distinction is less important than for diagnostic tests having less sensitivity towards CIN2+. Since most scientists have investigated the properties of HPV-DNA testing technologies having high sensitivity towards CIN2+, the distinction between sensitivity towards CIN2+ and cervical cancer has so far been ignored, also in the United States. We believe it is now utterly important to measure true diagnostic sensitivity in the correct way by investigating the sensitivity of different technologies towards invasive cervical cancer.

Sensitivity of cytology

Several reports estimating the sensitivity of cytology towards invasive cervical cancer have been published during the last decades. Most of them focus on the sensitivity towards CIN2+ indicating a sensitivity of about 50% (see section 'Positive predictive value towards CIN2+'). Several authors presume that existing screening programs using cytology have reduced the incidence of invasive cervical cancer by about 70%. This presumption is the same for both United States and for Norway,[20,23,84,124] but has inherent uncertainties since the situation today is compared to the situation 30-50 years ago not taking into the account the dramatic change in lifestyle during this period. It is also impossible to study the rate of invasive cervical cancer and corresponding deaths in today's society without preventive measures such as cytology.

The high-quality databases of the Norwegian Cancer Registry support the theory that cytology has a rather low sensitivity towards invasive cervical cancer. It is documented that 40-50% of women developing invasive cervical cancer (within the screening program) have a satisfactory screening history.[11,12] About 80% of the women developing invasive cervical cancer have one or more Pap smears during the preceding 24 months. For these women cytology did not prevent cervical cancer. These facts support the assumption that the sensitivity of cytology is between 20 and 50%.

Sensitivity of HPV-DNA testing technologies

There are many studies using HPV-DNA testing technologies on invasive cervical cancer cases.[41,221-224] Based on meta-analysis published by IARC the overall sensitivity is about 85%. However, recent studies comparing HPV-DNA testing technologies with E6/E7 mRNA technology indicate an overall sensitivity of about 90%. As mentioned above, many opinion leaders advocate the use of cytology to limit the number of HPV-positive women referred to colposcopy and for collection of biopsies.[220,225-227] This implies that the estimated sensitivity of HPV-DNA testing technologies will be counteracted by the subsequent cytology analysis. In any real-world application of HPV-DNA testing technologies triage with cytology is thus being used reducing the effective sensitivity to the sensitivity of cytology, which is estimated at about 50% (Figure 4).

Sensitivity of E6/E7 mRNA technology

Studies are available comparing the sensitivity of E6/E7 mRNA technology with HPV-DNA testing technologies on invasive cervical cancer. They show the diagnostic sensitivity of E6/E7 mRNA technology to be comparable to the HPV-DNA technologies tested. The sensitivity of E6/E7mRNA technology towards invasive cervical cancer is about 90%,[41,155] which is illustrated in Figure 5. This implies that a E6 mRNA negative test result will have the same negative predictive value compared to HPV-DNA testing technologies when looking at invasive cervical cancer cases.

Compared to HPV-DNA testing technologies, E6/E7 mRNA technology is the only technology available which has documented a lower sensitivity towards CIN2+.[37,43,44,154] With an optimal sensitivity towards invasive cervical cancer and at

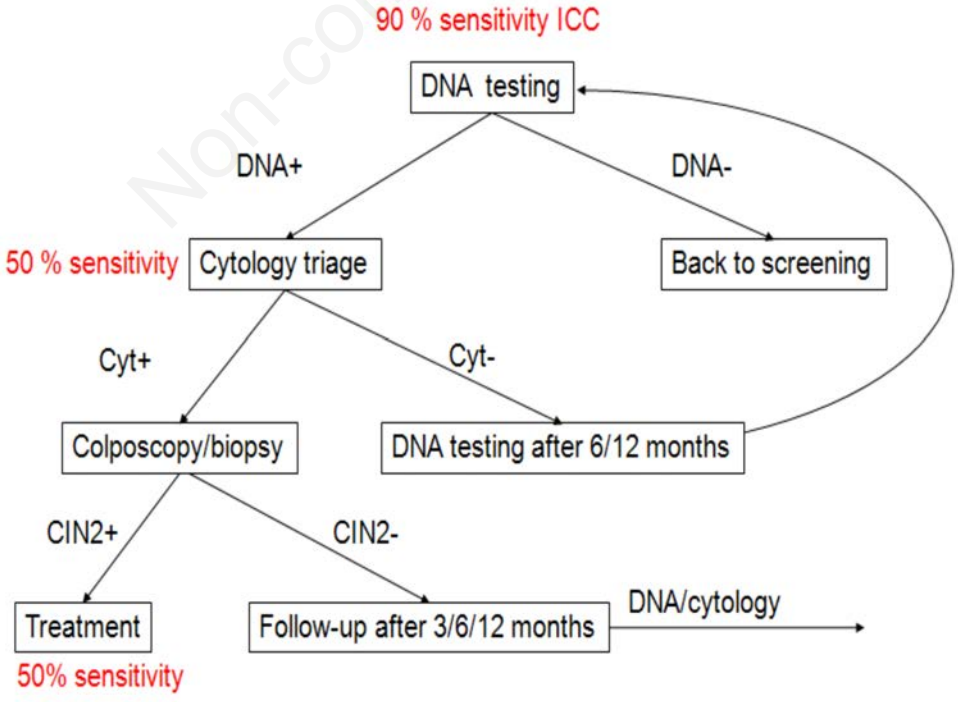

Figure 4. Sensitivity towards ICC using HPV-DNA testing technologies.

the same time a lower sensitivity towards CIN2+, E6/E7 mRNA technology is the only technology available to give both high assurance against the development of cervical cancer and at the same time reflect the "real world" knowledge of significant regression of CIN2+. This is strongly reflected in Figure 1, presenting the stage where women must be treated.[24,27,34,78]

Specificity in a screening population

Specificity is a way to measure false positives, *i.e.* patients without a disease positive by a diagnostic test. All patients enrolled in some sort of follow-up routine should be included when specificity is calculated.

Invasive cervical cancer and pre-cancerous lesions are rare conditions. Only about 11.270 (National Cancer Institute about cervical cancer, www.cancer.gov/cancertopics/types/cervical) women in the United States develop invasive cervical cancer annually (0.3 ‰ of the women tested annually). In addition, we expect that only about 1% or less are treated by conization annually. In a diagnostic setting, this implies that specificity is approximately 100% minus the positivity (abnormal) rate of the diagnostic test. If the positivity rates of different diagnostic tests are known (as exact numbers or as fixed ratios between the different tests), it is easy to calculate the expected diagnostic specificity (or relative specificity) in a screening population for these tests.

Specificity of cytology

The rate of abnormal cytology is estimated at about 6% in the United States. This implies a screening specificity of about 94%. In the literature, the specificity of cytology varies quite a bit depending on the study population at hand. A lower rate of abnormal cytology implies an increase in specificity. Some studies have also calculated specificity based on other cut-offs such as LSIL or HSIL resulting in very high specificities. This, however, does not reflect the basic requirement to include all patients enrolled in some sort of follow-up routine when calculating specificity.

Specificity of HPV-DNA testing technologies

As previously mentioned, the positivity rate of HPV-DNA testing technologies is estimated at about 3 times the rate of abnormal cytologies, *i.e.* 18%. This implies a screening specificity of about 82%. This specificity will be the same regardless of whether or not cytology is included to triage HPV-DNA positive cases. HPV-DNA testing technologies have a high number of false positives which will result in a multitude of women referred to follow-up routines.

Specificity of PreTect HPV-Proofer

As previously mentioned, we expect the positivity rate of E6/E7 mRNA technology to be about 25% less than the abnormal rate of cytology, *i.e.* 4.5%. This implies an expected screening specificity of about 95.5%.

Using the E6/E7 mRNA technology will avoid about 80% of the false positives detected by HPV-DNA testing technologies, decreasing the economic costs and psychological burdens of unnecessary follow-up.

Conclusions

In this paragraph we discussed the key properties of cytology, HPV-DNA testing technologies and E6/E7 mRNA technology. The key properties, *i.e.* PPV, sensitivity towards cervical cancer and specificity in a screening population including all referred clinical studies, are summarized in Table 2.

In addition, the follow-up rate is estimated as the positivity (abnormal) rate minus the number of identified CIN2+. With a rough per annum regression rate of 50% of test positives the accumulated follow-up rate is also indicated for the second year. The use of cytology has reduced the invasive cervical cancer incidence and mortality significantly during the last decades, but only in women older than 40 years of

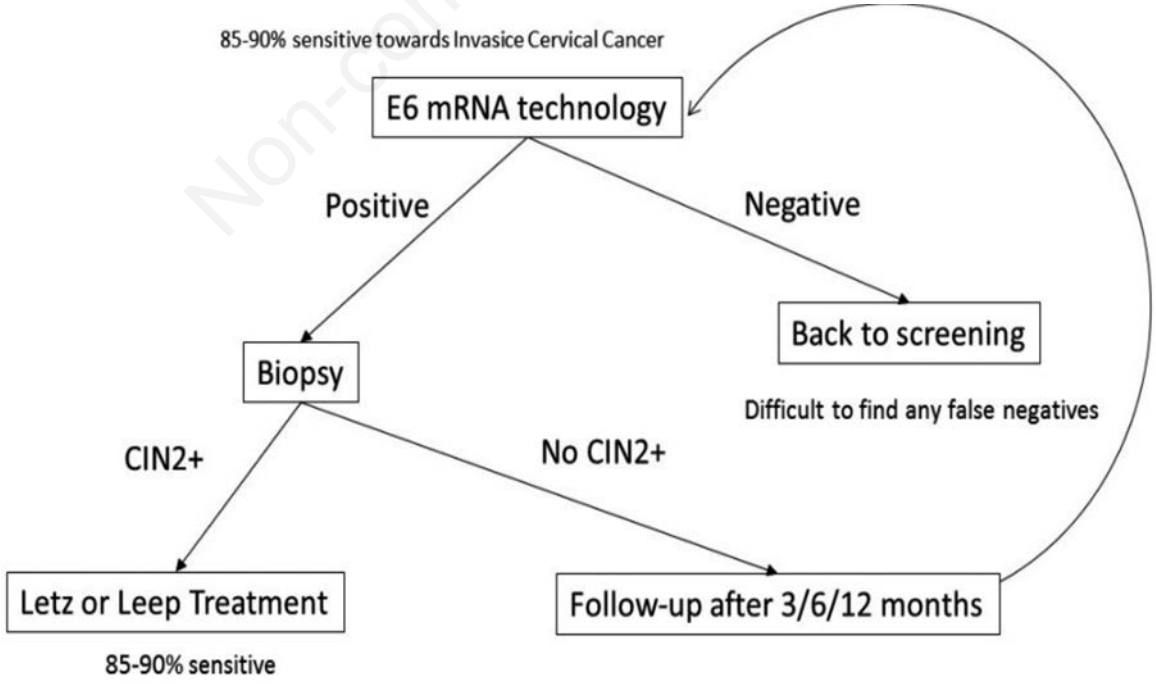

Figure 5. Sensitivity towards ICC using E6/E7 mRNA technologies.

age. Recently however, this rate of decline in cervical cancer mortality has leveled off and it may even be increasing slightly in young women. The reason is likely to be the limited sensitivity of on-time cytology, less coverage rate in the young female population or it may be general increased unprotected sexual activity. To be able to achieve further reduction in ICC incidence and mortality new technologies have to be implemented. The use of HPV-DNA testing technologies will most likely not increase the sensitivity towards ICC compared to the use of cytology due to the fact that cytology is included as a triage step for HPV-DNA testing technologies. In addition, screening specificity is dramatically reduced compared to the use of cytology only.

The E6 mRNA technology is likely to perform better than both cytology and HPV-DNA testing technologies (with cytology triage) in a screening setting (Figure 6). The number of E6 mRNA technology positives are comparable to the number of women referred to colposcopy in today's cytology-based screening. All E6/E7 mRNA technology positive women may then be referred directly to colposcopy and biopsies or direct treatment. The E6/E7 mRNA technology will result in superior PPV and sensitivity. In addition, the specificity will be comparable to the specificity of cytology.

Incident follow-up rate and the total number of women in a follow-up routine will be comparable when using E6/E7 mRNA technology or cytology. Using HPV-DNA testing technologies, about 16% of women screened will be directly referred to a follow-up routine. The size of the accumulated follow-up group is likely to exceed 20% when using HPV-DNA testing technologies resulting in unnecessary psychological strain and huge costs.

Based on all the discussions within this review, it is clear that the main driving engine and the cause of cervical pre-cancer and the main cause of invasive cervical cancer is the expression of E6 and E7 from HPV 16, 18, 31, 33 and 45. In some areas the HPV 35, 52 and 58 may be included, but it has been proved that the prevalence of these HPV types decline from cytological normal to confirmed invasive cervical cancer. There is a reason to believe that these HPV types may only be transient when discovered in cancer. However, it is very clear that it is only HPV 16, 18, 45 and maybe 33 that increase its prevalence from within cytological normal cases to histological confirmed invasive cervical cancer. The need for the detection and informing women about other HPV types is not clear. It is very clear that in a typical screening population including a high number of cytological normal cases the HPV DNA testing may miss from 14 to 40% of the women

Table 2. Summary of the estimated key properties.

Properties	Technologies		
	Cytology, %	DNA with cytology triage, %	PreTect HPV-Proofer, %
Positivity (abnormal) rate	6	18	4.5
Positive predictive value (PPV)	17	5	31
Sensitivity towards ICC	50	50	90
Specificity	94	82	95
Follow-up	5	16	3
Accumulated follow-up year 2	7.5	24	4.5

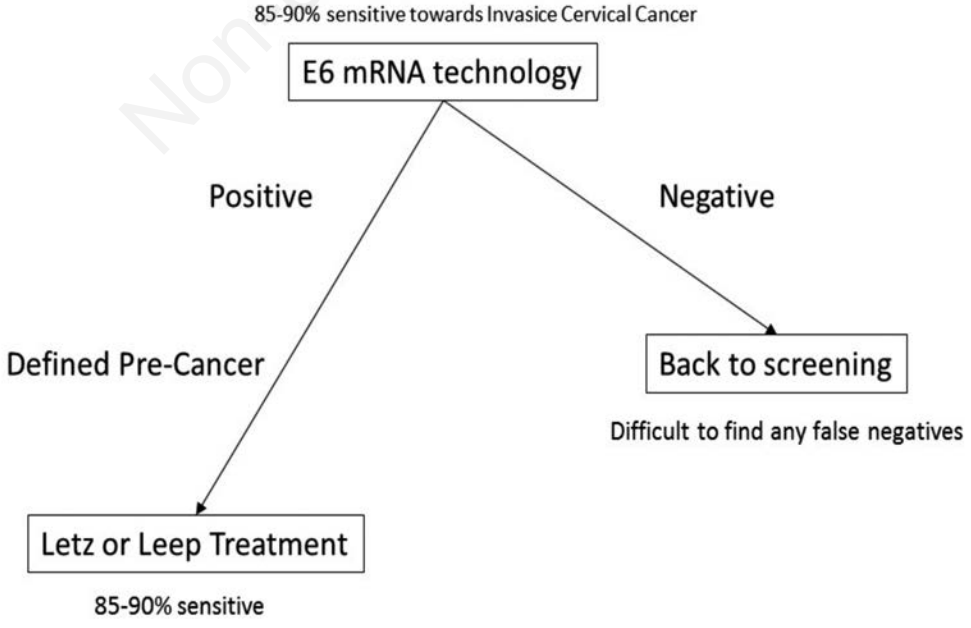

Figure 6. E6/E7 mRNA technology used in primary screening.

with carcinogenic infections. Blatt *et al.,* (2015) has proved this when more than 256 000 samples were evaluated missing 19% of the invasive cancers using "so called" gold standard HPV DNA technology; HC2. It may also be true that women may be more afraid of E6/E7 positive cases without a confirmed CIN2+ than CIN2+ confirmed cases without E6/E7 positive expression. Through many studies using the E6/E7 mRNA methods it has been clear that it is hard to confirmed that there do exist any false positive or any false negative using cancer or progressive disease as endpoint. This would cause the potentially most optimal screening scenario, selecting only women with a real pre-cancer disease, as illustrated in Figure 1 and 6. Therefore, it should be possible to select the method with the highest potential to be a medical gold standard for primary screening of cervical pre-cancer. The studies presented in this review, using the 3-5 biomarkers by the E6/E7 mRNA methods targeting the most carcinogenic activity ever discovered inside a well performed detection technology, have been shown to be similar or better than any other promising cancer or biomarker that are on its way into screening of breast, colon or other cancer types (Figure 1). As far as we have understood, none of these new biomarkers against other cancer types are able to predict more than 30% of the coming pre-cancer disease and none of them is able to detect higher than 90% of cases with invasive cancer.

References

1. Steinkamp RC, Harden VA, Deneke JS, Uyeda CK. Comparison of cervical Pap smears with subsequent diagnosis of cervical carcinoma an analysis of Arkansas health department Pap smears for 1970 and 1971. J Ark Med Soc 1975;72:168-71.
2. Sinykin MB. When the cervical Pap smear is positive. Minn Med 1966;49:1095.
3. Smith CW, Sealey RM. The clinical significance of class III cervical Pap smear. Am Surg 1962;28:420-2.
4. Dalla Palma P, Giorgi Rossi P, Collina G, et al. The risk of false-positive histology according to the reason for colposcopy referral in cervical cancer screening: a blind revision of all histologic lesions found in the NTCC trial. Am J Clin Pathol 2008;129:75-80.
5. Hellberg D, Nilsson S, Valentin J. Positive cervical smear with subsequent normal colposcopy and histology—frequency of CIN in a long-term follow-up. Gynecol Oncol 1994;53:148-51.
6. Korkolopoulou P, Kolokythas C, Kittas C, et al. Correlation of colposcopy and histology in cervical biopsies positive for CIN and/or HPV infection. Eur J Gynaecol Oncol 1992;13:502-6.
7. Husain O, Butler EB, Woodford FP. Combined external quality assessment of cytology and histology opinions: a pilot scheme for a cluster of five laboratories. J Clin Pathol 1984;37:993-1001.
8. Fowler W. Cervical cancer—intraepithelial neoplasia, severe dysplasia, invasive cancer of cervix, DES exposure. Proceedings, the annual meeting of the Medical Section of the American Council of Life Insurance. 1982:73-85.
9. Richart RM, Barron BA. Screening strategies for cervical cancer and cervical intraepithelial neoplasia. Cancer 1981;47:1176-81.
10. McCrory DC, Matchar DB, Bastian L, et al. Evaluation of cervical cytology. Evid Rep Technol Assess 1999:1-6.
11. Nygard JF, Nygard M, Skare GB, Thoresen SO. Screening histories of women with CIN 2/3 compared with women diagnosed with invasive cervical cancer: a retrospective analysis of the Norwegian Coordinated Cervical Cancer Screening Program. Cancer Causes Control 2005;16:463-74.
12. Nygard JF, Sauer T, Nygard M, et al. CIN 2/3 and cervical cancer in an organised screening programme after an unsatisfactory or a normal Pap smear: a seven-year prospective study of the Norwegian population-based screening programme. J Med Screen 2004;11:70-6.
13. Nygard JF, Sauer T, Skjeldestad FE, et al. CIN 2/3 and cervical cancer after an ASCUS pap smear. A 7-year, prospective study of the Norwegian population-based, coordinated screening program. Acta Cytol 2003;47:991-1000.
14. Nygard JF, Skare GB, Thoresen SO. The cervical cancer screening programme in Norway, 1992-2000: changes in Pap smear coverage and incidence of cervical cancer. J Med Screen 2002;9:86-91.
15. Fahey MT, Irwig L, Macaskill P. Meta-analysis of Pap test accuracy. Am J Epidemiol 1995;141:680-9.
16. Nanda K, McCrory DC, Myers ER, et al. Accuracy of the Papanicolaou test in screening for and follow-up of cervical cytologic abnormalities: a systematic review. Ann Intern Med 2000;132:810-9.
17. Wentzensen N, Zuna RE, Sherman ME, et al. Accuracy of cervical specimens obtained for biomarker studies in women with CIN3. Gynecol Oncol 2009;115:493-6.
18. Adamopoulou M, Kalkani E, Charvalos E, et al. Comparison of cytology, colposcopy, HPV typing and biomarker analysis in cervical neoplasia. Anticancer Res 2009;29:3401-9.
19. Wang SS, Dasgupta A, Sherman ME, et al. Towards improved biomarker studies of cervical neoplasia: effects of precolposcopic procedures on gene expression patterns. Am J Surg Pathol B 2005;14:59-64.
20. Patterson B, Domanik R, Wernke P, Gombrich M. Molecular biomarker-based screening for early detection of cervical cancer. Acta Cytol 2001;45:36-47.
21. Blatt AJ, Kennedy R, Luff RD, et al. Comparison of cervical cancer screening results among 256,648 women in multiple clinical practices. Cancer Cytopathol 2015;123:282-8.
22. Wang R, Guo XL, Wisman GB, et al. Nationwide prevalence of human papillomavirus infection and viral genotype distribution in 37 cities in China. BMC Infect Dis 2015;15:257.
23. Westre B, Giske A, Guttormsen H, et al. 5-type HPV mRNA versus 14-type HPV DNA test: test performance, overdiagnosis and overtreatment in triage of women with minor cervical lesions. BMC Clin Pathol 2016;16:9.
24. Sorbye SW, Fismen S, Gutteberg TJ, et al. HPV mRNA is more specific than HPV DNA in triage of women with minor cervical lesions. PloS One 2014;9:e112934.
25. Cuschieri K, Hardie A, Hovland S, et al. Comparison of the sensitivities of three commercial assays for detection of the high risk HPV types 16, 18 and 45. J Virol Methods 2013;193:147-50.
26. Alaghehbandan R, Fontaine D, Bentley J, et al. Performance of ProEx C and PreTect HPV-Proofer E6/E7 mRNA tests in comparison with the hybrid capture 2 HPV DNA test for triaging ASCUS and LSIL cytology. Diagn Cytopathol 2013;41:767-75.
27. Sorbye SW, Fismen S, Gutteberg TJ, et al. HPV mRNA testing in triage of women with ASC-US cytology may reduce the time for CIN2+diagnosis compared with repeat cytology. Curr Pharmaceut Design 2013;19:1401-5.
28. Rijkaart DC, Heideman DA, Coupe VM, et al. High-risk human papillomavirus (hrHPV) E6/E7 mRNA testing by PreTect HPV-Proofer for detection of cervical high-grade intraepithelial

neoplasia and cancer among hrHPV DNA-positive women with normal cytology. J Clin Microbiol 2012;50:2390-6.
29. Szarewski A, Mesher D, Cadman L, et al. Comparison of seven tests for high-grade cervical intraepithelial neoplasia in women with abnormal smears: the Predictors 2 study. J Clin Microbiol 2012;50:1867-73.
30. Gulliksen A, Keegan H, Martin C, et al. Towards a "Sample-In, Answer-Out" Point-of-Care Platform for Nucleic Acid Extraction and Amplification: Using an HPV E6/E7 mRNA Model System. J Oncol 2012;2012:905024.
31. Andersson E, Karrberg C, Radberg T, et al. Type-specific human papillomavirus E6/E7 mRNA detection by real-time PCR improves identification of cervical neoplasia. J Clin Microbiol 2011;49:3794-9.
32. Basu P, Chandna P, Bamezai RN, et al. MassARRAY spectrometry is more sensitive than PreTect HPV-Proofer and consensus PCR for type-specific detection of high-risk oncogenic human papillomavirus genotypes in cervical cancer. J Clin Microbiol 2011;49:3537-44.
33. Ratnam S, Coutlee F, Fontaine D, et al. Aptima HPV E6/E7 mRNA test is as sensitive as Hybrid Capture 2 Assay but more specific at detecting cervical precancer and cancer. J Clin Microbiol 2011;49:557-64.
34. Sorbye SW, Fismen S, Gutteberg T, Mortensen ES. Triage of women with minor cervical lesions: data suggesting a "test and treat" approach for HPV E6/E7 mRNA testing. PloS One 2010;5:e12724.
35. Sorbye SW, Fismen S, Gutteberg TJ, Mortensen ES. HPV mRNA test in women with minor cervical lesions: experience of the University Hospital of North Norway. J Virol Methods 2010;169:219-22.
36. Hovland S, Muller S, Skomedal H, et al. E6/E7 mRNA expression analysis: a test for the objective assessment of cervical adenocarcinoma in clinical prognostic procedure. Int J Oncol 2010;36:1533-9.
37. Hovland S, Arbyn M, Lie AK, et al. A comprehensive evaluation of the accuracy of cervical pre-cancer detection methods in a high-risk area in East Congo. Br J Cancer 2010;102:957-65.
38. Jeantet D, Schwarzmann F, Tromp J, et al. NucliSENS EasyQ HPV v1 test - Testing for oncogenic activity of human papillomaviruses. J Clin Virol 2009;45:S29-37.
39. Keegan H, Mc Inerney J, Pilkington L, et al. Comparison of HPV detection technologies: Hybrid capture 2, PreTect HPV-Proofer and analysis of HPV DNA viral load in HPV16, HPV18 and HPV33 E6/E7 mRNA positive specimens. J Virol Methods 2009;155:161-6.
40. Molden T, Kraus I, Skomedal H, et al. PreTect HPV-Proofer: real-time detection and typing of E6/E7 mRNA from carcinogenic human papillomaviruses. J Virol Methods 2007;142:204-12.
41. Kraus I, Molden T, Holm R, et al. Presence of E6 and E7 mRNA from human papillomavirus types 16, 18, 31, 33, and 45 in the majority of cervical carcinomas. J Clin Microbiol 2006;44:1310-7.
42. Molden T, Kraus I, Karlsen F, et al. Human papillomavirus E6/E7 mRNA expression in women younger than 30 years of age. Gynecol Oncol 2006;100:95-100.
43. Molden T, Kraus I, Karlsen F, et al. Comparison of human papillomavirus messenger RNA and DNA detection: a cross-sectional study of 4,136 women >30 years of age with a 2-year follow-up of high-grade squamous intraepithelial lesion. Cancer Epidemiol Biomarkers Prev 2005;14:367-72.
44. Molden T, Nygard JF, Kraus I, et al. Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV-proofer and consensus PCR: A 2-year follow-up of women with ASCUS or LSIL Pap smear. Int J Cancer 2005;114:973-6.
45. McCredie MR, Paul C, Sharples KJ, et al. Consequences in women of participating in a study of the natural history of cervical intraepithelial neoplasia 3. Austral New Zealand J Obstet Gynaecol 2010;50:363-70.
46. McCredie MR, Sharples KJ, Paul C, et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. Lancet Oncol 2008;9:425-34.
47. Ratnam S, Coutlee F, Fontaine D, et al. Clinical performance of the PreTect HPV-Proofer E6/E7 mRNA assay in comparison with that of the Hybrid Capture 2 test for identification of women at risk of cervical cancer. J Clin Microbiol 2010;48:2779-85.
48. Narimatsu R, Patterson BK. High-throughput cervical cancer screening using intracellular human papillomavirus E6 and E7 mRNA quantification by flow cytometry. Am J Clin Pathol 2005;123:716-23.
49. Pao CC, Hor JJ, Yang FP, et al. Detection of human papillomavirus mRNA and cervical cancer cells in peripheral blood of cervical cancer patients with metastasis. J Clin Oncol 1997;15:1008-12.
50. Holm R, Kraus I, Skomedal H, et al. Human papillomavirus DNA and e6/e7 mRNA status in relation to survival of patients treated for cervical squamous cell carcinoma. Open Virol J 2008;2:74-81.
51. Cuschieri KS, Beattie G, Hassan S, et al. Assessment of human papillomavirus mRNA detection over time in cervical specimens collected in liquid based cytology medium. J Virol Methods 2005;124:211-5.
52. Matsukura T, Sugase M. Pitfalls in the epidemiologic classification of human papillomavirus types associated with cervical cancer using polymerase chain reaction: driver and passenger. Int J Gynecol Cancer 2008;18:1042-50.
53. De Vuyst H, Mugo NR, Chung MH, et al. Prevalence and determinants of human papillomavirus infection and cervical lesions in HIV-positive women in Kenya. Br J Cancer 2012;107:1624-30.
54. De Vuyst H, Ndirangu G, Moodley M, et al. Prevalence of human papillomavirus in women with invasive cervical carcinoma by HIV status in Kenya and South Africa. Int J Cancer 2012;131:949-55.
55. Schiffman M, Castle PE. The promise of global cervical-cancer prevention. New Eng J Med 2005;353:2101-4.
56. Kuramoto H, Banno M, Hori M, et al. Optimal sampling devices for liquid-based procedure in screening for cervical cancer: comparison between cotton stick/Cytobrush and Cervex-Brush. Acta Cytol 2013;57:153-8.
57. Kohlberger PD, Stani J, Gitsch G, et al. Comparative evaluation of seven cell collection devices for cervical smears. Acta Cytol 1999;43:1023-6.
58. Solomon D, Davey D, Kurman R, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. Jama 2002;287:2114-9.
59. Chan PK, Picconi MA, Cheung TH, et al. Laboratory and clinical aspects of human papillomavirus testing. Crit Rev Clin Lab Sci 2012;49:117-36.
60. Getman D, Aiyer A, Dockter J, et al. Efficiency of the APTIMA HPV Assay for detection of HPV RNA and DNA targets. J Clin Virol 2009;45:S49-54.
61. Castle PE, Schiffman M, Burk RD, et al. Restricted cross-reactivity of hybrid

capture 2 with nononcogenic human papillomavirus types. Cancer Epidemiol Biomarkers Prev 2002;11:1394-9.
62. Castle PE, Cuzick J, Stoler MH, et al. Detection of human papillomavirus 16, 18, and 45 in women with ASC-US cytology and the risk of cervical precancer: results from the CLEAR HPV study. Am J Clin Pathol 2015;143:160-7.
63. Andersson S, Hansson B, Norman I, et al. Expression of E6/E7 mRNA from 'high risk' human papillomavirus in relation to CIN grade, viral load and p16INK4a. Int J Oncol 2006;29:705-11.
64. Andersson S, Mints M, Weiderpass E, Johansson B. Human papillomavirus DNA and E6/E7 mRNA testing as triage in liquid-based cytology samples from primary screening. Curr Pharmaceut Design 2013;19:1508-15.
65. Benevolo M, Terrenato I, Mottolese M, et al. Diagnostic and prognostic validity of the human papillomavirus E6/E7 mRNA test in cervical cytological samples of HC2-positive patients. Cancer Causes Control 2011;22:869-75.
66. Benevolo M, Vocaturo A, Caraceni D, et al. Sensitivity, specificity, and clinical value of human papillomavirus (HPV) E6/E7 mRNA assay as a triage test for cervical cytology and HPV DNA test. J Clin Microbiol 2011;49:2643-50.
67. Broccolo F, Fusetti L, Rosini S, et al. Comparison of oncogenic HPV type-specific viral DNA load and E6/E7 mRNA detection in cervical samples: results from a multicenter study. J Med Virol 2013;85:472-82.
68. Burger EA, Kornor H, Klemp M, et al. HPV mRNA tests for the detection of cervical intraepithelial neoplasia: a systematic review. Gynecol Oncol 2011;120:430-8.
69. Cuzick J, Cadman L, Mesher D, et al. Comparing the performance of six human papillomavirus tests in a screening population. Br J Cancer 2013;108:908-13.
70. Dixon EP, Gronn P, King LM, et al. Analytical performance of RNA isolated from BD SurePath cervical cytology specimens by the PreTect HPV-Proofer assay. J Virol Methods 2012;185:199-203.
71. Dixon EP, King LM, Adams MD, et al. Isolation of RNA from residual BD SurePath liquid-based cytology specimens and detection of HPV E6/E7 mRNA using the PreTectt HPV-Proofer assay. J Virol Methods 2008;154:220-2.
72. Frega A, Sesti F, Lombardi D, et al. Assessment of HPV-mRNA test to predict recurrent disease in patients previously treated for CIN 2/3. J Clin Virol 2014;60:39-43.
73. Giorgi Rossi P, Benevolo M, Vocaturo A, et al. Prognostic value of HPV E6/E7 mRNA assay in women with negative colposcopy or CIN1 histology result: a follow-up study. PloS one 2013;8:e57600.
74. Mesher D, Szarewski A, Cadman L, et al. Comparison of human papillomavirus testing strategies for triage of women referred with low-grade cytological abnormalities. Eur J Cancer 2013;49:2179-86.
75. Nygard M, Roysland K, Campbell S, Dillner J. Comparative effectiveness study on human papillomavirus detection methods used in the cervical cancer screening programme. BMJ Open 2014;4:e003460.
76. Ovestad IT, Vennestrom U, Andersen L, et al. Comparison of different commercial methods for HPV detection in follow-up cytology after ASCUS/LSIL, prediction of CIN2-3 in follow up biopsies and spontaneous regression of CIN2-3. Gynecol Oncol 2011;123:278-83.
77. Paolini F, Rollo F, Brandi R, et al. High risk human papillomavirus genotyping in clinical samples: evaluation of different commercial tests. Int J Immunopathol Pharmacol 2011;24:127-38.
78. Sorbye SW, Arbyn M, Fismen S, et al. HPV E6/E7 mRNA testing is more specific than cytology in post-colposcopy follow-up of women with negative cervical biopsy. PloS One 2011;6:e26022.
79. Sorbye SW, Arbyn M, Fismen S, et al. Triage of women with low-grade cervical lesions—HPV mRNA testing versus repeat cytology. PloS One 2011;6:e24083.
80. Szarewski A, Ambroisine L, Cadman L, et al. Comparison of predictors for high-grade cervical intraepithelial neoplasia in women with abnormal smears. Cancer Epidemiol Biomarkers Prev 2008;17:3033-42.
81. Thomsen LT, Dehlendorff C, Junge J, et al. Human papillomavirus mRNA and DNA testing in women with atypical squamous cells of undetermined significance: A prospective cohort study. Int J Cancer 2016.
82. Trope A, Jonassen CM, Sjoborg KD, et al. Role of high-risk human papillomavirus (HPV) mRNA testing in the prediction of residual disease after conisation for high-grade cervical intraepithelial neoplasia. Gynecol Oncol 2011;123:257-62.
83. Trope A, Sjoborg K, Eskild A, et al. Performance of human papillomavirus DNA and mRNA testing strategies for women with and without cervical neoplasia. J Clin Microbiol 2009;47:2458-64.
84. Trope A, Sjoborg KD, Nygard M, et al. Cytology and human papillomavirus testing 6 to 12 months after ASCUS or LSIL cytology in organized screening to predict high-grade cervical neoplasia between screening rounds. J Clin Microbiol 2012;50:1927-35.
85. Varnai AD, Bollmann M, Bankfalvi A, et al. Predictive testing of early cervical pre-cancer by detecting human papillomavirus E6/E7 mRNA in cervical cytologies up to high-grade squamous intraepithelial lesions: diagnostic and prognostic implications. Oncol Rep 2008;19:457-65.
86. Verdoodt F, Szarewski A, Halfon P, et al. Triage of women with minor abnormal cervical cytology: meta-analysis of the accuracy of an assay targeting messenger ribonucleic acid of 5 high-risk human papillomavirus types. Cancer Cytopathol 2013;121:675-87.
87. Vinokurova S, Wentzensen N, Kraus I, et al. Type-dependent integration frequency of human papillomavirus genomes in cervical lesions. Cancer Res 2008;68:307-13.
88. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92:709-20.
89. Francis DA, Schmid SI, Howley PM. Repression of the integrated papillomavirus E6/E7 promoter is required for growth suppression of cervical cancer cells. J Virol 2000;74:2679-86.
90. Daniel B, Rangarajan A, Mukherjee G, et al. The link between integration and expression of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions. J General Virol 1997;78:1095-101.
91. Jeon S, Lambert PF. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. Proc Natl Acad Sci USA 1995;92:1654-8.
92. Sanchez-Lanier M, Triplett C, Campion M. Possible role for human papillomavirus 16 in squamous cell

carcinoma of the finger. J Med Virol 1994;44:69-78.
93. Snijders PJ, Meijer CJ, van den Brule AJ, et al. Human papillomavirus (HPV) type 16 and 33 E6/E7 region transcripts in tonsillar carcinomas can originate from integrated and episomal HPV DNA. J General Virol 1992;73:2059-66.
94. Wagatsuma M, Hashimoto K, Matsukura T. Analysis of integrated human papillomavirus type 16 DNA in cervical cancers: amplification of viral sequences together with cellular flanking sequences. J Virol 1990;64:813-21.
95. Schneider-Maunoury S, Croissant O, Orth G. Integration of human papillomavirus type 16 DNA sequences: a possible early event in the progression of genital tumors. J Virol 1987;61:3295-8.
96. Wells SI, Aronow BJ, Wise TM, et al. Transcriptome signature of irreversible senescence in human papillomavirus-positive cervical cancer cells. Proc Natl Acad Sci USA 2003;100:7093-8.
97. Belaguli NS, Pater MM, Pater A. Splice sites of human papillomavirus type 16 E6 gene or heterologous gene required for transformation by E7 and accumulation of E7 RNA. J Med Virol 1995;47:445-53.
98. Ranki A, Lassus J, Niemi KM. Relation of p53 tumor suppressor protein expression to human papillomavirus (HPV) DNA and to cellular atypia in male genital warts and in premalignant lesions. Acta Dermatol Venereol 1995;75:180-6.
99. von Knebel Doeberitz M, Rittmuller C, Aengeneyndt F, et al. Reversible repression of papillomavirus oncogene expression in cervical carcinoma cells: consequences for the phenotype and E6-p53 and E7-pRB interactions. J Virol 1994;68:2811-21.
100. DiPaolo JA, Popescu NC, Alvarez L, Woodworth CD. Cellular and molecular alterations in human epithelial cells transformed by recombinant human papillomavirus DNA. Crit Rev Oncogen 1993;4:337-60.
101. Auvinen E, Hietanen S, Gissmann L, Alonso A. Human papillomavirus type 33 DNA and E6-E7 transcripts in late passages of the UT-DEC-1 vaginal keratinocyte cell line. Mol Cell Probes 1998;12:59-62.
102. Selinka HC, Sotlar K, Klingel K, et al. Detection of human papillomavirus 16 transcriptional activity in cervical intraepithelial neoplasia grade III lesions and cervical carcinomas by nested reverse transcription-polymerase chain reaction and in situ hybridization. Lab Investig 1998;78:9-18.
103. Thain A, Webster K, Emery D, et al. DNA binding and bending by the human papillomavirus type 16 E2 protein. Recognition of an extended binding site. J Biol Chem 1997;272:8236-42.
104. Shally M, Alloul N, Jackman A, et al. The E6 variant proteins E6I-E6IV of human papillomavirus 16: expression in cell free systems and bacteria and study of their interaction with p53. Virus Res 1996;42:81-96.
105. Stacey SN, Jordan D, Snijders PJ, et al. Translation of the human papillomavirus type 16 E7 oncoprotein from bicistronic mRNA is independent of splicing events within the E6 open reading frame. J Virol 1995;69:7023-31.
106. Yokoyama M, Nakao Y, Yang X, et al. Alterations in physical state and expression of human papillomavirus type 18 DNA following crisis and establishment of immortalized ectocervical cells. Virus Res 1995;37:139-51.
107. Czegledy J, Iosif C, Hansson BG, et al. Can a test for E6/E7 transcripts of human papillomavirus type 16 serve as a diagnostic tool for the detection of micrometastasis in cervical cancer? Int J Cancer 1995;64:211-5.
108. Yamada T, Yamashita T, Nishikawa T, et al. Biologic activity of human papillomavirus type 16 E6/E7 cDNA clones isolated from SiHa cervical carcinoma cell line. Virus Genes 1995;10:15-25.
109. Liu Z, Ghai J, Ostrow RS, et al. The E6 gene of human papillomavirus type 16 is sufficient for transformation of baby rat kidney cells in cotransfection with activated Ha-ras. Virology 1994;201:388-96.
110. Bosch FX, Schwarz E, Boukamp P, et al. Suppression in vivo of human papillomavirus type 18 E6-E7 gene expression in nontumorigenic HeLa X fibroblast hybrid cells. J Virol 1990;64:4743-54.
111. Cornelissen MT, Smits HL, Briet MA, et al. Uniformity of the splicing pattern of the E6/E7 transcripts in human papillomavirus type 16-transformed human fibroblasts, human cervical premalignant lesions and carcinomas. J General Virol 1990;71:1243-6.
112. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 1990;248:76-9.
113. Munger K, Phelps WC, Bubb V, et al. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol 1989;63:4417-21.
114. Bedell MA, Jones KH, Grossman SR, Laimins LA. Identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. J Virol 1989;63:1247-55.
115. Chesters PM, McCance DJ. Human papillomavirus types 6 and 16 in cooperation with Ha-ras transform secondary rat embryo fibroblasts. J General Virol 1989;70:353-65.
116. Watanabe S, Kanda T, Yoshiike K. Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. J Virol 1989;63:965-9.
117. Bedell MA, Jones KH, Laimins LA. The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and rat-1 cells. J Virol 1987;61:3635-40.
118. Takebe N, Tsunokawa Y, Nozawa S, et al. Conservation of E6 and E7 regions of human papillomavirus types 16 and 18 present in cervical cancers. Biochem Biophys Res Commun 1987;143:837-44.
119. Androphy EJ, Schiller JT, Lowy DR. Identification of the protein encoded by the E6 transforming gene of bovine papillomavirus. Science 1985;230:442-5.
120. Schwarz E, Freese UK, Gissmann L, et al. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature 1985;314:111-4.
121. O'Leary JJ, Browne G, Johnson MI, et al. PCR in situ hybridisation detection of HPV 16 in fixed CaSki and fixed SiHa cell lines. J Clin Pathol 1994;47:933-8.
122. Callahan DE, Karim A, Zheng G, et al. Quantitation and mapping of integrated human papillomavirus on human metaphase chromosomes using a fluorescence microscope imaging system. Cytometry 1992;13(:453-61.
123. Yim EK, Park JS. The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. Cancer Res Treat 2005;37:319-24.
124. Um SJ, Lee SY, Kim EJ, et al. Down-regulation of human papillomavirus E6/E7 oncogene by arsenic trioxide in cervical carcinoma cells. Cancer Lett 2002;181:11-22.
125. Kilic G, Cardillo M, Ozdemirli M, Arun B. Human papillomavirus 18 oncoproteins E6 and E7 enhance irra-

diation- and chemotherapeutic agent-induced apoptosis in p53 and Rb mutated cervical cancer cell lines. Eur J Gynaecol Oncol 1999;20:167-71.
126. Nindl I, Rindfleisch K, Lotz B, et al. Uniform distribution of HPV 16 E6 and E7 variants in patients with normal histology, cervical intra-epithelial neoplasia and cervical cancer. Int J Cancer 1999;82:203-7.
127. McLaughlin-Drubin ME, Munger K. Biochemical and functional interactions of human papillomavirus proteins with polycomb group proteins. Viruses 2013;5:1231-49.
128. Egawa N, Egawa K, Griffin H, Doorbar J. Human Papillomaviruses; Epithelial Tropisms, and the Development of Neoplasia. Viruses 2015;7:3863-90.
129. zur Hausen H. Human papillomaviruses and their possible role in squamous cell carcinomas. Curr Topics Microbiol Immunol 1977;78:1-30.
130. zur Hausen H. Papillomaviruses in human cancer. Cancer 1987;59:1692-6.
131. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002;2:342-50.
132. Togtema M, Jackson R, Richard C, et al. The human papillomavirus 16 European-T350G E6 variant can immortalize but not transform keratinocytes in the absence of E7. Virology 2015;485:274-82.
133. Clere N, Bermont L, Fauconnet S, et al. The human papillomavirus type 18 E6 oncoprotein induces Vascular Endothelial Growth Factor 121 (VEGF121) transcription from the promoter through a p53-independent mechanism. Exp Cell Res 2007;313:3239-50.
134. Cho NH, Kim YT, Kim JW. Alteration of cell cycle in cervical tumor associated with human papillomavirus: cyclin-dependent kinase inhibitors. Yonsei Med J 2002;43(6):722-8.
135. Gourronc FA, Robertson M, Herrig AK, et al. Proliferative defects in dyskeratosis congenita skin keratinocytes are corrected by expression of the telomerase reverse transcriptase, TERT, or by activation of endogenous telomerase through expression of papillomavirus E6/E7 or the telomerase RNA component, TERC. Exp Dermatol 2010;19:279-88.
136. Kim RH, Kang MK, Shin KH, et al. Bmi-1 cooperates with human papillomavirus type 16 E6 to immortalize normal human oral keratinocytes. Exp Cell Res 2007;313:462-72.
137. Nor Rashid N, Yusof R, Watson RJ. Disruption of repressive p130-DREAM complexes by human papillomavirus 16 E6/E7 oncoproteins is required for cell-cycle progression in cervical cancer cells. J Gen Virol 2011;92:2620-7.
138. Niebler M, Qian X, Hofler D, et al. Post-translational control of IL-1beta via the human papillomavirus type 16 E6 oncoprotein: a novel mechanism of innate immune escape mediated by the E3-ubiquitin ligase E6-AP and p53. PLoS Pathogens 2013;9:e1003536.
139. Bello JO, Nieva LO, Paredes AC, et al. Regulation of the Wnt/beta-Catenin Signaling Pathway by Human Papillomavirus E6 and E7 Oncoproteins. Viruses 2015;7:4734-55.
140. Ganti K, Broniarczyk J, Manoubi W, et al. The Human Papillomavirus E6 PDZ Binding Motif: From Life Cycle to Malignancy. Viruses 2015;7:3530-51.
141. Ben Khalifa Y, Teissier S, Tan MK, et al. The human papillomavirus E6 oncogene represses a cell adhesion pathway and disrupts focal adhesion through degradation of TAp63beta upon transformation. PLoS Pathogens 2011;7:e1002256.
142. Koss LG. Current concepts of intraepithelial neoplasia in the uterine cervix (CIN). Appl Pathol 1987;5:7-18.
143. Koss LG. Dysplasia. A real concept or a misnomer? Obstet Gynecol 1978;51:374-9.
144. Koss LG. Significance of dysplasia. Clin Obstet Gynecol 1970;13:873-88.
145. Munger K, Howley PM. Human papillomavirus immortalization and transformation functions. Virus Res 2002;89:213-28.
146. Munger K, Phelps WC. The human papillomavirus E7 protein as a transforming and transactivating factor. Biochim Biophys Acta 1993;1155:111-23.
147. Barron BA, Richart RM. Screening protocols for cervical neoplastic disease. Gynecol Oncol 1981;12:S156-67.
148. Barron BA, Richart RM. A statistical model of the natural history of cervical carcinoma based on a prospective study of 557 cases. J Natl Cancer Inst 1968;41:1343-53.
149. Hein K, Schreiber K, Cohen MI, Koss LG. Cervical cytology: the need for routine screening in the sexually active adolescent. J Pediatr 1977;91:123-6.
150. Koss LG, Phillips AJ. Summary and recommendations of the workshop on uterine-cervical cancer. Cancer 1974;33:1753-4.
151. Koss LG, Melamed MR, Daniel WW. In situ epidermoid carcinoma of the cervix and vagina following radiotherapy for cervical cancer. Cancer 1961;14:353-60.
152. Molden T, Kraus I, Hagmar B, Karlsen F. Multiplex real time NASBA, a new method for identification of active oncogenic human papilloma virus (HPV) mRNA. Int J Cancer 2002:319.
153. Nygard JF, Nygard M, Skare GB, Thoresen SO. Pap smear screening in women under 30 in the Norwegian Coordinated Cervical Cancer Screening Program, with a comparison of immediate biopsy vs Pap smear triage of moderate dysplasia. Acta Cytol 2006;50:295-302.
154. Kraus I, Molden T, Erno LE, et al. Human papillomavirus oncogenic expression in the dysplastic portio; an investigation of biopsies from 190 cervical cones. Br J Cancer 2004;90:1407-13.
155. Lie AK, Risberg B, Borge B, Sandstad B, Delabie J, Rimala R, et al. DNA-versus RNA-based methods for human papillomavirus detection in cervical neoplasia. Gynecol Oncol 2005;97:908-15.
156. Doorbar J. The papillomavirus life cycle. J Clin Virol 2005;32:S7-15.
157. Doorbar J. Model systems of human papillomavirus-associated disease. J Pathol 2016;238:166-79.
158. Maglennon GA, McIntosh P, Doorbar J. Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. Virology 2011;414:153-63.
159. Jeon S, Allen-Hoffmann BL, Lambert PF. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. J Virol 1995;69:2989-97.
160. zur Hausen H. Cervical carcinoma and human papillomavirus: on the road to preventing a major human cancer. J Natl Cancer Inst 2001;93:252-3.
161. Kraus I, Driesch C, Vinokurova S, et al. The majority of viral-cellular fusion transcripts in cervical carcinomas cotranscribe cellular sequences of known or predicted genes. Cancer Res 2008;68:2514-22.
162. Collins SI, Constandinou-Williams C, Wen K, et al. Disruption of the E2 gene is a common and early event in the natural history of cervical human papillomavirus infection: a longitudinal cohort study. Cancer Res 2009;69:3828-32.

163. Scarpini CG, Groves IJ, Pett MR, et al. Virus transcript levels and cell growth rates after naturally occurring HPV16 integration events in basal cervical keratinocytes. J Pathol 2014;233:281-93.
164. Schmitz M, Driesch C, Beer-Grondke K, et al. Loss of gene function as a consequence of human papillomavirus DNA integration. Int J Cancer 2012;131:E593-602.
165. Sathish N, Abraham P, Peedicayil A, et al. Human papillomavirus 16 E6/E7 transcript and E2 gene status in patients with cervical neoplasia. Mol Diagnosis 2004;8:57-64.
166. Luczak MW, Jagodzinski PP. Apicidin down-regulates human papillomavirus type 16 E6 and E7 transcripts and proteins in SiHa cervical cancer cells. Cancer Lett 2008;272:53-60.
167. Al Moustafa AE, Foulkes WD, Benlimame N, et al. E6/E7 proteins of HPV type 16 and ErbB-2 cooperate to induce neoplastic transformation of primary normal oral epithelial cells. Oncogene 2004;23:350-8.
168. Hall AH, Alexander KA. RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. J Virol 2003;77:6066-9.
169. Akerman GS, Tolleson WH, Brown KL, et al. Human papillomavirus type 16 E6 and E7 cooperate to increase epidermal growth factor receptor (EGFR) mRNA levels, overcoming mechanisms by which excessive EGFR signaling shortens the life span of normal human keratinocytes. Cancer Res 2001;61:3837-43.
170. Jones DL, Munger K. Analysis of the p53-mediated G1 growth arrest pathway in cells expressing the human papillomavirus type 16 E7 oncoprotein. J Virol 1997;71:2905-12.
171. Wazer DE, Liu XL, Chu Q, et al. Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. Proc Natl Acad Sci USA 1995;92:3687-91.
172. Morgan DM, Pecoraro G, Rosenberg I, Defendi V. Transformation by human papillomavirus type 16 (HPV16) DNA but not HPV6b DNA is enhanced by addition of the human cytomegalovirus enhancer. Virology 1992;189:687-94.
173. Kuner R, Vogt M, Sultmann H, et al. Identification of cellular targets for the human papillomavirus E6 and E7 oncogenes by RNA interference and transcriptome analyses. J Mol Med (Berl) 2007;85:1253-62.
174. Duensing S, Munger K. Centrosomes, genomic instability, and cervical carcinogenesis. Crit Rev Eukaryot Gene Expr 2003;13:9-23.
175. Duensing S, Munger K. The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. Cancer Res 2002;62:7075-82.
176. Burd EM. Human papillomavirus and cervical cancer. Clin Microbiol Rev 2003;16:1-17.
177. Schweizer J, Lu PS, Mahoney CW, et al. Feasibility study of a human papillomavirus E6 oncoprotein test for diagnosis of cervical precancer and cancer. J Clin Microbiol 2010;48:4646-8.
178. Castle PE, Schiffman M, Wheeler CM, et al. Human papillomavirus genotypes in cervical intraepithelial neoplasia grade 3. Cancer Epidemiol Biomarkers Prev 2010;19:1675-81.
179. Castle PE, Rodriguez AC, Burk RD, et al. Short term persistence of human papillomavirus and risk of cervical precancer and cancer: population based cohort study. BMJ 2009;339:b2569.
180. Castle PE. Beyond human papillomavirus: the cervix, exogenous secondary factors, and the development of cervical precancer and cancer. J Low Genit Tract Dis 2004;8:224-30.
181. Duensing A, Spardy N, Chatterjee P, et al. Centrosome overduplication, chromosomal instability, and human papillomavirus oncoproteins. Environ Mol Mutagen 2009;50:741-7.
182. Duensing S, Munger K. Centrosome abnormalities and genomic instability induced by human papillomavirus oncoproteins. Progress Cell Cycle Res 2003;5:383-91.
183. Duensing S, Duensing A, Flores ER, et al. Centrosome abnormalities and genomic instability by episomal expression of human papillomavirus type 16 in raft cultures of human keratinocytes. J Virol 2001;75:7712-6.
184. Duensing S, Lee LY, Duensing A, et al. The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. Proc Natl Acad Sci USA 2000;97:10002-7.
185. Griep AE, Lambert PF. Role of papillomavirus oncogenes in human cervical cancer: transgenic animal studies. Proc Soc Exp Biol Med Soc Exp Biol Med 1994;206:24-34.
186. Rodriguez AC, Burk R, Herrero R, et al. The natural history of human papillomavirus infection and cervical intraepithelial neoplasia among young women in the Guanacaste cohort shortly after initiation of sexual life. Sex Transm Dis 2007;34:494-502.
187. Safaeian M, Schiffman M, Gage J, et al. Detection of precancerous cervical lesions is differential by human papillomavirus type. Cancer Res 2009;69:3262-6.
188. Schiffman M, Castle PE, Jeronimo J, et al. Human papillomavirus and cervical cancer. Lancet 2007;370:890-907.
189. Schiffman M. Integration of human papillomavirus vaccination, cytology, and human papillomavirus testing. Cancer 2007;111:145-53.
190. Whiteside MA, Siegel EM, Unger ER. Human papillomavirus and molecular considerations for cancer risk. Cancer 2008;113:2981-94.
191. zur Hausen H. Papillomavirus and p53. Nature 1998;393:217.
192. zur Hausen H. Are human papillomavirus infections not necessary or sufficient causal factors for invasive cancer of the cervix? Int J Cancer 1995;63:315-6.
193. Nygard M, Daltveit AK, Thoresen SO, Nygard JF. Effect of an antepartum Pap smear on the coverage of a cervical cancer screening programme: a population-based prospective study. BMC Health Serv Res 2007;7:10.
194. Franceschi S, Rajkumar T, Vaccarella S, et al. Human papillomavirus and risk factors for cervical cancer in Chennai, India: a case-control study. Int J Cancer 2003;107:127-33.
195. Jacobs MV, Snijders PJ, Voorhorst FJ, et al. Reliable high risk HPV DNA testing by polymerase chain reaction: an intermethod and intramethod comparison. J Clin Pathol 1999;52:498-503.
196. de Roda Husman AM, Walboomers JM, van den Brule AJ, et al The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J General Virol 1995;76:1057-62.
197. Snijders PJ, van den Brule AJ, Schrijnemakers HF, et al. The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J General Virol 1990;71:173-81.
198. Cirpan T, Guliyeva A, Onder G, et al. Comparison of human papillomavirus testing and cervical cytology with colposcopic examination and biopsy in cervical cancer screening in a cohort of patients with Sjogren's syndrome. Eur J Gynaecol Oncol 2007;28:302-6.
199. Smits HL, Bollen LJ, Tjong AHSP, et

al. Intermethod variation in detection of human papillomavirus DNA in cervical smears. J Clin Microbiol 1995;33:2631-6.
200. Bosch FX, Burchell AN, Schiffman M, et al. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine 2008;26:K1-16.
201. Schiffman M, Khan MJ, Solomon D, et al. A study of the impact of adding HPV types to cervical cancer screening and triage tests. J Natl Cancer Inst 2005;97:147-50.
202. Peyton CL, Schiffman M, Lorincz AT, et al. Comparison of PCR- and hybrid capture-based human papillomavirus detection systems using multiple cervical specimen collection strategies. J Clin Microbiol 1998;36:3248-54.
203. Castle PE, Fetterman B, Poitras N, et al. Five-year experience of human papillomavirus DNA and Papanicolaou test cotesting. Obstet Gynecol 2009;113:595-600.
204. Castle PE, Follansbee S, Borgonovo S, et al. A comparison of human papillomavirus genotype-specific DNA and E6/E7 mRNA detection to identify anal precancer among HIV-infected men who have sex with men. Cancer Epidemiol Biomark Prev 2013;22:42-9.
205. Castle PE, Lorincz AT, Mielzynska-Lohnas I, et al. Results of human papillomavirus DNA testing with the hybrid capture 2 assay are reproducible. J Clin Microbiol 2002;40:1088-90.
206. Castle PE, Gutierrez EC, Leitch SV, et al. Evaluation of a new DNA test for detection of carcinogenic human papillomavirus. J Clin Microbiol 2011;49:3029-32.
207. Castle PE, Schiffman M, Herrero R, et al. PCR testing of pooled longitudinally collected cervical specimens of women to increase the efficiency of studying human papillomavirus infection. Cancer Epidemiol Biomark Prev 2005;14:256-60.
208. Schiffman M, Boyle S, Raine-Bennett T, et al. The Role of Human Papillomavirus Genotyping in Cervical Cancer Screening: A Large-Scale Evaluation of the cobas HPV Test. Cancer Epidemiol Biomark Prev 2015;24:1304-10.
209. Schiffman M, Hildesheim A, Herrero R, Bratti C. Human papillomavirus testing as a screening tool for cervical cancer. Jama 2000;283:2525-6.
210. Chan PK, Ho WC, Chan MC, et al. Meta-analysis on prevalence and attribution of human papillomavirus types 52 and 58 in cervical neoplasia worldwide. PloS One 2014;9:e107573.
211. Chan PK. Human papillomavirus type 58: the unique role in cervical cancers in East Asia. Cell Biosci 2012;2:17.
212. Arbyn M, Verdoodt F, Snijders PJ, et al. Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: a meta-analysis. Lancet Oncol 2014;15:172-83.
213. Racey CS, Gesink DC, Burchell AN, et al. Randomized Intervention of Self-Collected Sampling for Human Papillomavirus Testing in Under-Screened Rural Women: Uptake of Screening and Acceptability. J Womens Health. 2015.
214. Racey CS, Gesink DC. Barriers and Facilitators to Cervical Cancer Screening Among Women in Rural Ontario, Canada: The Role of Self-Collected HPV Testing. J Rural Health 2015.
215. Racey CS, Withrow DR, Gesink D. Self-collected HPV testing improves participation in cervical cancer screening: a systematic review and meta-analysis. Canad J Public Health 2013;104:e159-66.
216. Origoni M, Cristoforoni P, Carminati G, et al. E6/E7 mRNA testing for human papilloma virus-induced high-grade cervical intraepithelial disease (CIN2/CIN3): a promising perspective. Ecancermedicalscience 2015;9:533.
217. Zeng W, Tu Y, Zhu Y, et al. Predictive power of circulating miRNAs in detecting colorectal cancer. Tumour Biol 2015;36:2559-67.
218. Munkacsy G, Szasz MA, Menyhart O. Gene expression-based prognostic and predictive tools in breast cancer. Breast Cancer 2015;22:245-52.
219. Koliopoulos G, Arbyn M, Martin-Hirsch P, et al. Diagnostic accuracy of human papillomavirus testing in primary cervical screening: a systematic review and meta-analysis of non-randomized studies. Gynecol Oncol 2007;104:232-46.
220. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive women in cervical cancer screening. J Clin Virol 2015.
221. Clifford GM, Smith JS, Aguado T, Franceschi S. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. Br J Cancer 2003;89:101-5.
222. Clifford GM, Smith JS, Plummer M, et al. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br J Cancer 2003;88:63-73.
223. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12-9.
224. Karlsen F, Kalantari M, Jenkins A, et al. Use of multiple PCR primer sets for optimal detection of human papillomavirus. J Clin Microbiol 1996;34:2095-100.
225. Gustinucci D, Giorgi Rossi P, Cesarini E, et al. Use of Cytology, E6/E7 mRNA, and p16INK4a-Ki-67 to Define the Management of Human Papillomavirus (HPV)-Positive Women in Cervical Cancer Screening. Am J Clin Pathol 2016;145:35-45.
226. Gustinucci D, Passamonti B, Cesarini E, et al. Role of p16(INK4a) cytology testing as an adjunct to enhance the diagnostic specificity and accuracy in human papillomavirus-positive women within an organized cervical cancer screening program. Acta Cytol 2012;56:506-14.
227. Rijkaart DC, Berkhof J, van Kemenade FJ, et al. Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. Int J Cancer 2012;130:602-10.