**Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions**

Steenbergen, R.D.M., Snijders, P.J.F., Heideman, D.A.M., Meijer, C.J.L.M.

Nature Reviews Cancer, 14(6), 395-405

**NatureReviews Cancer. 2014 Jun;14(6):395-405. doi: 10.1038/nrc3728. Review.**

# Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions

*Renske D.M. Steenbergen, Peter J.F. Snijders, Daniëlle A.M. Heideman, and Chris J.L.M. Meijer*

Department of Pathology, VU University medical center, Amsterdam, The Netherlands

**Correspondence to:** R.D.M. Steenbergen, Dept. of Pathology, Unit of Molecular Pathology, VU University medical center, PO box 7057, 1007 MB Amsterdam, The Netherlands

Email: r.steenbergen@vumc.nl; telephone: +31-20-4442331; Fax: +31-20-4442964

**Preface**

Infection of cervical epithelium with high-risk human papillomavirus (hrHPV) might result in productive or transforming cervical intraepithelial neoplasia (CIN) lesions, the morphology of which can overlap. In transforming CIN lesions aberrations in host cell genes accumulate over time, which is necessary for ultimate progression to cancer. On the basis of (epi)genetic changes, early and advanced transforming CIN lesions can be distinguished. This paves the way for new molecular tools for cervical screening, diagnosis and management of cervical cancer precursor lesions.

**Introduction**

With about 530,000 new cases annually, cervical cancer is the third most common cancer in women worldwide, and the seventh most common cancer overall. In 2008, cervical cancer was responsible for 275,000 deaths, thereby being the fourth leading cause of cancer death in females worldwide [1, 2]. Virtually all cervical cancers result from a persistent infection with certain high-risk types of the human papillomavirus (hrHPV) family [3]. However, cervical cancer is a rare complication of a rather common viral infection; the lifetime risk of a hrHPV infection is estimated to be around 80% and fortunately [4], and the large majority of infections are cleared by the host immune system and do not give rise to lesions. Most of the remaining hrHPV infections develop into lesions that are thought to represent 'productive' infections that lead to the generation of new viral progeny. Although such infections display no signs of cellular transformation, morphologically they can show dysplastic features that overlap with those seen in progressive precancers. Only a minority of hrHPV infections become 'transforming' infections, characterized by the altered expression of two viral genes, E6 and E7 (see below). Such a condition may ultimately lead to cancer if the respective precursor lesion is left untreated. It is still poorly understood which factors determine the malignant fate of a hrHPV infection.

Here, we address recent advances that shed more light on the development and progression of transforming hrHPV infections. The focus is on cellular genetic and epigenetic alterations underlying the progression to cancer. Their implications for development of new molecular diagnostic tools for cervical screening, diagnosis and management of patients with cervical precancer is also discussed.

**Cervical cancer and HPV**

According to their epidemiological association with cervical cancer and consolidated by biological studies, twelve HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) have now been consistently classified as hrHPV (also known as IARC class I). HPV 68 has been classified as probable high-risk (also known as IARC class 2A) and another seven types have been classified as possible high-risk (HPV 26, 53, 66, 67, 70, 73 and 82;also known as IARC class 2B) [5].

Following a hrHPV infection, cervical cancer develops through a series of subsequent steps: hrHPV persistence, hrHPV mediated epithelial transformation, development of precancerous lesions (cervical intraepithelial neoplasia graded 1 to 3 (CIN1-3)), and finally progression to invasive cervical cancer (**FIG. 1**). Cervical cancer development, in particular the step from precancer to invasive cancer takes a long time in most patients. Whereas high-grade precancerous CIN2 and CIN3 lesions can develop within 3-5 years following an hrHPV infection [6], further progression to invasive cancer can take up to 20-30 years [7, 8]. This long period offers many opportunities for intervention and has probably contributed to the success of frequent Pap screening to reduce the incidence and mortality of cervical cancer in the Western world [9].

Histomorphologically, most cervical cancers are squamous cell carcinomas (SCC; accounting for 80% of cervical cancers). Adenocarcinomas (AdCA; accounting for 10-20%) represent the second most common histotype, followed by a rather small fraction of adeno-squamous carcinomas and other rare histotypes including neuro-endocrine carcinomas.

**Features of productive versus transforming infections**

A productive infection begins when viral particles gain access to the epithelial basement membrane, most likely via micro-abrasions, and subsequently enter the basal cells of squamous epithelium. In infected basal cells, the viral genome is replicated in conjunction with cellular DNA during S-phase and maintained as stable episomes [10]. In these cells, expression of the viral proteins occurs at very low levels, which likely facilitates escape from immune surveillance [11-13]. Following cell division, one of the daughter cells undergoes a differentiation process and exits the cell cycle. Subsequently, viral differentiation-dependent promoters become upregulated, resulting in an increased expression of viral genes, including the viral early genes E6 and E7. Expression of the E6 and E7 genes drives the differentiated cells into S-phase, thereby creating environmental conditions supporting vegetative viral genome replication. In the upper layer of the squamous epithelium, the last stage of the viral life cycle involves generation of new viral particles that are released from shedding terminally differentiated cells (reviewed in [14]). Productive infections in the cervix may give rise to mild to moderate cellular abnormalities, and histologically such conditions are manifested as CIN1 or 2 (CIN1/CIN2). In order to distinguish this condition from true cancer precursor lesions, such lesions are here referred to as productive CIN lesions. Usually, these lesions regress spontaneously within 1-2 years, a process accompanied with viral clearance resulting from cell-mediated immune responses to E2, E6 and E7. Immune evasion accompanied with viral and lesion persistence may result from various mechanisms, such as virus-mediated suppression of innate immunity, suppression of T-cell effector function, increase in regulatory T cells and frequent loss of HLA expression resulting from genetic events (reviewed in [15]). Loss of immune control facilitating viral persistence is crucial for HPV-mediated carcinogenesis as HPV infections are not only essential for the initiation, but also maintenance of the transformed phenotype (reviewed in [16]).

Morphologically, CIN3 and a subset of CIN2 lesions typify transforming CIN lesions, in which the normal viral life cycle is aborted and the viral early genes E6 and E7 are overexpressed in proliferating cells. Morphologically, however CIN2 lesions resulting from a productive infection can not be distinguished from CIN2 lesions resulting from a transforming infection. In the context of dividing cells, the E6 and E7 encoded proteins act as oncoproteins and the respective genes are therefore referred to as viral oncogenes. A direct result from E6 and E7 deregulation in a transforming infection is the altered expression of cell cycle and DNA repair regulators. The exact mechanism contributing to this rather unnatural E6 and E7 expression pattern has not been understood, but altered intraviral control of E6 and E7 expression by genetic (viral DNA integration, for example) and epigenetic (methylation of viral promoter regions, for example) alterations of the viral genome have been suggested [17-19]. Alternatively, a different host cell environment that is non-permissive for viral replication could favour non-canonical regulation of E6 and E7 expression. A candidate cell type that could be highly susceptible to HPV transformation is the squamo-columnar junction (SCJ) cell. Herfs *et al.*[20] recently reported that this discrete population of single layered, cuboidal epithelial cells of embryonic origin, which are localized between ectocervical squamous and endocervical glandular epithelium, represents the likely cellular precursor of most cervical cancers and their precursor lesions. By contrast, productive infections might arise exclusively from infection of basal cells of the squamous epithelium lining the ectocervix or adjacent transformation zone [21].

SCJ cells display a unique gene expression profile for several genes, including *Krt7*, *AGR2*, *MMP7*, and *GDA* [20]. The proteins encoded by these genes can serve as an SCJ-specific protein biomarker panel. The expression of these proteins is not induced by HPV E6 or E7 *in vitro* in squamous epithelial cells, and expression of these proteins is lost if the SCJ is removed by cone biopsy or loop electrical excision. Therefore, it seems that that the SCJ-specific expression profile in CIN lesions and cervical cancers is not acquired during the transformation process and instead reflects the embryonal origin of the cells. Interestingly, for all cervical cancers analysed (both SCC and AdCA), the majority of CIN2/CIN3 lesions, and one third of CIN1 lesions were positive for a SCJ expression profile[20, 22]. The presumed high transformation susceptibility of these SCJ cells compared to squamous cells of the ectocervix and transformation zone is supported by the fact that HPV-related high-grade precancerous lesions are up to 20 times more common in the cervix (which contains a SCJ) than in other genital sites lacking a SCJ, such as vagina and vulva [23].

The net result of deregulated expression of E6 and E7 in proliferating cells is chromosomal instability [14], which likely provides the driving force for accumulation of alterations in cancer genes of the host cell and consequently progression towards cancer.

In the following sections, the primary and secondary consequences of deregulated E6 and E7 expression on host cell genes and gene products will be discussed in the context of cervical cancer development.

**Primary effects of E6 and E7 deregulation**

It is now widely accepted that combined hyperactivity of E6 and E7 in proliferating cells represents the trigger for HPV-induced malignant transformation. Initially, binding of tumour suppressor gene products RB by E7 and p53 by E6 were thought to be the primary events responsible for malignant transformation. Targeting of RB by E7 leads to uncontrolled cell proliferation, primarily resulting from increased E2F activity as evident through the upregulation of E2F responsive genes, such as PCNA, Ki-67, minichromosome maintenance proteins (MCMs), cyclin E and p21 (reviewed in [24, 25]). Formation of a complex between the ubiquitin ligase E6AP and E6 results in ubiquitin-mediated degradation of p53, thereby interfering with the normal p53-mediated apoptosis- and cell cycle control mechanisms induced by genotoxic stress[24, 25].

Nowadays, it has become evident that complex formation of E6 and E7 with other cellular proteins also contributes to the virus-mediated transformation process. Some of the interactions result in chromatin remodelling (reviewed in [26]). Both E6 and E7 can modulate the DNA methylation machinery, thereby influencing cellular and viral gene expression. HPV16 E6 can induce upregulation of the DNA methyltransferase DNMT1 via suppression of p53 [27], whereas HPV16 E7 can directly bind and activate DNMT1 [28]. In support of these *in vitro* findings, both DNMT1 and DNMT3b were shown to be upregulated in CIN3 lesions and cervical carcinomas [29-31]. A further modulating effect on epigenetic reprogramming can be accomplished by E7 via induction of histone lysine demethylases KDM6A and/or KDM6B. This leads to histone demethylation of genes that were silenced by polycomb repressive complex (PRC)-mediated histone H3 lysine 27 (K27) tri-methylation[32, 33]. One of these genes encodes the cyclin-dependent kinase inhibitor $p16^{INK4a}$ [33]. Although this induction does not effect proliferation because of the downstream targeting of RB by E7, overexpression of $p16^{INK4a}$ is nowadays widely considered as a hallmark of hrHPV activity (**FIG. 2**) [34].

In addition, HPV16 E6 and E7 are also known to alter the expression of miRNAs **(BOX 1**), through direct and indirect effects. HPV16 E6 can downregulate miR-218, miR-23b and miR-34a expression [35-37], the latter two being linked to E6-induced p53 degradation. Reduced miR-203 and increased expression of the miR-15a/16-1 cluster is attributed to E2F release upon RB inactivation by hrHPV E7 [38]. Vice versa, miRNAs may also regulate viral gene expression [39], and first indications for the existence of HPV-encoded miRNAs have been reported [40]. These findings, however, await further confirmation.

**Secondary effects of deregulated E6 and E7 expression**

Although E6 and E7 are necessary for initiation and maintenance of the transformed phenotype, the long duration of progression from precancer to invasive cancer indicates that several additional oncogenic events are pivotal for malignant progression. A well known consequence of deregulated E6 and E7 expression is chromosomal instability (reviewed in [24]). This genomic instability likely contributes to the accumulation of aberrations in host cell genes over time (**FIG.2**). Such

acquired aberrations can be both genetic and epigenetic, and some of them result in functional abrogation of human tumour suppressor genes or activation of oncogenes. Host cell aberrations observed in cervical (pre)cancers include deletions, copy number alterations, DNA mutations, and epigenetic alterations, such as DNA methylation affecting both protein coding genes and non-coding genes like miRNAs. An overview is available in the form of a recently established database of genes that have been found to be altered in cervical cancer [41]. In the following sections, various aberrations in cervical cancers and CIN lesions are described.

*Chromosomal aberrations*

A meta-analysis of 12 array comparative genomic hybridization (aCGH) studies covering a total of 293 samples showed that the most frequent DNA copy number alterations in cervical SCC include gain at 3q (rate 0.55), loss at 3p (rate 0.36) and loss at 11q (rate 0.33) [42]. Gain at 3q was particularly frequent in HPV16-positive SCC (rate 0.84). Gain at 17q (rate 0.36) was most frequent in AdCA (4 studies, 58 samples). Gain at 1p was the most frequent aberration in high-grade CIN (rate 0.34). This was followed in decreasing order of frequency (from 0.27 to 0.08) by gain at 3q, loss at 4q, 2q, 4p, 11p and 3p. From these regions candidate driver genes can be extracted by analysis of recurrent focal aberrations and/or expression profiling supplemented with functional analysis. This approach has led to the identification of *EYA2* and *hsa-mir-375* as novel onco- and tumour suppressor genes, respectively, in cervical cancer [43]. In support of these findings, *EYA2* has recently been identified as a target of viral integration and a tumour suppressive function of miR-375 has also been corroborated in other studies [44, 45]. These data provide a proof-of-concept that specific chromosomal aberrations can contribute to HPV-induced carcinogenesis.

*DNA mutations*

To date, relatively few reports on mutations in oncogenes or tumour suppressor genes have been described for cervical cancer or its precursor lesions. Because p53 and RB are inactivated by E6 and E7 they are only rarely mutated in cervical cancer (5% and 3%, respectively) [Cosmic catalogue of somatic mutations][46]. Other somatic mutations found in cervical cancers mainly involve members of signalling pathways. Highest mutation rates are reported for *PIK3CA* in both SCC and AdCA, as corroborated in two recent papers (i.e., mutations rates in SCC: 37.5% and 14%, respectively; mutation rates in AdCA: 25% and 16%, respectively) [47, 48]. Wright *et al.* [47] also identified *KRAS* mutations in AdCA only (17.5%), and *EGFR* mutations in SCC only (7.5%). Ojesina *et al.* [48] in addition showed recurrent mutations in *EP300* (16%), *FBXW7* (15%), *HLA-B* (9%), *MAPK1* (8%), *PTEN* (6%), *STK11* (4%) and *NFE2L2* (4%) in SCC, and *ELF3* (13%) and *CBFB* (8%) in AdCA. So far, CIN lesions have neither been studied, nor been analysed at substantial sample size.

*Aberrant DNA methylation*

Epigenetic mediators include histone modifications, nucleosome occupancy and positioning, protein and non-coding RNA interactions as well as direct DNA modifications (reviewed in [49]). In cervical lesions, DNA methylation has gained most attention. This involves the covalent binding of a methyl-group (CH3) at the carbon-5 position of cytosine located 5' of a guanine, to generate a 5-methylcytosine. In general, increased methylation of CpG-rich human gene promoters represses gene transcription, and often involves (candidate) tumour suppressor genes. On the other hand, methylation of viral DNA is thought to both negatively and positively regulate viral gene transcription.

A rapidly growing number of studies have analysed the occurrence and role of viral DNA methylation in the development of cervical cancer. Although an altered HPV methylation pattern with disease progression is a common finding, being most pronounced in the L1 and L2 regions, data are inconsistent (reviewed in [50, 51]). Besides technical differences and differences in the CpG sites analysed, the nature of the samples may account for the discrepant findings. It is currently unclear whether viral methylation is of any biological significance to malignant transformation in terms of providing the infected cell with a growth advantage. It has been suggested that viral DNA methylation represents a generic phenomenon of *de novo* methylation of foreign DNA, serving as a host defence mechanism to silence viral replication and transcription [52, 53]. DNA methylation of the viral upstream regulatory region (URR) has been associated with latent infection, which is proposed to facilitate and preserve a long-latency infection [54]. Methylation of the four E2 binding sites (E2BS; each containing one or two CpG dinucleotides) in the viral URR reduces E2 binding [55], thereby contributing to deregulated E6 and E7 expression, the driving force of a transforming HPV infection. A gradual increase in E2BS methylation is thought to result in a further increase in E6 and E7 expression during disease progression. In line with this concept, methylation of the E2BS has been reported to increase with disease progression, with methylation at E2BS2 in the HPV16 enhancer region being the most consistent finding across the various methylation studies [50, 51].

Aberrant methylation patterns have been described for a diverse number of (candidate) tumour suppressor genes in CIN lesions and cervical cancers (reviewed in [56, 57]). The methylation patterns are in part histotype dependent, with *CADM1*, *CDH1*, *DAPK1*, *EPB4L3 FAM19A4*, *MAL*, *PAX1*, *PRDM14* and *TERT* belonging to the most frequently methylated genes in both SCC and AdCA. Of these genes in transforming CIN lesions, the weighted mean methylation frequencies were highest for *CADM1*, followed by *CDH1*, *DAPK1* and *TERT* [56]. A number of recent genome-wide methylation profiling studies have identified a substantial number of additional genes that are methylated in CIN lesions and cervical cancers, findings that warrant further validation studies [58-63]. For a small subset of genes, including *CADM1, DKK3, MAL, SFRP2* and *C13orf18*, tumour suppressive activity in cervical cancer cells has been demonstrated [64-69]. The biological relevance of most other methylation events described in cervical lesions remains elusive.

*miRNAs*

Several genome wide studies on miRNA expression in cervical carcinomas have resulted in the identification of a relatively low number of miRNAs that are consistently altered across studies. These include miR-126, miR-143, and miR-145 down-regulation and miR-15b, miR-16, miR-146a, and miR-155 up-regulation (reviewed in REFs[39, 70, 71]). For a larger number of miRNAs that might have altered expression further independent validation studies are required. Another future challenge includes the identification of the target genes affected by the altered miRNAs and determination of their functional relevance in HPV-induced transformation. Only for a small fraction of miRNAs (miR-9, -203, -375, -143, -145, -146a and -199a), has a mechanistic role been shown in cervical cancer cells or HPV-immortalized cells [43, 72-76]. Four studies that included transforming CIN lesions in their analysis showed that altered expression of a number of miRNAs represents a rather early event in HPV-induced carcinogenesis detectable in CIN lesions **(BOX 1** and **Supplementary TABLE 1)** [77-80]. Most miRNA alterations, however, are not directly induced following an HPV-infection, and are secondary alterations [77] that might in part be a consequence of a copy number gain at chromosome 5p encoding the microRNA processor Drosha [81, 82]. Downregulation of miRNAs could be accomplished by methylation of the CpG rich regulatory sequences. Indeed, downregulation of *hsa-miR-124-1*, *hsa-miR-124-2*, *hsa-miR-124-3*, *hsa-miR-149*, *hsa-miR-203*, *hsa-miR-375*, *hsa-miR-641* and *hsa-miR-1287* in cervical cancers has been linked to increased promoter methylation of respective genes [73, 74, 83, 84].

**Duration of existence of transforming CIN is reflected by molecular profile**

As indicated above, CIN3 lesions and a subset of CIN2 lesions constitute transforming CIN lesions. Whereas CIN3 is morphologically regarded as the immediate, most advanced cervical cancer precursor, it in fact represents a rather heterogeneous disease [85-87]. This heterogeneity likely reflects variable duration of lesion existence relative to the long time line of 20-30 years necessary for progression to invasive carcinoma in most patients [8]. In addition, natural history studies have revealed that, if not treated, only a subset of CIN3 lesions would progress to invasive cancer [7, 88]. Therefore, the short-term risk of progression of transforming CIN to cancer is highly variable. Cross-sectional studies have revealed variable frequencies of (epi)genetic alterations in CIN lesions and cervical scrapings thereof (reviewed in [42, 57, 89], **Supplementary TABLE 2)**. Since some of the observed molecular aberrations overlap with those found in cervical cancers, it seems obvious that these molecular changes represent more advanced transforming CIN lesions having a longer duration of existence. This is supported by recent findings showing that a longer duration of preceding HPV infection, considered as a surrogate for duration of existence of a transforming CIN, is associated with an increase in the number of chromosomal aberrations [90]. Transforming CIN found in women with long-term preceding hrHPV infections (≥5 years) had a significantly higher average percentage of chromosomal aberrations (i.e.16.5% of microarrayCGH (maCGH) probes deviated from normal state) than women with a preceding HPV infection of less than 5 years (2.8% deviating maCGH probes). By comparison, CIN3 lesions adjacent to cervical SCC, considered representatives of most advanced, transforming CIN lesions, had on average 28.8% deviating maCGH probes. The genomic profiles of most CIN3 with a long-term preceding hrHPV infection were similar to those of invasive carcinomas and tumour adjacent CIN3. More recently, it was also found that methylation levels of two host cell genes, *CADM1* and *MAL*, in cervical scrapings were increased in CIN3 lesions of women with long-term preceding hrHPV infections, and reached the highest values in women with cervical cancer [91]. These data are fully in line with the concept that an increase in specific genetic and epigenetic alterations reflects a longer duration of existence of the underlying lesion.

**Biomarkers for cervical cancer screening**

Due to its high sensitivity for detecting CIN2, CIN3 and cervical cancer (i.e. CIN2 + lesions), testing for hrHPV-DNA is likely to become the predominant method for cervical screening in the western world in the near future [92, 93]. The main drawback of this screening tool is a 2-4% lower specificity for CIN2+ than cytology, since the hrHPV test also detects transient HPV infections, resulting in overdiagnosis and overtreatment. To compensate for this limitation different triage algorithms have been suggested in order to keep the follow-up procedures, and associated costs, within acceptable limits. Cytology, with and without HPV 16/18 genotyping is a currently widely-accepted triage tool for HPV-positive women [94, 95 96]. Alternative algorithms to triage HPV-positive women for colposcopy are based on morphological or molecular biomarkers. For biomarker validation in cervical screening a 5-phase framework has been proposed [97] based on recommendations made by Pepe et al. on biomarker development for early detection of cancer [98, 99].The designated phases are: 1) preclinical exploratory studies; 2) clinical assay development for clinical disease and assessment in non-invasive samples; 3) retrospective longitudinal repository studies; 4) prospective screening studies and 5) prospective intervention studies. Phase 5 preferentially concerns a population-based randomised controlled trial where a new biomarker test is applied and evaluated against the reference.
At present most biomarkers are in phase 1 or 2 and only a few have achieved later phases (see below).

*Morphological biomarkers for triage of HPV-positive women*

Cross-sectional and longitudinal studies have shown that $p16^{INK4A}$ or $p16^{INK4A}$ and Ki-67 dual immunostaining on cytological preparations provides a promising triage strategy for HPV-positive women [100, 101]. Other candidates explored by immunostaining include overexpression of topoisomerase 2A (TOP2A) and MCM2, which reflects aberrant S-phase induction and correlates with severity of cervical disease (reviewed in [102] and [103]).

These immunohistochemical candidate triage tests, however, are microscopy-dependent and require the use of a well fixed specimen with preserved morphology and a skilled (cyto)pathologist. Recently, self-sampling of cervico-vaginal material has proved to be a promising new sampling technique for hrHPV testing. However, these specimens have shown decreased number of cervical cells with often poor morphology in a background of excess vaginal cells resulting in low sensitivity of cytology for transforming CIN. A systematic review and a meta-analysis showed that hrHPV testing on self-samples can be similarly accurate as on physician-taken cervical scrapings when a validated combination of sampling device and HPV test is used [104, 105], whereas cytology has shown to be inferior on self-samples [106]. Accordingly, triage of women with an hrHPV-positive self-sample by cytology-based tests would require an extra visit to the physician for making a cervical smear for cytological examination. Therefore, molecular, non-morphology-based triage tools, which are also directly applicable to self-samples, are of great interest for future cervical screening programs.

*Molecular biomarkers for triaging HPV-positive women*

To date, molecular biomarkers based on DNA methylation have gained most attention, because altered DNA methylation in cervical cancer has been well established and DNA methylation can be easily detected in both histological and cytological cervical specimens. Other cellular gene alterations, such as DNA mutations and DNA copy number aberrations, are currently less attractive as molecular triage markers. DNA mutations in transforming CIN are not sufficiently well defined to be used as a triage marker. Moreover, studies on cancers indicate that mutations in proto-oncogenes or tumour suppressor genes are insufficiently prevalent to enable the identification of all cancers [47, 48]. Although being better defined, the detection of DNA copy number aberrations in cervical scrapings is expected to suffer from relatively limited sensitivity for advanced disease by current assays due to a dilution of cells from the lesion, and therefore awaits further technical developments and clinical evaluation. Conversely, a number of sensitive methods are available to analyse DNA methylation in cervical scrapings and cervico-vaginal self-samples (**BOX 2**).

Current data on methylated host cell gene promoters investigated in cervical scrapings are shown in **supplementary TABLE 2**. Studies on HPV DNA methylation have recently been reviewed elsewhere [50, 51], and combinations based on viral and host cell gene promoter methylation are currently being explored [107].

So far, only a limited number of the host cell methylation markers have been extensively tested for their use as triage marker of HPV-positive women. These studies indicate that a panel of methylation markers is needed to reach high sensitivities for transforming CIN. These include various combinations of the markers *SOX1*, *PAX1*, *LMX1A* and *NKX6-1* [108], the four-marker panel *JAM3-EPB41L3-TERT-C13ORF18* [109], and the bi-marker panel *CADM1-MAL* [110]. With respect to the 5-phase frame work of biomarker validation [97], most markers or marker panels tested on cervical scrapings have so far only reached early phases. One biomarker panel (i.e. *CADM1-MAL*) has been validated in a population-based screening setting, thereby reaching phases 3 and 4 of the biomarker validation framework. On HPV-positive cervical scrapings this panel was, depending on the threshold setting, equally discriminatory for CIN3+ as cytology at similar specificity [111].

For HPV-positive self-samples, methylation analysis various marker combinations, such as *JAM3-EPB41L3-TERT-C13ORF18* and *MAL-hsa-miR-124-2,* appeared a feasible triage tool [109, 112]. The use of methylation analyses would obviate the need for HPV-positive women to make an extra visit to a physician for a subsequent cervical sample for morphology-based triage testing. The *MAL-hsa-miR-124-2* panel recently passed the later phases of biomarker validation by first a test-definition on self-samples collected in a prospective screening study [112],and subsequently a prospective, randomised clinical trial with intervention among non-attendees of the regular cervical screening programme [113]. In this latter trial DNA methylation analysis using the *MAL-hsa-miR124-2* panel on HPV-positive self-samples (intervention arm) was compared with an additional physician-collected

cervical scraping (control arm) for CIN2+ detection. The results indicate that direct DNA methylation-based molecular triage was at least as sensitive as cytology triage in the detection of CIN2+ [113]. Unlike cytology, methylation analysis on self-samples scored all women with cervical carcinoma positive. The results also showed a better compliance and shorter diagnostic track, but at the cost of a higher colposcopy referral rate.

The question can be raised whether methylation markers can be used in clinical practice since these markers do not detect all CIN3 lesions and tend to detect less CIN2 lesions than cytology at the same specificity [111]. However, these markers can still be considered eligible for triage when 1) they at least detect all invasive cancers and advanced transforming CIN with a high short-term progression risk for cancer, and 2) test-negative women have a sufficiently low risk of cervical cancer that they can be dismissed from direct colposcopy referral.

In this context the following observations are important. Increasing methylation levels of genes like *CADM1* and *MAL* have been shown to parallel the increasing severity and duration of CIN disease. High methylation levels of *CADM1* and *MAL* were detected in cervical scrapings of women with advanced transforming CIN lesions and methylation levels in scrapings of women with cervical cancer were exceptionally high [91]. Consistent with these findings several studies showed that all (100%) cervical scrapings of women with underlying cervical cancer were positive for DNA methylation using *PAX1* (n=14), *TIMP3* (n=11), or a tri-marker panel consisting of *CADM1*, *MAL* and *hsa-miR124-2* genes (n=79) (L., de Strooper and M., van Zummeren, personal communication) [114, 115]. From these findings it can be concluded that methylation analysis detects with a high sensitivity cancer and advanced lesions with a high short-term progression risk for cancer, thereby missing less advanced lesions with a likely low short-term probability of progression to cancer. Cytology, on the other hand, detects with a moderate sensitivity all morphological cellular abnormalities associated with most CIN2, CIN3 and cancer (schematically depicted in **FIG. 3**), but misses a fraction of advanced transforming CIN lesions and cancers [116, 117].

This concept implicates that HPV positive women with a positive methylation test should be sent for colposcopy because of the presence of cancer or advanced transforming CIN lesions with a high short-term progression risk for cancer. It follows that methylation negative women are not in need of immediate colposcopy because of a very low short-term progression risk for cancer. Instead, these women could be offered a repeat test after 12-18 months. For pregnant women this approach appears particularly important as only treatment of methylation positive lesions is indicated thereby limiting the risk of preterm delivery resulting from treatment. [118-120].

The above mentioned methylation studies also point to the possibility of using methylation analysis as primary screening tool in cervical screening. When these findings, in particular the high sensitivity for cancer, can be confirmed by others, primary methylation testing may provide a screen and treat approach in developing countries. This is particularly attractive, since in such countries

quality controlled cytology is absent and implementation of follow-up algorithms for HPV-positive women is very complicated.

**Molecular markers in management of CIN lesions**

In most European countries, women treated for CIN2 and CIN3 are monitored by cervical cytology at 6, 12 and 24 months after treatment. After three consecutive negative test results, women return to the screening programme (interval 3-5 years), or are recalled within 5 years. Recently, the risk of recurrent CIN2+ disease proved to be similar when combined cytology and hrHPV testing at 6 and 24 months only was used [121, 122].

An interesting perspective is the surveillance of women treated for CIN2+ disease using a combination of hrHPV testing and methylation marker analysis. Residual advanced CIN2 or CIN3 lesions that result from incomplete excision of the original CIN lesion are expected to have higher methylation levels compared to de-novo or incident recurrent CIN2+ lesions because of their longer duration of existence. Awaiting clinical confirmation, this would imply that methylation marker testing could be helpful in differentiating between cervical cancer and advanced CIN2 and CIN3 lesions that result from residual disease, and de-novo or incident CIN2 and CIN3 disease. The clinical value of post-treatment monitoring by combined HPV and methylation marker testing is currently being evaluated (M., Uijterwaal, personal communication). If successful, it is anticipated that in the future women treated for CIN2/CIN3 could self-collect a cervico-vaginal specimen for post-treatment surveillance by combined HPV and methylation marker testing.

**Post-vaccination and therapeutic options**

Prophylactic vaccination against HPV 16 and 18 has been introduced in many countries. In post-vaccination screening cohorts, the probability of a high-grade lesion after a positive screening result, either by cytology or an HPV test, will be lower. In this context, the use of a methylation marker assay might help to identify women with progressive CIN lesions with a high short-term cancer risk in need of treatment, and to prevent overtreatment.

Current advances in genome-wide analyses uncovering of the molecular alterations driving cervical carcinogenesis will also provide the opportunity for targeted drug development, such as small molecules targeting altered cancer genes, and personalized treatment regimens. The reversible nature of the epigenetic alterations in transforming CIN and cervical cancers offers alternative options for pharmaceutical intervention. Demethylating agents, such as 5-azacytidine and decitabin (5-aza-2'-deoxycytidine), have been approved by the US Food and Drug administration for treatment of haematological malignancies, and are in phase I clinical trials for the treatment of solid tumours. Their application is limited by a high toxicity and poor chemical stability. DNMT inhibitors, such as zebularine and small non-nucleoside analogs, are being developed, but await clinical testing [123].

**Future perspectives**

The distinction between productive CIN1/CIN2 lesions and transforming CIN2/CIN3 lesions has consequences for clinical management of women with these lesions. At present, a productive CIN2 and a transforming CIN2 cannot be distinguished morphologically, resulting in overtreatment of these lesions. Uncovering the molecular alterations that are associated with the transition from viral infection to cervical cancer can be used for a molecular classification of cervical lesions over and above the currently existing morphological (histological) one, i.e., CIN1, CIN2 and CIN3. The histological changes currently reported as CIN1/CIN2 lesions that coincide with viral production (productive CIN), which has a very low cancer progression rate, can be distinguished from CIN2/CIN3 representing viral transformation (transforming CIN), by molecular means. Transforming CIN can in turn be subdivided by the level of genetic and epigenetic alterations, such as DNA copy number aberrations and DNA methylation, into early and advanced transforming CIN. Women with early transforming CIN, characterized by low levels of molecular aberrations, have a low short-term progression risk for cancer and could be managed by close surveillance. Women with advanced transforming CIN, characterized by increased levels of molecular aberrations, have a high short-term progression risk for cancer and are in need of immediate treatment. Accordingly, the detection of increased DNA methylation provides an indication for treatment of CIN2/CIN3 lesions. This molecular distinction allows for better management of women diagnosed with CIN lesions, and may particularly be beneficial to women of reproductive age, as treatment of CIN lesions coincides with some degree of morbidity of the cervix and can give rise to pre-term delivery [118-120].

HPV testing is likely to become the primary screening tool for cervical cancer. Due to the slightly lower specificity compared to cytology-based screening, triage of hrHPV-positive women is required in order to keep follow-up procedures and associated costs within acceptable limits. The increase in DNA methylation of tumor suppressor genes associated with the development of advanced transforming CIN and cervical cancer provides valuable objective molecular triage markers. Such markers are likely to replace current triage algorithms based on cytology, with or without HPV 16/18 genotyping. DNA methylation can be easily detected in cervical scrapings and self-samples and methylation analysis has a high sensitivity for cervical cancer and advanced transforming CIN lesions in both sample types. The compatibility of methylation markers with HPV testing and self-sampling allows for full molecular cervical screening in the near future. In addition, the methylation markers could provide molecular tools to monitor women for post-treatment CIN2+.

In conclusion, recent insight in genetic and epigenetic changes associated with cervical cancer development has offered opportunities for molecular distinction of cervical cancer precursor lesions, paving the way for new biomarkers useful for screening, diagnosis and management of cervical cancer precursor lesions.

**Key Points Summary**

- CIN lesions can be divided into productive CIN (CIN1/2) and transforming CIN (CIN2/3). Morphologically, productive CIN2 can not be distinguished from transforming CIN2.
- Transforming CIN reflects a heterogeneous disease. Early and advanced transforming CIN lesions, displaying a low- and high short-term progression risk for cancer, respectively, can be distinguished on the basis of molecular host cell alterations.
- When applied to cervical scrapings specific methylation markers, such as *CADM1*, *MAL* and *miR124-2*, detect advanced transforming CIN and cancer with a high sensitivity.
- CIN 2/3 lesions detected by specific methylation markers are in need of immediate treatment given their high short-term progression risk for cancer.
- Cytology detects with a moderate sensitivity morphological cellular abnormalities associated with CIN2, CIN3 and cancer, but may miss cancer and advanced transforming CIN with a high short-term progression risk for cancer.
- HPV testing will replace cytology as primary screening tool for cervical cancer.
- Clinically validated panels of methylation markers, such as *CADM1*, *MAL* and *miR124-2*, can be used as triage marker for HPV positive women.
- Methylation marker panels with a high sensitivity for cancer, such as *CADM1*, *MAL* and *miR124-2*, have the potential to serve as primary screening tool.
- DNA methylation marker panels may in addition be used for management of women with CIN lesions to prevent overtreatment of CIN2/3 lesions.
- The compatibility of methylation markers with HPV testing and self-sampling has the potential for full molecular cervical screening in the near future.

**BOX1: microRNAs and their differential expression in transforming CIN**

MicroRNAs (miRNAs) are noncoding regulatory RNAs of 18-25 nucleotides in length, that can bind to the 3′ untranslated regions (3′UTR) of target mRNAs, thereby inhibiting protein translation, mRNA degradation, or both. As such, altered expression of miRNAs may affect tumour suppressor or oncogene protein expression. To date, more than 2500 human mature miRNAs have been annotated in the miRNA database (miRBase 20, release date June 2013; www.mirbase.org). miRNA expression profiles are highly tissue- and/or differentiation-specific, and often altered in cancers, which may at least in part result from DNA copy number alterations as well as epigenetic alterations [124].

A summary of differentially expressed miRNAs in transforming cervical intraepithelial neoplasia (CIN) lesions compared to normal cervical biopsies, and of which altered expression persists or increases in cervical carcinomas, is listed in **TABLE 1**. At present, there is relatively little overlap in altered miRNAs detected in the various studies and further research using independent platforms is warranted to extract the most powerful miRNA signature(s) predicting cervical cancer risk. Nonetheless, preliminary data indicate that miRNA expression analysis of a subset of differentially expressed miRNAs in cervical scrapings enables the detection of underlying transforming CIN (S., Wilting, personal communication)

**BOX2: Methods for DNA methylation detection applicable to cervical scrapings and self-samples**

Sensitive methods to detect aberrant DNA methylation in cervical scrapings or self-samples include (quantitative) methylation specific PCR ((q)MSP), MethyLight, methylation-specific high-resolution melting (MS-HRM) analysis and pyrosequencing. Each of these techniques is based on sodium bisulfite treatment of DNA resulting in conversion of unmethylated cytosines into uracils, while leaving methylated cytosines unaffected. This allows for DNA methylation mapping at single base resolution. which can be detected by PCR amplification and sequencing. qMSP, MethyLight and HS-HRM have similar analytical sensitivities and can detect as little as 0.1–1.0% of methylated DNA in a background of unmethylated DNA [125-127]. The sensitivity for bisulfite pyrosequencing is approximately 5% [128]. Although the sensitivity of bisulfite sequencing analysis can be increased when converted to a massive parallel sequencing-by-synthesis approach [129], its high-throughput application on large sample series awaits further developments.

A major advantage of the quantitative real-time PCR technologies is the option to analyse multiple methylation targets and an internal control in a multiplex reaction using a single aliquot of sample material, thereby saving material, time, costs and improving quality control, as recently developed for *CADM1-MAL-hsa-miR-124-2* and the reference gene *β-actin* [126].

**Glossary**

Cervical intraepithelial neoplasia (CIN) also known as cervical dysplasia is a premalignant condition of the uterine cervix, which histologically can be subdivided into CIN1, CIN2 and CIN3.

DNA methylation: Addition of a methyl-group at a cytosine in a CG dinucleotide pair. DNA methylation of CG-rich areas in gene promoters can result in gene silencing.

Epigenetic changes: Changes in DNA methylation and chromatin, that do not involve a change in the DNA sequence

Episome: An extrachromosomal DNA element that can replicate independently from host chromosomal DNA

Methylation marker panel: A panel of genes, most often involving gene promoter sequences, in which methylation of CG sites represent a biomarker for a specific condition, such as a (pre)cancerous lesion of the cervix.

Microarray comparative genomic hybridization (microarrayCGH): a platform on which at a genome wide level DNA copy number aberrations can be assessed in a single experiment

Self-sample: A self (at home)-collected cervico-vaginal specimen using a lavage or brush-based sampler. The self-collected cells can be used for cervical cancer screening by HPV detection and triage by methylation marker analysis.

**Acknowledgements**

The research in the laboratory of R.D.M.S., P.J.F.S., D.A.M.H. and C.J.L.M.M. is supported by grants from the Dutch Cancer Society (KWF2007-3771, KWF2009-4413, KWF2009-4522 and KWF2010-4668), Zorgonderzoek Nederland-Medische Wetenschappen (ZON-MW), the European community Program Health-FP7 Program (PreHdIct FP7-Health 2009-24206- and CoheaHr-Health-F3-2013-603019) and the European Research Council (ERC advanced 2012-AdG, proposal 322986 Mass-care).

**Legends to the Figures**

**Figure 1. HPV-mediated cervical carcinogenesis.**

The various outcomes of exposure of cervical epithelial cells to high-risk human papillomavirus (hrHPV) are represented as a transient infection (no pathology), productive infection (productive cervical intraepithelial neoplasia (CIN); mainly representing CIN1 and subset of CIN 2) and transforming infection (transforming CIN; mainly representing the remaining subset of CIN2 and CIN 3). Morphologically, CIN2 associated with a productive HPV infection can not be distinguished from CIN2 associated with a transforming HPV infection. Similarly, CIN1 lesions that occasionally may represent transforming infections are morphologically not distinguishable from productive counterparts. From the onset of a transforming CIN it can take another 20-30 years before invasive cancer will develop. Transforming CIN represents a heterogeneous disease with varying duration of existence, which may either regress or progress to cancer. The risk of cancer progression is dependent on molecular host cell alterations.

A new concept suggests that most of the transforming CIN and cervical cancers arise from exposure of embryonic squamo-columnar junction (SCJ) cells to hrHPV [20], suggesting a high susceptibility of these cells for HPV transformation. The SCJ cells and corresponding lesions are characterized by a specific protein expression pattern (expression of Krt7, AGR2, MMP7 and GDA) and precursor lesions arising from these SCJ cells are unlikely to be preceded by a productive CIN. The latter are suggested to arise from infection of cells in the ectocervix or transformation zone.

**Figure 2. Cellular changes required for progression of transforming cervical intraepithelial neoplasia (CIN) to cancer.**

The human papillomavirus (HPV)-related and host cell aberrations associated with disease progression are indicated below the concept of HPV-induced cervical carcinogenesis. Colour intensities indicate their level or frequency of detection and dashed lines indicate their infrequent or unknown detection. The potential application of the viral and host cell aberrations as markers for screening, diagnosis and treatment strategies is listed on the right. tCIN: transforming CIN; CxCa: cervical cancer

**Figure 3. Triage tools in cervical scrapings of human papillomavirus (HPV)-positive women.**

A schematic representation of the sensitivity (y-axis) of different triage methods for women with HPV-test positive cervical scrapings (blue line, host cell DNA methylation; black line, cytology) along the time line of transforming CIN towards invasive cervical cancer (x-axis).

Reference List


1. Arbyn,M. *et al.* Worldwide burden of cervical cancer in 2008. *Ann. Oncol.* **22**, 2675-2686 (2011).

2. Ferlay,J. *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* **127**, 2893-2917 (2010).

3. Walboomers,J.M. *et al.* Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189**, 12-19 (1999).

4. Baseman,J.G. & Koutsky,L.A. The epidemiology of human papillomavirus infections. *J. Clin. Virol.* **32 Suppl 1**, S16-S24 (2005).

5. IARC. A review of human carcinogens-PartB: biological agents. 100B. 2011.

6. Snijders,P.J., Steenbergen,R.D., Heideman,D.A., & Meijer,C.J. HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J. Pathol.* **208**, 152-164 (2006).

7. McCredie,M.R. *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.* **9**, 425-434 (2008).

8. Vink,M.A. *et al.* Clinical progression of high-grade cervical intraepithelial neoplasia: estimating the time to preclinical cervical cancer from doubly censored national registry data. *Am. J. Epidemiol.* **178**, 1161-1169 (2013).


**References 7 and 8 describe the natural history of transforming CIN and cervical cancer and provide an estimate of the duration of progression to cervical cancer.**


9. Peto,J., Gilham,C., Fletcher,O., & Matthews,F.E. The cervical cancer epidemic that screening has prevented in the UK. *Lancet* **364**, 249-256 (2004).

10. Doorbar,J. Molecular biology of human papillomavirus infection and cervical cancer. *Clin. Sci. (Lond)* **110**, 525-541 (2006).

11. Stoler,M.H. *et al.* Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. *Hum. Pathol.* **23**, 117-128 (1992).

12. Greenfield,I., Nickerson,J., Penman,S., & Stanley,M. Human papillomavirus 16 E7 protein is associated with the nuclear matrix. *Proc. Natl. Acad. Sci. U. S. A* **88**, 11217-11221 (1991).

13. Crum,C.P., Nagai,N., Levine,R.U., & Silverstein,S. In situ hybridization analysis of HPV 16 DNA sequences in early cervical neoplasia. *Am. J. Pathol.* **123**, 174-182 (1986).

14. Korzeniewski,N., Spardy,N., Duensing,A., & Duensing,S. Genomic instability and cancer: lessons learned from human papillomaviruses. *Cancer Lett.* **305**, 113-122 (2011).

15. Stern,P.L. *et al.* Therapy of human papillomavirus-related disease. *Vaccine* **30 Suppl 5**, F71-F82 (2012).

16. zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J. Natl. Cancer Inst.* **92**, 690-698 (2000).

17. Schwarz,E. *et al.* Structure and Transcription of Human Papillomavirus Sequences in Cervical-Carcinoma Cells. *Nature* **314**, 111-114 (1985).

18. Duensing,S. & Munger,K. The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. *Cancer Res.* **62**, 7075-7082 (2002).

19. Bhattacharjee,B. & Sengupta,S. CpG methylation of HPV 16 LCR at E2 binding site proximal to P97 is associated with cervical cancer in presence of intact E2. *Virology* **354**, 280-285 (2006).

20. Herfs,M. *et al.* A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. *Proc. Natl. Acad. Sci. U. S. A* **109**, 10516-10521 (2012).

**Reports on a new type of cells, referred to as squamo-columnar junction (SCJ) cells that may represent the cells of origin of cervical cancer**.

21. Mcnairn,A.J. & Guasch,G. Epithelial transition zones: merging microenvironments, niches, and cellular transformation. *Eur. J. Dermatol.* **21 Suppl 2**, 21-28 (2011).

22. Herfs,M. *et al.* Cervical squamocolumnar junction-specific markers define distinct, clinically relevant subsets of low-grade squamous intraepithelial lesions. *Am. J. Surg. Pathol.* **37**, 1311-1318 (2013).

23. de Martel,C. *et al.* Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol.* **13**, 607-615 (2012).

24. Moody,C.A. & Laimins,L.A. Human papillomavirus oncoproteins: pathways to transformation. *Nat. Rev. Cancer* **10**, 550-560 (2010).

25. Klingelhutz,A.J. & Roman,A. Cellular transformation by human papillomaviruses: lessons learned by comparing high- and low-risk viruses. *Virology* **424**, 77-98 (2012).

26. McLaughlin-Drubin,M.E. & Munger,K. Biochemical and functional interactions of human papillomavirus proteins with polycomb group proteins. *Viruses.* **5**, 1231-1249 (2013).

27. Au Yeung,C.L. *et al.* HPV-16 E6 upregulation of DNMT1 through repression of tumor suppressor p53. *Oncol. Rep.* **24**, 1599-1604 (2010).

28. Burgers,W.A. *et al.* Viral oncoproteins target the DNA methyltransferases. *Oncogene* **26**, 1650-1655 (2007).

29. Wilting,S.M. *et al.* Increased gene copy numbers at chromosome 20q are frequent in both squamous cell carcinomas and adenocarcinomas of the cervix. *J. Pathol.* **209**, 220-230 (2006).

30. Sawada,M. *et al.* Increased expression of DNA methyltransferase 1 (DNMT1) protein in uterine cervix squamous cell carcinoma and its precursor lesion. *Cancer Lett.* **251**, 211-219 (2007).

31. Leonard,S.M. *et al.* Oncogenic human papillomavirus imposes an instructive pattern of DNA methylation changes which parallel the natural history of cervical HPV infection in young women. *Carcinogenesis* **33**, 1286-1293 (2012).

32. Hyland,P.L. *et al.* Evidence for alteration of EZH2, BMI1, and KDM6A and epigenetic reprogramming in human papillomavirus type 16 E6/E7-expressing keratinocytes. *J. Virol.* **85**, 10999-11006 (2011).

33. Laughlin-Drubin,M.E., Crum,C.P., & Munger,K. Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. *Proc. Natl. Acad. Sci. U. S. A* **108**, 2130-2135 (2011).

**Reports on the mechanisms underlying p16 overexpression in transforming CIN and cervical cancer**

34. Wentzensen,N. & von Knebel,D.M. Biomarkers in cervical cancer screening. *Dis. Markers* **23**, 315-330 (2007).

35. Martinez,I. *et al.* Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. *Oncogene* **27**, 2575-2582 (2008).

36. Wang,X. *et al.* Oncogenic HPV infection interrupts the expression of tumor-suppressive miR-34a through viral oncoprotein E6. *RNA.* **15**, 637-647 (2009).

37. Au Yeung,C.L., Tsang,T.Y., Yau,P.L., & Kwok,T.T. Human papillomavirus type 16 E6 induces cervical cancer cell migration through the p53/microRNA-23b/urokinase-type plasminogen activator pathway. *Oncogene* **30**, 2401-2410 (2011).

38. Melar-New,M. & Laimins,L.A. Human papillomaviruses modulate expression of microRNA 203 upon epithelial differentiation to control levels of p63 proteins. *J. Virol.* **84**, 5212-5221 (2010).

39. Zheng,Z.M. & Wang,X. Regulation of cellular miRNA expression by human papillomaviruses. *Biochim. Biophys. Acta* **1809**, 668-677 (2011).

40. Qian,K. *et al.* Identification and validation of human papillomavirus encoded microRNAs. *PLoS. One.* **8**, e70202 (2013).

41. Agarwal,S.M., Raghav,D., Singh,H., & Raghava,G.P. CCDB: a curated database of genes involved in cervix cancer. *Nucleic Acids Res.* **39**, D975-D979 (2011).

42. Thomas,L.K. *et al.* Chromosomal gains and losses in human papillomavirus-associated neoplasia of the lower genital tract - A systematic review and meta-analysis. *Eur. J. Cancer* **50**, 85-98 (2014).

43. Bierkens,M. *et al.* Focal aberrations indicate EYA2 and hsa-miR-375 as oncogene and tumor suppressor in cervical carcinogenesis. *Genes Chromosomes. Cancer* **52**, 56-68 (2013).

44. Wang,F. *et al.* miR-375 is down-regulated in squamous cervical cancer and inhibits cell migration and invasion via targeting transcription factor SP1. *Am. J. Pathol.* **179**, 2580-2588 (2011).

45. Yan,J.W., Lin,J.S., & He,X.X. The emerging role of miR-375 in cancer. *Int. J. Cancer*(2013).

46. Forbes,S.A. *et al.* COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res.* **39**, D945-D950 (2011).

47. Wright,A.A. *et al.* Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix. *Cancer* **119**, 3776-3783 (2013).

48. Ojesina,A.I. *et al.* Landscape of genomic alterations in cervical carcinomas. *Nature*(2013).

**First landscape paper on cervical carcinomas describing genome-wide analysis of DNA mutations**

49. Shen,H. & Laird,P.W. Interplay between the cancer genome and epigenome. *Cell* **153**, 38-55 (2013).

50. Clarke,M.A. *et al.* Human Papillomavirus DNA Methylation as a Potential Biomarker for Cervical Cancer. *Cancer Epidemiol. Biomarkers Prev.* **21**, 2125-2137 (2012).

51. Johannsen,E. & Lambert,P.F. Epigenetics of human papillomaviruses. *Virology* **445**, 205-212 (2013).

**References 50 and 51 provide a detailed overview on current literature regarding HPV DNA methylation and its biological and clinical implications**

52. Doerfler,W. *et al.* The fate of foreign DNA in mammalian cells and organisms. *Dev. Biol. (Basel)* **106**, 89-97 (2001).

53. Fernandez,A.F. *et al.* The dynamic DNA methylomes of double-stranded DNA viruses associated with human cancer. *Genome Res.* **19**, 438-451 (2009).

54. Vinokurova,S. & von Knebel,D.M. Differential methylation of the HPV 16 upstream regulatory region during epithelial differentiation and neoplastic transformation. *PLoS. One.* **6**, e24451 (2011).

55. Thain,A., Jenkins,O., Clarke,A.R., & Gaston,K. CpG methylation directly inhibits binding of the human papillomavirus type 16 E2 protein to specific DNA sequences. *J. Virol.* **70**, 7233-7235 (1996).

56. Wentzensen,N., Sherman,M.E., Schiffman,M., & Wang,S.S. Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. *Gynecol. Oncol.* **112**, 293-299 (2009).

57. Szalmas,A. & Konya,J. Epigenetic alterations in cervical carcinogenesis. *Semin. Cancer Biol.* **19**, 144-152 (2009).

58. Chen,Y.C. *et al.* Methylomics analysis identifies epigenetically silenced genes and implies an activation of beta-catenin signaling in cervical cancer. *Int. J. Cancer*(2013).

59. Lendvai,A. *et al.* Genome-wide methylation profiling identifies hypermethylated biomarkers in high-grade cervical intraepithelial neoplasia. *Epigenetics.* **7**, 1268-1278 (2012).

60. Farkas,S.A., Milutin-Gasperov,N., Grce,M., & Nilsson,T.K. Genome-wide DNA methylation assay reveals novel candidate biomarker genes in cervical cancer. *Epigenetics.* **8**, 1213-1225 (2013).

61. Brebi,P. *et al.* Genome-wide methylation profiling reveals Zinc finger protein 516 () and FK-506-binding protein 6 () promoters frequently methylated in cervical neoplasia, associated with HPV status and ethnicity in a Chilean population. *Epigenetics.* **9**, (2013).

62. Steenbergen,R.D. *et al.* Methylation-specific digital karyotyping of HPV16E6E7-expressing human keratinocytes identifies novel methylation events in cervical carcinogenesis. *J. Pathol.* **231**, 53-62 (2013).

63. Huang,R.L. *et al.* Methylomic analysis identifies frequent DNA methylation of zinc finger protein 582 (ZNF582) in cervical neoplasms. *PLoS. One.* **7**, e41060 (2012).

64. Chung,M.T. *et al.* SFRP1 and SFRP2 suppress the transformation and invasion abilities of cervical cancer cells through Wnt signal pathway. *Gynecol. Oncol.* **112**, 646-653 (2009).

65. Steenbergen,R.D. *et al.* TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. *J. Natl. Cancer Inst.* **96**, 294-305 (2004).

66. Overmeer,R.M. *et al.* Association between dense CADM1 promoter methylation and reduced protein expression in high-grade CIN and cervical SCC. *J. Pathol.* **215**, 388-397 (2008).

67. Overmeer,R.M. *et al.* Repression of MAL tumour suppressor activity by promoter methylation during cervical carcinogenesis. *J. Pathol.* **219**, 327-336 (2009).

68. Huisman,C. *et al.* Functional validation of putative tumor suppressor gene C13ORF18 in cervical cancer by Artificial Transcription Factors. *Mol. Oncol.* **7**, 669-679 (2013).

69. Lee,E.J. *et al.* Dkk3, downregulated in cervical cancer, functions as a negative regulator of beta-catenin. *Int. J. Cancer* **124**, 287-297 (2009).

70. Kaczkowski,B., Morevati,M., Rossing,M., Cilius,F., & Norrild,B. A Decade of Global mRNA and miRNA Profiling of HPV-Positive Cell Lines and Clinical Specimens. *Open. Virol. J.* **6**, 216-231 (2012).

71. Saavedra,K.P., Brebi,P.M., & Roa,J.C. Epigenetic alterations in preneoplastic and neoplastic lesions of the cervix. *Clin. Epigenetics.* **4**, 13 (2012).

72. Lee,J.W. *et al.* Altered MicroRNA expression in cervical carcinomas. *Clin. Cancer Res.* **14**, 2535-2542 (2008).

73. Wilting,S.M. *et al.* Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol. Cancer* **9**, 167 (2010).

74. Wilting,S.M. *et al.* Methylation-mediated transcriptional repression of microRNAs during cervical carcinogenesis. *Epigenetics.* **8**, 220-228 (2013).

75. Wang,X. *et al.* Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. *PLoS. One.* **3**, e2557 (2008).

76. Lee,J.W. *et al.* Altered MicroRNA expression in cervical carcinomas. *Clin. Cancer Res.* **14**, 2535-2542 (2008).

77. Wilting,S.M. *et al.* Altered microRNA expression associated with chromosomal changes contributes to cervical carcinogenesis. *Oncogene* **32**, 106-116 (2013).

78. Li,Y. *et al.* Progressive miRNA expression profiles in cervical carcinogenesis and identification of HPV-related target genes for miR-29. *J. Pathol.* **224**, 484-495 (2011).

79. Cheung,T.H. *et al.* Dysregulated microRNAs in the pathogenesis and progression of cervical neoplasm. *Cell Cycle* **11**, 2876-2884 (2012).

80. Pereira,P.M., Marques,J.P., Soares,A.R., Carreto,L., & Santos,M.A. MicroRNA expression variability in human cervical tissues. *PLoS. One.* **5**, e11780 (2010).

81. Scotto,L. *et al.* Identification of copy number gain and overexpressed genes on chromosome arm 20q by an integrative genomic approach in cervical cancer: potential role in progression. *Genes Chromosomes. Cancer* **47**, 755-765 (2008).

82. Muralidhar,B. *et al.* Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNA profiles. *J. Pathol.* **224**, 496-507 (2011).

83. Yao,T. *et al.* Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in cervical cancer. *Virol. J.* **10**, 175 (2013).

84. Botezatu,A. *et al.* Quantitative analysis of the relationship between microRNA124a, -34b and -203 gene methylation and cervical oncogenesis. *Mol. Med. Rep.* **4**, 121-128 (2011).

85. Yang,H.P. *et al.* Clinical and pathological heterogeneity of cervical intraepithelial neoplasia grade 3. *PLoS. One.* **7**, e29051 (2012).

86. Wentzensen,N. *et al.* Heterogeneity of high-grade cervical intraepithelial neoplasia related to HPV16: implications for natural history and management. *Int. J. Cancer* **132**, 148-154 (2013).

**References 85 and 86 provide insight in the clinical/colposcopical and pathological heterogeneity of transforming CIN**

87. Castle,P.E., Gravitt,P.E., Wentzensen,N., & Schiffman,M. A descriptive analysis of prevalent vs incident cervical intraepithelial neoplasia grade 3 following minor cytologic abnormalities. *Am. J. Clin. Pathol.* **138**, 241-246 (2012).

88. Ostor,A.G. Natural history of cervical intraepithelial neoplasia: a critical review. *Int. J. Gynecol. Pathol.* **12**, 186-192 (1993).

89. Wentzensen,N., Sherman,M.E., Schiffman,M., & Wang,S.S. Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. *Gynecol. Oncol.* **112**, 293-299 (2009).

90. Bierkens,M. *et al.* Chromosomal profiles of high-grade cervical intraepithelial neoplasia relate to duration of preceding high-risk human papillomavirus infection. *Int. J. Cancer* **131**, E579-E585 (2011).

91. Bierkens,M. *et al.* CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional to degree and duration of underlying cervical disease. *Int. J. Cancer* **133**, 1293-1299 (2013).

**References 89 and 90 are first studies indicating that the number of chromosomal aberrations and levels of DNA methylation are not only associated with severity of CIN disease but with also duration of transforming CIN.**

92. Arbyn,M. *et al.* Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer. *Vaccine* **30 Suppl 5**, F88-F99 (2012).

93. Ronco,G. *et al.* Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet* **383**, 524-532 (2014).

**A pooled analysis showing that the HPV test provides a 70% greater protection against cervical cancer compared with cytology test**

94. Katki,H.A. *et al.* Benchmarking CIN 3+ risk as the basis for incorporating HPV and Pap cotesting into cervical screening and management guidelines. *J. Low Genit. Tract. Dis.* **17**, S28-S35 (2013).

95. Dijkstra,M.G. *et al.* Primary hrHPV DNA Testing in Cervical Cancer Screening: How to Manage Screen-Positive Women? A POBASCAM Trial Substudy. *Cancer Epidemiol. Biomarkers Prev.*(2013).

96. Rijkaart,D.C. *et al.* HPV DNA testing in population-based cervical screening (VUSA-Screen study): results and implications. *Br. J. Cancer*(2012).

97. Arbyn,M., Ronco,G., Cuzick,J., Wentzensen,N., & Castle,P.E. How to evaluate emerging technologies in cervical cancer screening? *Int. J. Cancer* **125**, 2489-2496 (2009).

98. Pepe,M.S. *et al.* Phases of biomarker development for early detection of cancer. *J. Natl. Cancer Inst.* **93**, 1054-1061 (2001).

99. Pepe,M.S., Feng,Z., Janes,H., Bossuyt,P.M., & Potter,J.D. Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design. *J. Natl. Cancer Inst.* **100**, 1432-1438 (2008).

100. Carozzi,F. *et al.* Risk of high-grade cervical intraepithelial neoplasia during follow-up in HPV-positive women according to baseline p16-INK4A results: a prospective analysis of a nested substudy of the NTCC randomised controlled trial. *Lancet Oncol.* **14**, 168-176 (2013).

101. Petry,K.U. *et al.* Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology. *Gynecol. Oncol.* **121**, 505-509 (2011).

102. Conesa-Zamora,P. Role of cell cycle biomarkers in human papillomavirus related uterine lesions. *Curr. Pharm. Des* **19**, 1412-1424 (2013).

103. Reuschenbach,M. & von Knebel,D.M. Diagnostic tests for the detection of human papillomavirus-associated cervical lesions. *Curr. Pharm. Des* **19**, 1358-1370 (2013).

104. Snijders,P.J. *et al.* High-risk HPV testing on self-sampled versus clinician-collected specimens: a review on the clinical accuracy and impact on population attendance in cervical cancer screening. *Int. J. Cancer* **132**, 2223-2236 (2013).

105. Arbyn,M. *et al.* Accuracy of human papillomavirus testing on self-collected versus clinician-collected samples: a meta-analysis. *Lancet Oncol.* **15**, 172-183 (2014).

106. Brink,A.A. *et al.* High concordance of results of testing for human papillomavirus in cervicovaginal samples collected by two methods, with comparison of a novel self-sampling device to a conventional endocervical brush. *J. Clin. Microbiol.* **44**, 2518-2523 (2006).

107. Kalantari,M. *et al.* Methylation of human papillomavirus 16, 18, 31, and 45 L2 and L1 genes and the cellular DAPK gene: Considerations for use as biomarkers of the progression of cervical neoplasia. *Virology* **448**, 314-321 (2014).

108. Lai,H.C. *et al.* Quantitative DNA methylation analysis detects cervical intraepithelial neoplasms type 3 and worse. *Cancer* **116**, 4266-4274 (2010).

109. Eijsink,J.J. *et al.* Detection of cervical neoplasia by DNA methylation analysis in cervico-vaginal lavages, a feasibility study. *Gynecol. Oncol.* **120**, 280-283 (2011).

110. Overmeer,R.M. *et al.* Combined CADM1 and MAL promoter methylation analysis to detect (pre-)malignant cervical lesions in high-risk HPV-positive women. *Int. J. Cancer* 129:2218-2225 (2011).

111. Hesselink,A.T. *et al.* Combined Promoter Methylation Analysis of CADM1 and MAL: An Objective Triage Tool for High-Risk Human Papillomavirus DNA-Positive Women. *Clin. Cancer Res.* **17**, 2459-2465 (2011).

112. Hesselink,A.T. *et al.* Methylation marker analysis of self-sampled cervico-vaginal lavage specimens to triage high-risk HPV-positive women for colposcopy. *Int. J. Cancer* Epub **doi: 10.1002/ijc.28723**, (2014).

113. Verhoef,V.M. *et al.* A randomised controlled trial on triage of HPV positive women on self-collected cervico-vaginal specimens: direct methylation marker testing versus indirect cytology. *Lancet Oncol.* **15**, 315-322 (2014).

**The first randomised controlled clinical trial on implementation of methylation markers in cervical screening using self-samples**

114. Widschwendter,A. *et al.* Analysis of aberrant DNA methylation and human papillomavirus DNA in cervicovaginal specimens to detect invasive cervical cancer and its precursors. *Clin. Cancer Res.* **10**, 3396-3400 (2004).

115. Huang,T.H. *et al.* Quantitative analysis of methylation status of the PAX1 gene for detection of cervical cancer. *Int. J. Gynecol. Cancer* **20**, 513-519 (2010).

116. Cuzick,J. *et al.* Overview of the European and North American studies on HPV testing in primary cervical cancer screening. *Int. J. Cancer* **119**, 1095-1101 (2006).

117. Kitchener,H.C., Castle,P.E., & Cox,J.T. Chapter 7: Achievements and limitations of cervical cytology screening. *Vaccine* **24 Suppl 3**, S3-63-S3/70 (2006).

118. Bruinsma,F.J. & Quinn,M.A. The risk of preterm birth following treatment for precancerous changes in the cervix: a systematic review and meta-analysis. *BJOG.* **118**, 1031-1041 (2011).

119. Arbyn,M. *et al.* Perinatal mortality and other severe adverse pregnancy outcomes associated with treatment of cervical intraepithelial neoplasia: meta-analysis. *BMJ* **337**, a1284 (2008).

120. Kyrgiou,M. *et al.* Obstetric outcomes after conservative treatment for intraepithelial or early invasive cervical lesions: systematic review and meta-analysis. *Lancet* **367**, 489-498 (2006).

121. Kocken,M. *et al.* Risk of recurrent high-grade cervical intraepithelial neoplasia after successful treatment: a long-term multi-cohort study. *Lancet Oncol.* **12**, 441-450 (2011).

122. Kocken,M. *et al.* High-risk human papillomavirus testing versus cytology in predicting post-treatment disease in women treated for high-grade cervical disease: a systematic review and meta-analysis. *Gynecol. Oncol.* **125**, 500-507 (2012).

123. Gnyszka,A., Jastrzebski,Z., & Flis,S. DNA methyltransferase inhibitors and their emerging role in epigenetic therapy of cancer. *Anticancer Res.* **33**, 2989-2996 (2013).

124. Lopez-Serra,P. & Esteller,M. DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer. *Oncogene* **31**, 1609-1622 (2012).

125. Wojdacz,T.K. & Dobrovic,A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res.* **35**, e41 (2007).

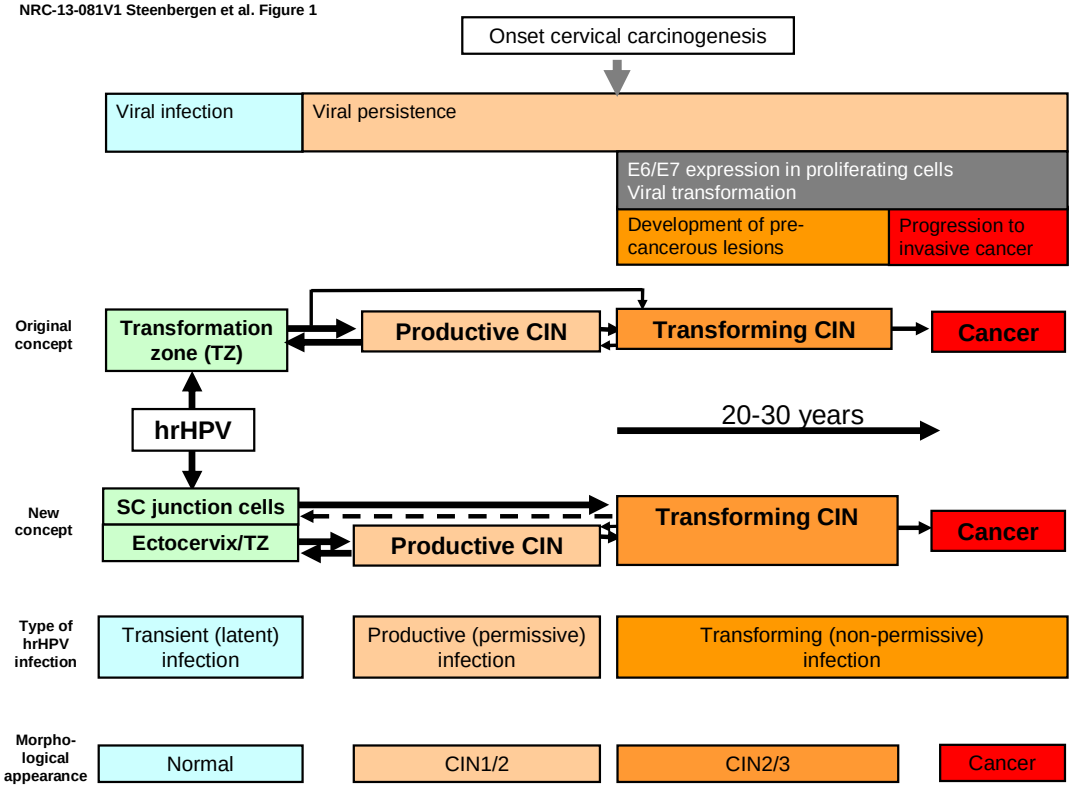
126. Snellenberg,S. *et al.* Development of a multiplex methylation-specific PCR as candidate triage test for women with an HPV-positive cervical scrape. *BMC. Cancer* **12**, 551 (2012).

127. Eads,C.A. *et al.* MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* **28**, E32 (2000).

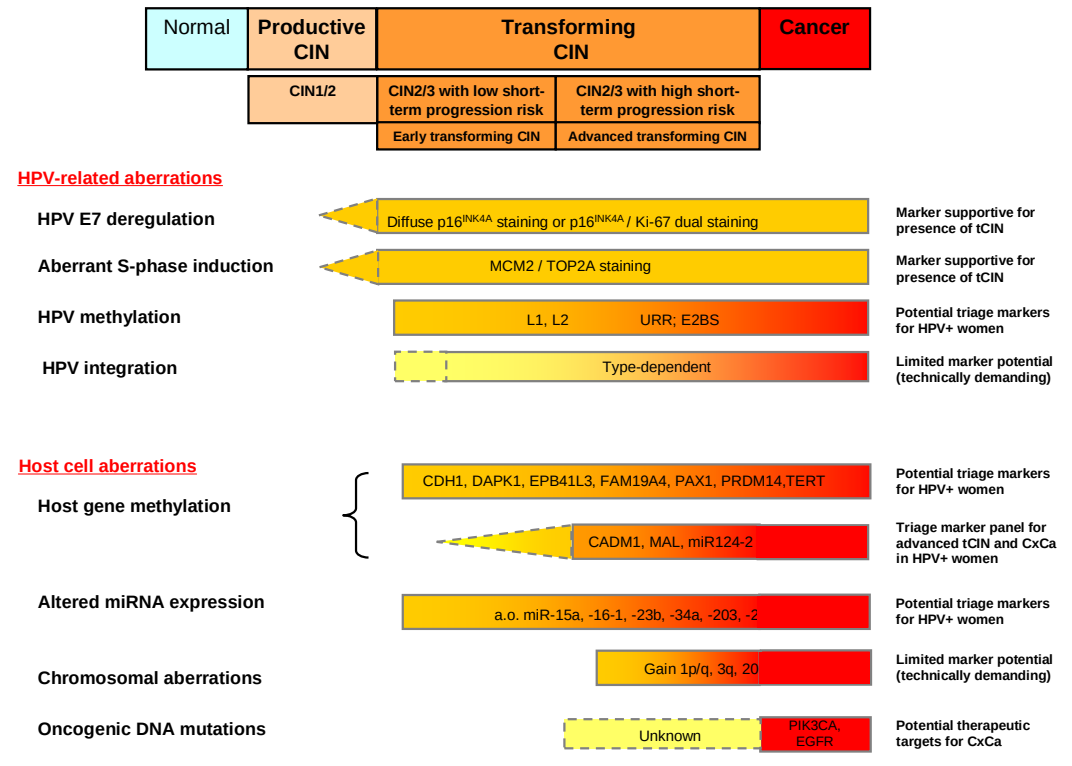
128. Mikeska,T., Felsberg,J., Hewitt,C.A., & Dobrovic,A. Analysing DNA methylation using bisulphite pyrosequencing. *Methods Mol. Biol.* **791**, 33-53 (2011).

129. Taylor,K.H. *et al.* Ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters by 454 sequencing. *Cancer Res.* **67**, 8511-8518 (2007).

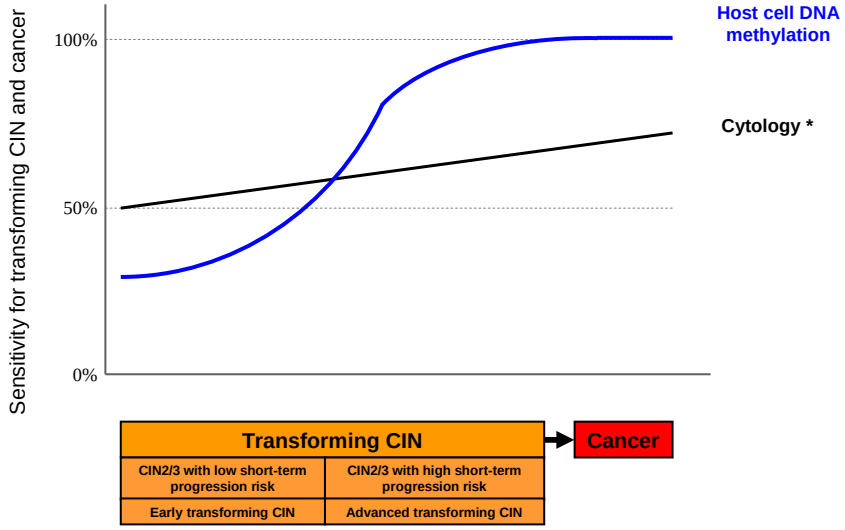
NRC-13-081V1 Steenbergen et al. Figure 1




NRC-13-081V1 Steenbergen et al. Figure 2



NRC-13-081V2 Steenbergen et al. Figure 3




*) depending on quality