

GENETIC, EPIGENETIC AND ENVIRONMENTAL DETERMINANTS OF CERVICAL CANCER RISK AND IMPLICATIONS FOR EARLY DETECTION AND SCREENING

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Declaration of originality

This thesis presents original work of the titled authors. That which is not is referenced accordingly.

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ABSTRACT

Introduction: Cervical cancer remains a leading cause of cancer mortality worldwide. Infection with human papillomavirus (HPV) is necessary for the development of cervical cancer but the factors related to persistent infection are poorly understood. Many potential environmental and behavioural exposures related to the development of cervical cancer have been studied, but randomised control trials are uncommon, and the reliability of evidence gained from observational studies remains unclear. Meanwhile heritability of cervical cancer has been estimated to approach 36% but genome-wide association exploration has been limited. There is increasing evidence for an association between DNA methylation and cervical carcinogenesis, with potential for use as a molecular marker for early detection of high-grade cervical lesions.

Methods: We performed an umbrella review of the literature regarding any environmental or behavioural exposure relating to cervical outcomes. We did a genome-wide association study (GWAS) in women with invasive cervical cancer (ICC) and CIN3 (N=4769) and female controls (N=145,545) in the UK Biobank. Epigenetic signatures were examined in an epigenome-wide study on the Illumina 850K array in liquid-based cytology samples from a cohort of 241 women. Through a systematic review and meta-analysis, we examined the diagnostic test accuracy of HPV DNA methylation as a test for cervical cancer.

Results: From 171 meta-analyses we found strong evidence relating to HIV positivity (RR=2.20, (95%CI=1.89-2.54)), vaginal dysbiosis (RR=1.59(1.40-1.81)) and chronic immunosuppressive therapy (RR=1.33(1.89-2.54)) for cervical cancer outcomes. Novel genetic loci rs1017546 (*PAX8*; $P=1.07 \times 10^{-9}$) and rs27069 (*CLPTMIL*; $P=1.51 \times 10^{-9}$), and previously reported rs9272050 (*HLA-DQA1*; $P=2.51 \times 10^{-28}$) were strongly associated to cervical cancer. EWAS further identified two independent CpG sites in *PAX1* ($P=3.90 \times 10^{-12}$) and *NREP-ASI* ($P=2.45 \times 10^{-10}$) genes that were able to detect CIN3 or Cancer with an AUC=0.92(0.92-0.92) in the discovery cohort and AUC=0.77(0.76-0.79) in an independent cohort. The HPV16 L1 gene had the strongest association with CIN2 or worse, with a pooled sensitivity of 77%(63-87%), specificity 64%(55–71%), and AUC of 0.73(0.69–0.77).

Conclusions: This work evaluates and presents the strongest environmental risk factors and substantial new evidence for genetic susceptibility to cervical cancer, mediated through disruption in immune function and apoptotic pathways. *PAX1*, *NREP-ASI* and HPV16 L1 are identified as promising methylation markers for triage of women identified as hrHPV positive at cervical screening.

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Publications, Presentations, Posters and Prizes relating to the thesis

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3. Bowden S, Kyrgiou M, 2020, Human papillomavirus, *Obstetrics, Gynaecology & Reproductive Medicine*, Vol: 30, Pages: 109-118, ISSN: 1751-7214
4. Athanasiou A, Bowden S, Paraskevaidi M, Fotopoulou C, Martin-Hirsch P, Paraskevaides E, Kyrgiou M et al., 2020, HPV vaccination and cancer prevention, *Best Practice and Research: Clinical Obstetrics and Gynaecology*, Vol: 65, Pages: 109-124, ISSN: 1521-6934
5. Bowden SJ, Lathouras K, Kyrgiou M, 2020, Can DNA methylation tests improve the accuracy of cervical screening?, *British Journal of Obstetrics and Gynaecology*, Vol: 128, Pages: 515-515, ISSN: 1470-0328
6. Bowden SJ, Kalliala I, Veroniki AA, Arbyn M, Mitra A, Lathouras K, Mirabello L, Chadeau-Hyam M, Paraskevaides E, Flanagan JM, Kyrgiou M. The use of human papillomavirus DNA methylation in cervical intraepithelial neoplasia: A systematic review and meta-analysis. *EBioMedicine*. 2019 Dec;50:246-259.

Prizes

1. Victor Bonney Prize. RCOG, London. December 2019
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Presentations

1. Genetic and epigenetic contributions in cervical disease. Hellenic Cervical Pathology & Colposcopy. Ioannina, Greece (virtual) November 2021.
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1. Kyrgiou M*, Bowden S*, Athanasiou A, Paraskevaidi M, Kechagias K, Zikopoulos A, Terzidou V, Martin-Hirsch P, Arbyn M, Bennett P, Paraskevaidis E et al., 2021, Morbidity after local excision of the transformation zone for cervical intra-epithelial neoplasia and early cervical cancer, *Best Practice & Research Clinical Obstetrics & Gynaecology* (joint first authors)
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1. SUMMARY

Improving and tailoring national cervical screening programmes has been a major concerted effort in high-resource countries over the last two decades. Screening programmes are based on a call-recall system tailored to individuals based upon individual risk factors which currently includes age, HIV status and previous screening results.

The relationship between HPV infection, HPV persistence, CIN and progression to cancer, is incompletely understood. Environmental and behavioural factors are thought to play a role in the risk of HPV persistence and progression to cervical cancer, however the majority of associations have been reported via observational studies which are subject to inherent biases. The body of literature exploring potential risk and protective associations for cervical cancer is continuously growing. Some widely accepted risk factors for cervical cancer may in fact be biased and based upon weak evidence, while lesser-known risk factors may have strong evidence but not yet reached public dissemination. Uncovering the strength of evidence and degree of bias in the associations between environmental and behavioural risk factors and cervical cancer, may increase our understanding for the risk of cervical cancer and influence public health information and policy decisions.

Traditionally, screening programmes have relied on a cytology test to detect the presence of dyskaryotic cells. However, the emergence of genomic exploration has opened the opportunity to develop molecular tests. Large genetic databases with genome-wide coverage and linked epidemiological metadata, such as the UK Biobank are allowing researchers to examine associations between common genetic variants and cancer phenotypes, which may inform future risk prediction models and personalised screening programmes. An additional benefit of examining cervical neoplasia at the molecular level, is the potential for enhanced accuracy detection markers. It is possible that molecular markers may even predict progression or regression of preinvasive cervical disease. Such insights could increase the early detection of progressive CIN, whilst reducing unnecessary treatments of CIN lesions that are regressing. Further potential benefits of molecular tests include opportunities for robotic automation of screening, development of point-of-care testing for low-resource settings, and translation to self-sampling for screening non-attenders.

2. Epidemiology of cervical intraepithelial neoplasia (CIN) and Cervical Cancer

Cancer of the uterine cervix (cervical cancer) is the 4th most common female cancer globally, and the leading cause of female cancer mortality in many developing countries. In 2020 cervical cancer contributed to an estimate of 604,127 new cases and 341,831 deaths, nearly a ten percent increase from

2018 figures⁶. There is a wide variation in incidence by geographical region, with over 84% of cervical cancers and 88% of deaths being detected in women in living low-resource countries, and the highest rates observed in sub-Saharan Africa; China and India together account for around a third of all cervical cancer cases⁷. This variation is largely due to economic poverty, poor access to healthcare and organised screening, combined with increased high-risk HPV (hrHPV) infection prevalence rates⁸. In many low-resource countries cervical cancer is the leading female cancer in terms of incidence and mortality. An exception to this is the lower rates of cervical cancer observed in West Asia and the Middle East where lower rates are thought to be associated with female sexual behaviours conferring a reduced hrHPV exposure. Age-standardised incidence varies from 10 cases per 100,000 women in many high-resource countries to over 40 cases per 100,000 women in some low-resource countries; survival rates vary greater, with cervical cancer age-standardised mortality averaging at three deaths per 100,000 women in many high-resource countries to more than 30 deaths per 100,000 women in some low-resource counties⁹. These differences are largely attributed to the introduction of national cervical screening programmes in more developed countries^{10,11}. Aimed at detecting and treating pre-invasive lesions, cervical screening programmes have been highly successful in reducing the overall incidence of cervical cancer and also increasing detection of cancers at an early stage with early small-volume disease. Around 8-10% of women in the UK receive an abnormal smear test in their lifetime; they are referred for colposcopy and follow-up in a call-recall system. Introduction of screening in the UK in 1988 has led to an estimated 24% reduction in cervical cancer incidence; cervical cancer is now the 14th most common cancer in UK females¹². Although the number of countries with certified cancer registries is increasing, only around 24% of countries provide national incidence data, and 44% provide mortality data; accurate comparisons across countries or time is therefore limited in many geographical regions⁷.

3. Risk and pathogenesis of CIN and cervical cancer

3.1 HPV infection

In the 1960's it was hypothesised that cervical cancer was a sexually transmitted disease, based on much earlier observations that celibate women including nuns, never developed cervical cancer. In 1976-77, several scientific teams isolated HPV from abnormal cervical squamous epithelial cells¹³. Harald Zur Hausen was the first to confirm the association between HPV and cervical cancer¹⁴; HPV infection was recognised as the first tumour virus in 1983 and Zur Hausen was later awarded the Nobel prize in medicine.

Papillomaviruses are ancient and ubiquitous small double-stranded DNA viruses. They lack the necessary enzymatic machinery to replicate independently and so have evolved over millions of years to infect and replicate through a range of animal hosts including humans¹⁵. Human papillomavirus (HPV) primarily infects epithelial cutaneous and mucosal surfaces of oral and anogenital areas; it is spread through direct, sexual or vertical transmission^{16,17}. HPV infections are extremely common; rapidly acquired after sexual debut with a lifetime incidence exceeding 80% in most populations.

Extensive study over the last four decades has furthered our understanding of the causal association of hrHPV with primarily cervical, but also other cancers. hrHPV is thought to be responsible for 10% of all malignancies in women and 5% in men: 99.7% of cervical cancers, a large proportion of other anogenital cancers (vaginal, vulval, penile, anal), and a subset of oropharyngeal cancers¹⁸. A small number of primary cervical cancers (<1%) are not caused by hrHPV and include rare neuroendocrine tumours, melanoma, sarcoma, lymphoma and cancers of unspecified histology.

The natural history of hrHPV infection is relatively well understood due to accessibility of the cervix allowing serial sampling in prospective cohorts. Cervical cancer has provided the best model for study of all hrHPV neoplastic processes. Viral transmission between sexual partners is thought to be related to both viral load and duration of exposure, while microtrauma in the epithelial surface is thought to be required for an initial active infection. Development of an initial active infection is likely to be influenced greatly by both the host immune response and the surrounding microenvironment, including the presence of inflammation secondary to concomitant infection¹⁷. More recently it has been found that even in the absence of occult vaginal infection, a vaginal microbiome with low *Lactobacillus* or bacterial vaginosis may predispose to hrHPV infection and cervical disease^{19,20}.

While persistent infection with high-risk HPV subtypes is necessary for the development of cervical cancer, this is not sufficient²¹. The majority of hrHPV infections (~90%) are thought to be transient and cleared through an incompletely understood immune response, usually within 12 to 24 months²². Longitudinal natural history studies of hrHPV incident infections in cohorts of young women have shown that clearance is higher in women under 30 years, where ~50% of hrHPV infections clear within 6 months, with ~90% clearing within a few years after the initial infection²³. It is thought that clearance of a transient hrHPV infection occurs largely through activation of the local innate immune response, including immunological cells present in cervical mucous. Longer infections usually activate the adaptive immune response, which involves antigen-presenting cells and antibody production, after which a level of natural immunity occurs²⁴.

HPV genotypes

To date, over 150 HPV have been identified, sequenced, and classified into five major phylogenetic types (Alpha, Beta, Gamma, Mu and Nu) (Figure 1), of which the most common are Alpha or Beta/Gamma¹. The majority of HPV infections from Beta and Gamma types are transient and benign, allowing for evolutionary fitness through efficient viral transmission in the population. Where chronic persistent infections occur from these types, continued virion production, in the absence of visible disease may continue for months to years. Conversely Alpha types have developed effective immune evasion strategies, likely through slow co-evolution with humans, to cause visible and persistent benign warts (papillomatosis) on anogenital and other mucocutaneous surfaces.

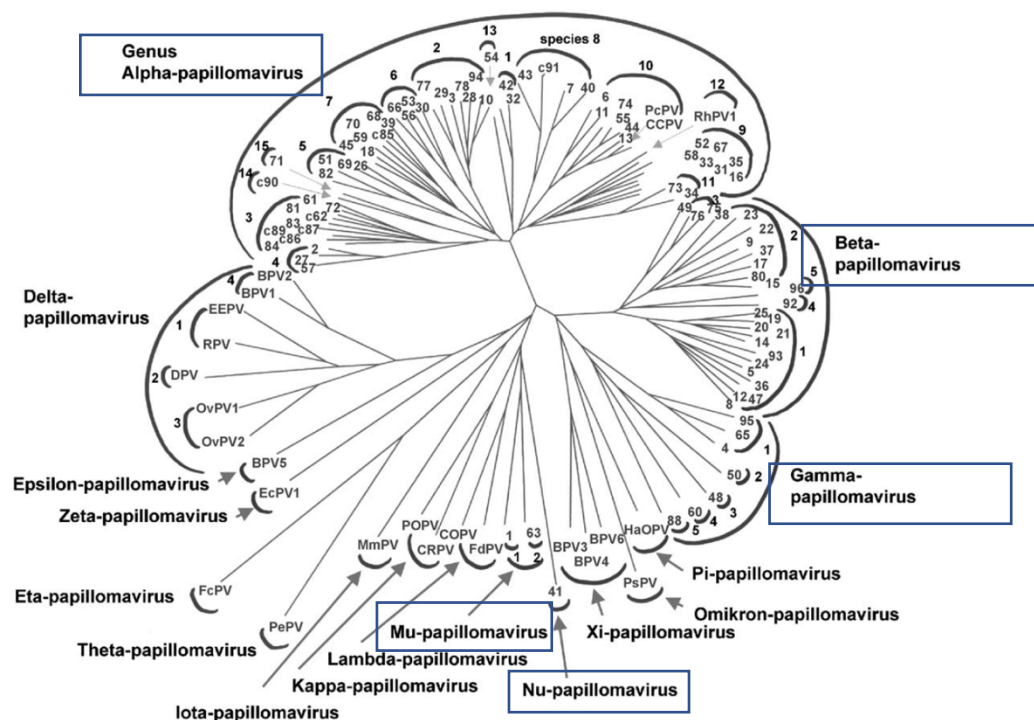


Figure 1. HPV phylogenetic tree, major types involved in cervical cancer are shown in blue boxes. Adapted from de Villiers *et al* (2004)¹

HPV16 and 18 are thought to cause over 70% of cases of invasive cervical cancer, with a further five HPV genotypes (31, 33, 45, 52, 58) responsible for the additional 20%. hrHPV predominates in squamous cell cancers, whilst HPV 18 may be the most common type in adenocarcinoma of the cervix. HPV type-specific prevalence varies globally; in populations that account for the majority of global cervical cancer deaths (e.g. South America and Sub-Saharan Africa), type-specific prevalence of HPV is largely unknown. Rates of HPV transmission between heterosexual couples also vary greatly²⁵.

Age-specific prevalence of HPV varies, with the peak incidence of HPV infection in adolescents, and incident infections subsequently decreasing steadily over time. Higher rates of HPV in young populations are thought to be related not only to higher age-specific HPV prevalence, but also a naïve immune response and possibly an immature cervical epithelium of the transformation zone in pubescent adolescents²⁶.

HPV genome and viral life-cycle

HPV is a double-stranded DNA virus with a circular genome of 7900-8000bp made up of 8 genes (or open reading frames (ORFs) and a long control region (LCR) of promoters and enhancers²⁷. Over 40 HPV subtypes have been recognised to infect the anogenital tract but only 12-13 of these are known to have the potential to instigate a neoplastic process and are therefore classified as high-risk HPV: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 (HPV68 is probably carcinogenic)²⁸. These types cause inapparent infection before visible precancerous lesions become evident. The remaining disease-causing HPV genotypes are considered low-risk. The most common of which are HPV 6 and 11 (alpha papillomaviruses) – responsible for visible genital warts and laryngeal papillomatosis².

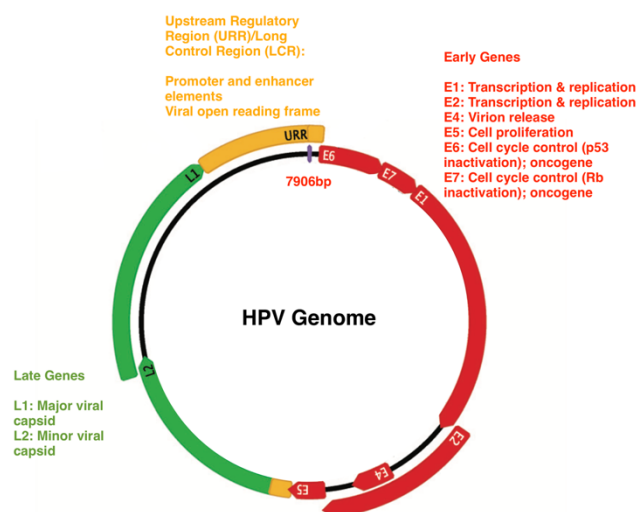


Figure 2. HPV16 viral genome and summary of known gene functions.

The first early genes, E1 and E2 are transcription factors and involved in maintaining viral replication²⁹. While E4 proteins contribute to genome amplification and viral synthesis, they may also facilitate virion release and transmission³⁰. E5 is not coded for in all hrHPVs; in Alpha types E5 interacts with host

factors to control viral replication and persistence as well as cell proliferation - such mechanisms include reducing surface major histocompatibility (MHC) 1 expression in infected basal cells to aid immune avoidance³¹. E6 and E7 are known as the oncogenes and are thought to mediate viral proliferation through cell cycle control; they induce disruption to normal keratinocyte differentiation through inhibition of tumour suppressor genes (particularly P53 and retinoblastoma protein (pRB)), allowing viral amplification and influencing cell senescence and apoptosis. These genes are particularly important in the expansion of lesion size seen with high-risk types^{32,33}. An important difference between low-risk and high-risk types is the ability of high-risk E7 to associate with retinoblastoma protein. Additionally, high-risk E7 expression is thought to cause extensive epigenetic reprogramming and cell cycle stimulation. The late genes, L2 and L1 code for the minor and major viral capsid proteins respectively - necessary for viral packaging and viral release and have formed the basis for the first prophylactic vaccines. Variation in the size of open reading frames varies between papillomaviruses; E1, E2, L1 and L2 genes tend to be well conserved between hrHPV types, the remaining genes (E6, E7, E4 and E5) are more variable by genotype.

3.2 Natural history of the disease

In 2-5% of women (>30 years), persistent hrHPV infection leads to the development of pre-invasive cervical lesions, known as cervical intraepithelial neoplasia (CIN) (Figure 3). In cases of CIN with hrHPV clearance, disease regresses spontaneously. Regression rates decrease as CIN grade increases. In hrHPV persistence, molecular carcinogenic processes continue that can lead to CIN progression. The progression to cancer is much less common). In women who develop cervical cancer, around 3200 cases a year in the UK⁷, the median interval between the initial hrHPV infection to invasive disease approximates 10-15 years²⁶. In rare cases, the infection can be more aggressive, with fast progression to cancer.

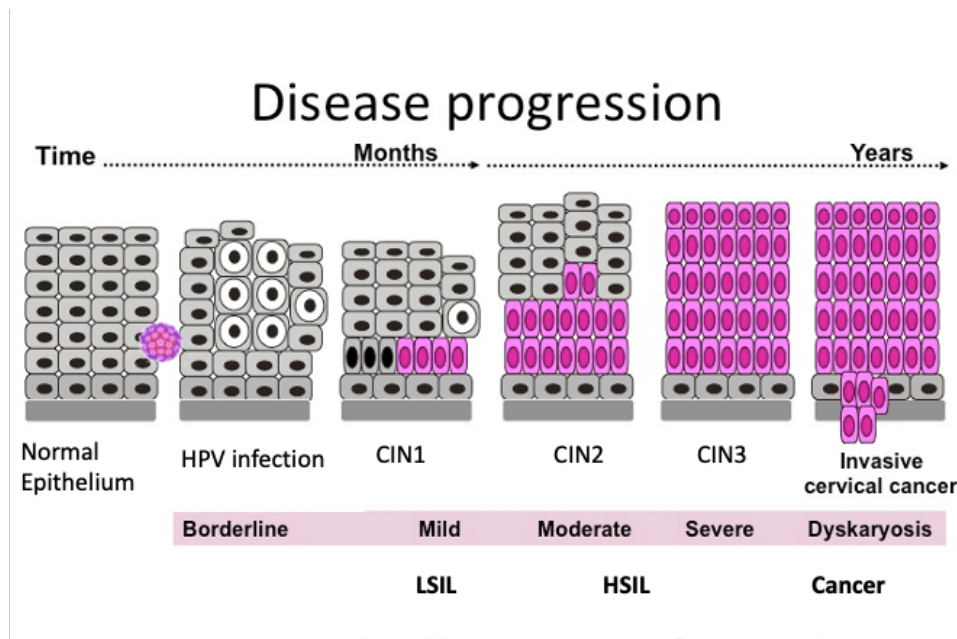


Figure 3. The natural history of cervical carcinogenesis.

Most cervical cancers arise in the transformation zone (Figure 4). The epithelial transformation zone of the cervix is a boundary of metaplastic change in cell type from endocervical columnar epithelium to stratified squamous epithelium. This process of squamous metaplasia is naturally occurring in a woman's lifetime - beginning at puberty where eversion of the endocervix causes exposure to the acidic vaginal pH and local irritation. The resultant squamous metaplasia is immature and particularly vulnerable to hrHPV infection and lesion formation. A similar process of squamous metaplasia also occurs in the anal and tonsillar epithelia.

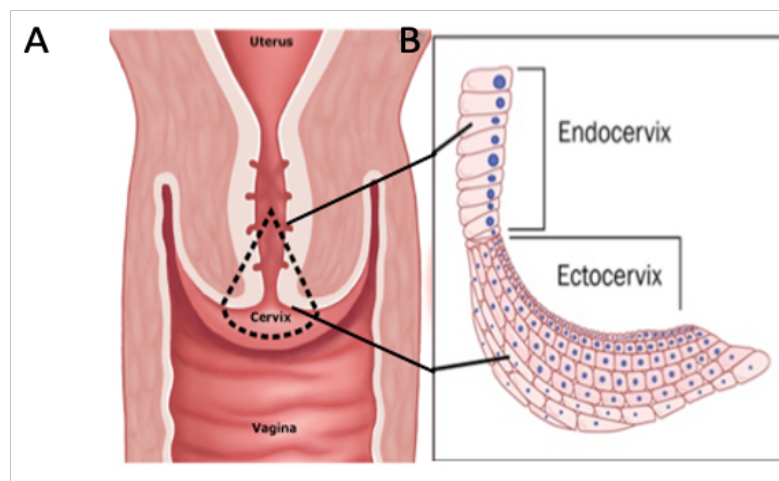


Figure 4. A) Representation of the uterine cervix, and approximate location of the endocervix and ectocervix. B) illustration of the columnar cells of the endocervical epithelia next to the flattened squamous cells of the ectocervical epithelium.

Once the viral episome has reached the basal keratinocyte cells (the last layer of the cervical epithelium) viral DNA replication occurs. The wound healing pathway is thought to drive the spread of infected cells - mediated by E6/E7 proliferation, epithelial cell differentiation allows spread of hrHPV virions from basal layers through suprabasal, to the epithelial surface³⁴. In established infection, new virions in exfoliated cervical cells allow spread of infection to other cells and also transmission. Persistence of infection with or without progression to precancerous lesions, is maintained by hrHPV infection of basal keratinocytes. Frequency of re-infection after hrHPV clearance has been debated, Moscicki et al. demonstrated that in a longitudinal American cohort of 295 unvaccinated women, where clearance was confirmed by a negative hrHPV test, HPV16 redetection rate is 18% at 8.5 years³⁵.

Although the majority of cervical pre-invasive disease arise in the squamous epithelium, infection of the endocervix also occurs and can lead to the development of glandular lesions in 20-30% of cases – namely cervical glandular intraepithelial neoplasia (cGIN) and adenocarcinoma. The endocervix consists of a single layer of columnar cells organised into crypts. Here the epithelial layer is unable to stratify and therefore the papillomavirus cell cycle differs. In columnar cells, hrHPV gene expression deregulation is thought to drive carcinogenesis. As well as being more difficult to detect in screening, adenocarcinoma is thought to be able to undergo a faster neoplastic process – not necessarily requiring a low-grade precursor. It generally has a poorer prognosis, which may reflect in part a delay in diagnosis. Together HPV 16, 18 and 45 are responsible for almost 90% of cases in most populations. Adenocarcinomas first detected in the endocervix may occasionally be non-HPV driven and can represent spread from endometrial cell types.

3.3 Microenvironment of CIN

High-risk HPV infection of basal keratinocytes initiates a cascade of inflammatory and immune responses. While keratinocytes are equipped with immune surveillance factors such as pathogen recognition receptors (PRR), human papillomaviruses have multiple adaptive strategies for immune evasion. In the presence of HPV persistence, this interaction forms the post-infection microenvironment of CIN. HPV-infected keratinocytes initiate transcription and secretion of pro-inflammatory cytokines as part of innate immunity responses, which are partially downregulated by E6 and E7 HPV oncogenes as demonstrated by *in vitro* studies³⁶. E6 and E7 have additionally been shown to recruit DNA methylation enzyme (DNMT1) to methylate promoter regions and therefore induce downregulation of other chemokines such as CXCL14³⁷. It is thought that upregulation of interleukins such as IL-6 by HPV E6, E7 and E2, disrupts normal cell signalling within the immune pathway to facilitate malignant progression³⁸. Langerhans cells are the main antigen presenting cells (APC) found in the cervical epithelium. Downregulation of interferons and other chemokines is thought to prevent recruitment of

CD4⁺ and CD8⁺ T cells by APCs for further adaptive immune responses. HPV E7 and E5 are additionally thought to bind with and disrupt the function of major histocompatibility complex (MHC) class 1 processing and downstream interactions within the endoplasmic reticulum and Golgi apparatus, therefore preventing adequate viral antigen processing for antigen-specific cytotoxicity³⁹. HPV may also downregulate natural killer cell activity, therefore preventing the destruction of virus infected cells⁴⁰. T cell responses are particularly important in epithelial viral infection. In response to antigen-presenting cells, cytotoxic CD8⁺ T cells migrate to sites of infection and CD4⁺ cells activate T helper 1 and 2 subsets. HPV appears to persist despite high levels of T cell infiltration, which is thought to be due to an ability by HPV to disrupt T cell function. There also appears to be an element of metabolic reprogramming in CIN - similar to cervical tumour cells, reduced glutamine concentrations have been observed in high-grade CIN, consistent with excessive cell growth⁴¹. HPV appears to switch cellular metabolism to glycolysis pathways which is likely to promote viral persistence and aid ongoing malignant transformation. Chronic inflammatory processes induced by HPV are likely key to malignant transformation of infected cells. Chronic inflammation causes oxidative stress and DNA damage induce carcinogenesis, while COX-2 inhibition by HPV oncogenes has been shown to increase prostaglandins which are in turn related to cell proliferation and anti-apoptosis⁴². Despite these insights, there is currently no available immunomodulatory treatment for CIN and surgical treatment remains the mainstay for cancer prevention.

3.4 Environmental and behavioural risk factors

Persistent hrHPV infection is a necessary but not sufficient condition for the development of cervical preinvasive disease and cancer. Large observational studies suggest associations between several risk and protective factors that modulate the risk of hrHPV persistence and cervical cancer. The most evidence exists for smoking, long-term hormonal contraceptive use, immunocompromise (including HIV infection) and high parity (more than three pregnancies)^{43,44}, all of which have plausible biological explanations.

Chemicals from cigarette smoke are thought to influence both acquisition and persistence of hrHPV infection along with progression to cancer. Smoking has been shown to mediate innate, adaptive and barrier immune responses to hrHPV infection. Nicotine is detectable in cervical mucous and may modulate local immune markers allowing hrHPV persistence⁴⁵. Additionally, carcinogens in cigarette smoke cause genomic instability through which many cancers are mediated⁴⁶. It has been estimated that the additional risk of smoking results in 21% of cervical cancers in the UK^{45,47}.

Use of oral contraceptives has been linked to increased cervical cancer rates; oestrogens in particular are thought to increase the eversion of the endocervical canal into the vagina, causing more exposure of vulnerable immature cells of the cervical transformation zone to hrHPV infection, and an acidic pH which allows DNA damage⁴⁸. Oestrogen and progesterone exposure also increase cell proliferation at the cervix. Higher parity has been associated with a higher incidence of cervical cancer, which may be related to a higher lifetime hormone exposure or repeated trauma.

Sexual behaviour is thought to influence lifetime risk of cervical cancer; an increased incidence is observed in accordance with earlier sexual debut and increased number of sexual partners, which increases hrHPV exposure⁴⁹. Condom use is only partially effective against hrHPV infection as transmission can also occur from direct contact with anogenital skin¹⁶. Additionally, the role of condoms in assisting clearance of persistent infection is not known, but it is hypothesised that they may aid regression through reduction of the viral load⁵⁰. Studies suggest that co-infection with other sexually transmitted infections, in particular *Chlamydia trachomatis* may increase the acquisition of hrHPV through promotion of a pro-inflammatory environment⁵¹, or an effect on the vaginal pH and cervical epithelial barrier, allowing for hrHPV entry to basal keratinocytes⁵².

It has been reliably demonstrated that in immunocompromised individuals such as in HIV infection, the risk of cervical cancer is higher. Such individuals are invited to annual screening in the UK.

Cervical cancer in England is more common in females living in the most deprived areas, independently of other risk factors; higher incidence is also observed in women of Black ethnicity, with a lower incidence in White Caucasian and Asian females^{53,54}. Worldwide, lower incidence is observed in North Africa and the Middle East, which may be associated with societal practices related to sexual behaviour. These countries also have low rates of other sexually transmitted infections such as HIV.

Due to the nature of proposed risk and protective factors, randomised studies are rare and further research into the causality of associated life course exposures are warranted.

3.5 Heritability and genetic risk

Although infection by hrHPV is common, only a fraction of infections have true carcinogenic potential⁵⁵. Factors affecting risk of progression are largely unknown. There is emerging evidence from large population studies that inherited genetic factors may explain a proportion of risk. Cervical cancer is likely a multi-factorial disease trait characterised by a complex interplay between the host, the virus and vaginal microenvironment, which promotes disease progression in some but not others. Identifying

genetic factors impacting the susceptibility to the disease and its progression could have major clinical applications the progression of hrHPV infection to cervical cancer in some women and not others.

It is likely that most human traits that exhibit variability have a genetic component, however the degree of heritability differs between traits, which reflects the effect of both genetic variation and environmental and behavioural factors which segregate into families⁵⁶. While the study of heritability of cervical cancer has been limited, the best estimate of heritability (defined as the proportion of total phenotypic variation that is attributable to variation in genetic factors i.e. $H^2 = V_G/V_P$) is 27-36%⁵⁷⁻⁵⁹. Initial Swedish family-based studies have reported family aggregation of cervical cancer, but were not able to discriminate between the effect of shared genes and that of shared environmental factors^{58,60}. The first study to support, with strong evidence, a significant genetic risk factor for the development of cervical tumours was published in 2000, used linkage between the Swedish Cancer Registry and the National Family Register⁵⁷ to estimate, in a family-based set-up that genetic variation accounted for 27% of total phenotypic variance. A second study of 2,866 cervical cancer cases and 6,481 controls reported common variant based heritability used a large GWAS and a liability threshold method and genetic relatedness in a non-familial dataset to estimate heritability at 36%⁵⁹.

A number of candidate gene studies have explored genetic regions related to plausible immunological (Human leukocyte antigen (*HLA*)⁶¹ and interleukins^{62,63}) and carcinogenic pathways (altered cell cycle control, DNA repair, apoptosis⁶⁴⁻⁶⁸). The most evidence exists for the *HLA* region and cervical neoplasia including studies as early as 1994 (Apple et al Nature Genetics, 1994 and JNCI 1995; Wang et al J Inf Dis 2001; Cuzick et al Br J Ca 2000; Odunsi et al Mol Med 1995 and Int J Ca 1996) but other many other genes that have been identified have showed inconsistent results that frequently failed to replicate in larger cohorts. The advent of hypothesis-free genome-wide sequencing and association studies (GWAS) has greatly expanded the ability to explore such complex traits. To date, seven such studies have examined genetic variation in cervical precancer or cancer; three included women of European ancestry^{59,69,70}, one of Chinese Han⁷¹ and three of Japanese ancestry⁷²⁻⁷⁴ (Table 1). The most consistently identified allelic variation was at the 6p21.3 locus, within the *HLA* region, where 44 variants have been reported across six studies^{59,69-72,74} (with both protective and risk haplotypes described), followed by the major histocompatibility complex (MHC) class I polypeptide-related sequence A gene (*MICA*) gene in three studies^{59,69,70}, as well as EXOC1 (chr4) and GSDMB (chr17) genes⁷¹.

Table 1. Summary characteristics of previous known genome-wide association studies (GWAS) published in CIN and Cervical Cancer

Study	Country	Genotyping Assay/Array	Study design	Phenotype in discovery set	Genes associated with phenotype
Chen 2014	Sweden	TaqMan of four SNPs	Targeted case-control study	951 cases (827 CIN3, 134 ICC), 1725 controls	HLA-DRB1, HLA-DPB2, MICA
Leo 2017	Australia	Illumina Human660-Quad BeadChips (cases) and Illumina Human610-Quad BeadChips (controls)	GWAS	2866 cases (CIN2-3 1336, ICC 693), 6762 controls	HLA-DQA1, HLA-DRB1, HLA-DQB1, MICA
Chen 2013	Sweden	Illumina HumanOmniExpress BeadChip	GWAS	1075 cases (1008 CIN3, 67 ICC), 4014 controls	HLA-DRB1, HLA-DQA1, HLA-DPB2, MICA
Shi 2013	China	Affymetrix Axiom Genome-Wide CHB1 Array	GWAS	1364 cases, 3028 controls	HLA-DPB2, HLA-DPB1, EXOC1, GSDMB.
Takeuchi 2019	Japan	Unclear	MA of GWAS	2609 cases, 4712 controls	ARRDC3, VSTM2A
Miura 2014	Japan	Affymetrix Genome-Wide Human SNP Array 6.0	GWAS	226 cases, 192 controls	ZNF804B
Masuda 2020	Japan	Illumina Human OmniExpressExome BeadChip, Illumina HumanOmniExpress and HumanExome Bead- Chip		538 cases, 39,556 controls	MHC region, INS-IGF2, SOX9

Abbreviations: MA: meta-analysis.

To date, the largest GWAS in cervical cancer has been performed by Leo et al ⁵⁹(2017), consisting of 2866 cases and 6481 controls. However, 821 cases included CIN2, which is not necessarily a cancer precursor. Large cohorts of genetic data that have been linked to cancer registries, such as the UK Biobank offer the opportunity for further exploration of genetic variation in cervical cancer, to better understand genetic determinants that may influence the persistence of hrHPV infection and risk of cervical oncogenesis⁷⁵.

4. Classification and staging

4.1 CIN/cGIN

Cytology reporting of cervical disease is based on a classification system which evaluates the presence of hrHPV infection and severity of dyskaryosis. Staging systems have evolved since the first Bethesda system devised in 1988. In the UK the most widely accepted cytology classification system has shifted from the NHSCSP/BAC 2013 system which was based on degrees of cellular dyskaryosis, to the Bethesda 2014 which groups pre-invasive lesions atypical squamous cells, low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) (Table 2).

In cervical histopathology, three stages in the disease process of squamous pre-invasive lesions are recognised, these are usually asymptomatic but can be detected through cervical screening⁷⁶. The definition of CIN (which is based on the number of thirds of the epithelium that are affected by diseased cells) is reliant on a full-thickness specimen of the transformation zone. CIN1 is defined as disease affecting the basal third of the epithelium only and is now recognised as a state of continuous viral replication rather than a true invasive precursor, CIN2 affects two thirds of the epithelium, and CIN3 a state with the full epithelium affected and over 30% risk for disruption of the basal membrane and stromal invasion⁷⁷. Invasive cervical cancer represents disease which has broken through the basement membrane of the epithelium, allowing access to lymph and blood vessels for metastatic spread.

The pre-invasive precursor CIN has been traditionally categorised in 3 grades: CIN1, CIN2 and CIN3. In 2012, the American Society for Colposcopy and cervical pathology (ASCCP) together with the College of American Pathologists, established the Lower Anogenital Squamous Terminology (LAST) Project in order to develop a uniform, two-tier system to describe all hrHPV-associated squamous disease across anogenital tissues⁷⁸. The recommended terminology splits squamous intraepithelial lesions (SIL) into low- or high i.e. LSIL (low-grade squamous intraepithelial neoplasia) and HSIL (high-grade squamous intraepithelial neoplasia) similar to the Bethesda 2014 system, but with the additional aim of highlighting the difficulties in characterising CIN2 lesions and the poor intra-observer agreement of pathologists when classifying CIN2⁷⁹. The LAST project considers whether CIN2 is really a separate biological entity with a valid clinical meaning, or simply an underdetermined diagnosis between a low-grade and high-grade lesion; they suggest the use of additional biomarkers such as P16^{INK41} immunohistochemical staining to improve consistency of histopathological interpretation between low- and high-grade lesions⁸⁰. In many European screening settings, both terminologies are

reported, such as ‘HSIL (CIN2)’ or ‘HSIL (CIN3)’. Additionally, the CIN classification is still commonly used for research purposes and equivalence between terminologies is described in Table 2.

Table 2. Summary of cytology and histology classification systems in use by NHSCSP for squamous and glandular lesions.

<u>Cytology</u>		<u>Histology</u>	
NHSCSP/BAC 2013	Bethesda 2014	NHSCSP 2012	LAST 2012 (ASCCP and CAP)
Squamous cell (ectocervix)			
Borderline changes in squamous cells	ASC-US ASC-H		
Low-grade dyskaryosis	LSIL	CIN1	LSIL
High-grade dyskaryosis (moderate)	HSIL	CIN2	HSIL (CIN2)
High-grade dyskaryosis (severe)	HSIL	CIN3	HSIL (CIN3)
SCC	SCC	SCC	SCC
Glandular (endocervix)			
Borderline changes in endocervix	AGC NOS		
Glandular neoplasia	Endocervical	L-cGIN	NA
Endocervical type	Endometrial		
Non-cervical	Glandular		
	AGC favours neoplastic	H-cGIN	NA
	Endocervical		
	Glandular		
	Endocervical AIS		
	Adenocarcinoma	ACC	NA
	Endocervical		
	Endometrial		
	Extrauterine		
	NOS		

Abbreviations: ACC: adenocarcinoma; AGC: Atypical glandular cells; NOS: atypical glandular cells, not otherwise specified; AIS: adenocarcinoma in situ ASCUS: atypical squamous cells of undetermined significance; ASC-H: atypical squamous cells cannot exclude HSIL; ASCCP: American Society for Colposcopy and Cervical Pathology; CAP: College of American Pathologists; CIN: cervical intra-epithelial neoplasia; L-CGIN: low grade cervical glandular intraepithelial neoplasia; H-CGIN: high grade cervical glandular intraepithelial neoplasia; LAST: Lower Anogenital Squamous Terminology; LSIL: low-grade squamous intra-epithelial lesion; HSIL: high-grade squamous intra-epithelial lesion; SCC: squamous cell carcinoma.

4.2 Cervical cancer

FIGO system

Cervical cancer is widely staged according to the FIGO system. Most recently updated in 2018, the FIGO staging ranges from stage I-IV with squamous cell carcinoma representing over 90% of cases⁴. Briefly, stage IA includes invasive carcinoma diagnosed only by microscopy, with a maximum depth of invasion <5mm; stage IB is invasive carcinoma with a measured deepest invasion of greater or equal to 5mm, but limited to the cervix uteri; stage IIA includes tumours involving limited to the upper two-thirds of the vagina without parametrial involvement whereas stage IIB includes parametrial involvement but not up to the pelvic wall; stage IIIA includes the lower third of the vagina but not the pelvic wall, while IIB extends to the pelvic side wall and/or results in hydronephrosis or causes non-functioning kidney, IIIC involves the pelvic and/or paraaortic lymph nodes; stage IVA is disease which has spread to adjacent pelvic organs and stage IVB includes spread to distant organs. These stages include further subclassifications, which along with lymphovascular space involvement (LVSI) is used to guide management (Table 3). Surgery is suitable for early stages, while in later stages primary chemoradiotherapy is recommended⁸¹. Where radiotherapy is not available, neoadjuvant chemotherapy may be considered but the outcome is unclear⁸². In the absence of organised screening, many of women with tumours present with symptoms and therefore locally advanced cervical cancer (LACC) or advanced cancer at diagnosis i.e. FIGO 2018 stage IB3/IA2/IIB/IIIA-B/IVA⁴, which is associated with a poorer overall survival.

Table 3: FIGO staging of cancer of the uterine cervix (2018)⁴

Stage	Description
I	The carcinoma is strictly confined to the cervix (extension to the uterine corpus should be disregarded)
IA	Invasive carcinoma that can be diagnosed only by microscopy, with maximum depth of invasion <5 mm ^a
IA1	Measured stromal invasion <3 mm in depth
IA2	Measured stromal invasion ≥3 mm and <5 mm in depth
IB	Invasive carcinoma with measured deepest invasion ≥5 mm (greater than Stage IA), lesion limited to the cervix uteri ^b
IB1	Invasive carcinoma ≥5 mm depth of stromal invasion, and <2 cm in greatest dimension
IB2	Invasive carcinoma ≥2 cm and <4 cm in greatest dimension
IB3	Invasive carcinoma ≥4 cm in greatest dimension
II	The carcinoma invades beyond the uterus, but has not extended onto the lower third of the vagina or to the pelvic wall
IIA	Involvement limited to the upper two-thirds of the vagina without parametrial involvement
IIA1	Invasive carcinoma <4 cm in greatest dimension
IIA2	Invasive carcinoma ≥4 cm in greatest dimension
IIB	With parametrial involvement but not up to the pelvic wall
III	The carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or nonfunctioning kidney and/or involves pelvic and/or para-aortic lymph nodes ^c
IIIA	The carcinoma involves the lower third of the vagina, with no extension to the pelvic wall
IIIB	Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney (unless known to be due to another cause)
IIIC	Involvement of pelvic and/or para-aortic lymph nodes, irrespective of tumor size and extent (with r and p notations) ^c
IIIC1	Pelvic lymph node metastasis only
IIIC2	Para-aortic lymph node metastasis
IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. (A bullous edema, as such, does not permit a case to be allotted to Stage IV)
IVA	Spread to adjacent pelvic organs
IVB	Spread to distant organs

When in doubt, the lower staging should be assigned.

^a*Imaging and pathology can be used, where available, to supplement clinical findings with respect to tumor size and extent, in all stages.*

^b*The involvement of vascular/lymphatic spaces does not change the staging. The lateral extent of the lesion is no longer considered.*

^c*Adding notation of r (imaging) and P (pathology) to indicate the findings that are used to allocate the case to Stage IIIC.*

5. Prevention and early detection

5.1 HPV vaccination

HPV vaccines represent a major public health breakthrough and immunisation programmes of prepubescent girls (and some boys) have been implemented across many developed countries. Methods of delivery, uptake, gender-policy and monitoring systems vary greatly, as does the choice of vaccines and dosing schedule.

Natural infection with hrHPV induces a response from epithelial antigen-presenting cells (APCs) and antigen-producing B-cells⁸³. Interaction with cytotoxic T-cells aims to restrict the infection to the epithelium, while T-helper cells enhance the antigen response. The cell-mediated immune response becomes detectable within weeks of infection, whereas seroconversion takes 8-9 months. Resultant

antibody concentrations after natural hrHPV infection are low and only roughly 50-70% of women with incident hrHPV infection seroconvert. Even in the presence of seroconversion following natural infection, there is minimal protection against new HPV infection by the same type⁸⁴.

Currently available vaccines (Table 4) are comprised of virus-like particles (VLPs), which are based upon the L1 viral capsid protein which self-assemble into VLPs. VLPs do not contain the viral genome and therefore are not infectious but are highly immunogenic and can generate high levels of anti-HPV L1 IgG antibodies against included subtypes if administered before the age of 15^{85,86}. The adjuvant used in the vaccines is known to further enhance antibody production and generation of B memory cells⁸⁷. There is emerging evidence that vaccines offer considerable cross-protection against phylogenetically similar HPV types⁸⁸.

The high prevalence and oncogenicity of HPV 16 and 18 led to their selection for the first prophylactic vaccines, with addition of types 6 and 11 to protect against genital warts (Table 4). In the UK, the quadrivalent Gardasil® vaccine is now freely offered to girls and boys aged 12-13, men who have sex with men (aged under 45), sex workers and HIV positive individuals. Gardasil9® is also licensed and available in many countries.

Table 4. Types of currently licensed HPV vaccines.

	Bivalent	Quadrivalent	Nonavalent
Manufacturer	GlaxoSmithKline Cervarix®	Merck	Merck
Trade Name		Gardasil®	Gardasil9®
Vaccine type	HPV L1 VLP	HPV L1 VLP	HPV L1 VLP
HPV types	16, 18	6, 11, 16, 18	6, 11, 16, 18, 31, 33, 45, 52, 58
Age range	FDA: 9-25y (females) EMA: from 9y without upper limit (females-males); recommended up to 25y	FDA: 9-26y (females-males) EMA: from 9y without upper limit (females-males); recommended up to 45y	FDA: 9-45y (females-males) EMA: from 9y without upper limit (females-males); recommended up to 26y
Administration*	≥15y: 0, 1, 6 <15y: 0, 5-13	≥14y: 0, 2, 6 <14y: 0, 6-12	≥15y: 0, 2, 6 <15y: 0, 6-12
Contraindications	Severe allergic reaction to previous dose	Hypersensitivity to yeast; severe allergic reaction to previous dose	Hypersensitivity to yeast; severe allergic reaction to previous dose

* The 2-dose schedule for girls or boys <15y is FDA-approved only for Gardasil9

Abbreviations: *EMA*: European medicines agency; *FDA*: food and drug administration; *VLP*: virus-like particle

Bi- and quadrivalent vaccines are proven to be very safe⁸⁹, with an effective long-term immune protection, and some cross-protection for non-vaccine types. Some of the largest RCTs are the PATRICIA trial^{90,91} (bivalent vaccine vs Hepatitis A vaccine control), the FUTURE I⁹² and FUTURE II⁹³ trials (quadrivalent vaccine vs placebo) and Broad Spectrum HPV Vaccine Study⁹⁴ (nonavalent vaccine vs quadrivalent vaccine). These trials recruited hrHPV-naïve women aged 15 to 26 (identified via a negative hrHPV DNA test and seronegativity at baseline for hrHPV). Long term follow-up show efficacy above 95% and protection 12 years after vaccination. Side-effects are similar to other vaccines and include predominantly injection site local reactions, and mild to moderate systemic effects. HPV vaccines are considered safe for breastfeeding mothers⁹⁵. Consistent public health promotion will be necessary to maintain existing coverage in existing countries where vaccination is offered. At 80% vaccine coverage, it is estimated that herd immunity can reduce HPV 16 prevalence by 93% in females and 83% in males within 70 years⁹⁶. For the majority of the world's population the HPV vaccine is not routinely available or affordable; international effort and funding is still needed to provide global protection from HPV-related disease and move towards elimination.

Recommendation was for two vaccine doses^{97,98} however emerging evidence suggests that in boys and girls younger than 15 years, only one dose of Gardasil9 may be required (JCVI ref). Current recommendations promote a prepubescent vaccination programme at age 13, however the stronger immune response during childhood and potential for increased coverage through established childhood vaccination regimes, justifies further investigation of vaccinating younger children⁹⁹. Trials investigating vaccination under 9 years are underway¹⁰⁰.

Vaccination may be partially effective in sexually active individuals with previous but cleared natural infections, as the immunity level induced by vaccination may offer greater protection against reinfection (although cost-effectiveness is unclear). A randomised-control trial NOVEL is examining the value of HPV vaccination in preventing disease recurrence after local treatment for CIN¹⁰¹.

5.2 Cervical Screening Programmes

5.2.1 The role of cervical screening

Where implemented, organised screening programmes have substantially reduced the incidence and mortality from cervical cancer. In the UK, incidence rates have dropped 44% since the early 1970s; while introduction of the 'call and recall' system in 1988 led mortality rates to drop from 7.1 to 2.4 per 100 000 in 2007. The largest benefits of screening are seen when this is centrally organised, population-

wide, and has high coverage. Most new cases of cervical cancer are found in women non-compliant with screening (60%), while 10% are in women managed inappropriately. One-third of cancer cases (30%) are found in women that attended screening and received a negative result secondary to incorrect sampling or analysis of results¹⁰². Maintaining a well-developed screening organisation is expensive and requires substantial infrastructure and quality assurance programmes. These challenges are obvious when reviewing worldwide statistics. Within Europe, the majority of cases (almost 60%) are still diagnosed in countries of Eastern Europe where screening programmes are not implemented. To address this the World Health Organisation (WHO) have announced a call to action including recommendations that all countries should initially aim for a minimum standard of twice-lifetime screening, particularly targeting the highest risk age-group (women aged 30-49) and adopting a screen-and-treat approach as an acceptable and realistic first step where call-recall are currently infeasible⁸¹. Although the introduction of HPV vaccination is anticipated to greatly reduce the incidence and mortality of cervical cancer, screening programmes have a large role to play in the prevention of disease until a substantial expansion of vaccination is observed.

5.2.2 Primary screening

Cytology

Exfoliated cytology has been the main approach to cervical screening since the introduction of organised screening programmes in the 1980's. Traditionally the cells were taken by a spatula or brush and smeared on to glass slides, then stained, as first described by Papanicolaou in 1943¹⁰³. Slides are then examined by a trained cytopathologist. This process was replaced in most developed countries with a liquid-based cytology (LBC) approach, which involved the collection of cells but a plastic brush and direct suspension into a fixative solution. This development leads to a uniform spread of epithelial cells making it possible to remove red cells, white cells and debris, thereby improving efficiency and quality over conventional cervical cytology. LBC has been shown to reduce the rate of unsatisfactory smears from 9.1% to 1.6% in some settings, while the solution can be used for reflex testing for hrHPV test and other markers¹⁰⁴. However, cytology-based screening however, has inherent limitations including a low sensitivity - which ranges from 50-70% depending on the quality of the cytopathological laboratory, resulting in 20–25% of false-negative results. Meanwhile, intra-observer agreement has been recorded to be low in many laboratories. While the majority of cervical cancers are squamous, glandular cell abnormalities represent 4% of abnormal smears¹⁰⁵. An additional limitation of cytology is a decreased ability to detect glandular lesions located within endocervical glands because the glands are more difficult to reach and do not shed epithelial cells on exfoliation, these cancers are therefore associated with a poorer prognosis in part due to detection at a later stage¹⁰⁶. Training and

maintaining quality-assured cytopathologists and laboratories is expensive and a logistical challenge for many national screening programmes.

HPV testing

Concerted global efforts to study HPV over the last two decades has led to the development of high-accuracy HPV assays for the detection of viral DNA and mRNA¹⁰⁷⁻¹⁰⁹. The introduction of hrHPV as an add-on test to cytology was found to increase the accuracy of cervical screening, as well as reducing inter- and intra-observer variability. Following on from this, randomised head-to-head trials began comparing hrHPV to cytology, as the primary screening test. Follow-up of four European randomised controlled trials provided Level A evidence to suggest that hrHPV-based screening offers a 60-70% greater protection against invasive cervical cancer when compared to cytology in women over 30¹¹⁰⁻¹¹². Even greater protection was seen for glandular disease¹¹³. As a result, hrHPV testing has been introduced to many national screening programmes across Europe and the USA, including the UK (Figure 5). Improved accuracy of hrHPV-based screening may permit the prolongation of screening intervals from three, to five years following a negative result¹¹⁴. Other roles for hrHPV tests include as a ‘test of cure’, where hrHPV testing has been shown to predict more accurately the risk of treatment failure than previous methods such as margin involvement¹¹⁵ and cytology. Additionally, the role of hrHPV testing in vaginal self-sampling for non-responders or areas of poor access to healthcare is increasingly being considered; self-sampling has been found to have a similar sensitivity to physician samples, although with a slightly reduced specificity^{116,117}. HrHPV testing also necessitates less training for quality assurance allowing easier expansion to lower resource settings¹¹⁸. Laboratory trials of other molecular markers, such as methylation tests, have also begun¹¹⁹⁻¹²¹.

Despite its superiority over cytology¹²², hrHPV testing has a modest specificity and positive predictive value, while the HPV point-prevalence in the UK screened population is as high as 15% and >30% in women aged under 30¹²³. The high prevalence of HPV in women under 30 means the trials did not support its use for primary screening in this age group. hrHPV testing is unable to differentiate transient hrHPV infections from those with true carcinogenic potential. As a result, an add on test is required in most screening settings; in the field of cervical screening this is referred to as a triage test, meaning women identified as hrHPV positive in primary testing have a second test to determine whether they need referral to colposcopy; this is ideally performed on the same sample. Triage tests are required to select the women who would benefit from colposcopic referrals and reduce over investigation of women with transient hrHPV infections or low-grade lesions, and the associated anxiety and potential over-intervention^{124,125}.

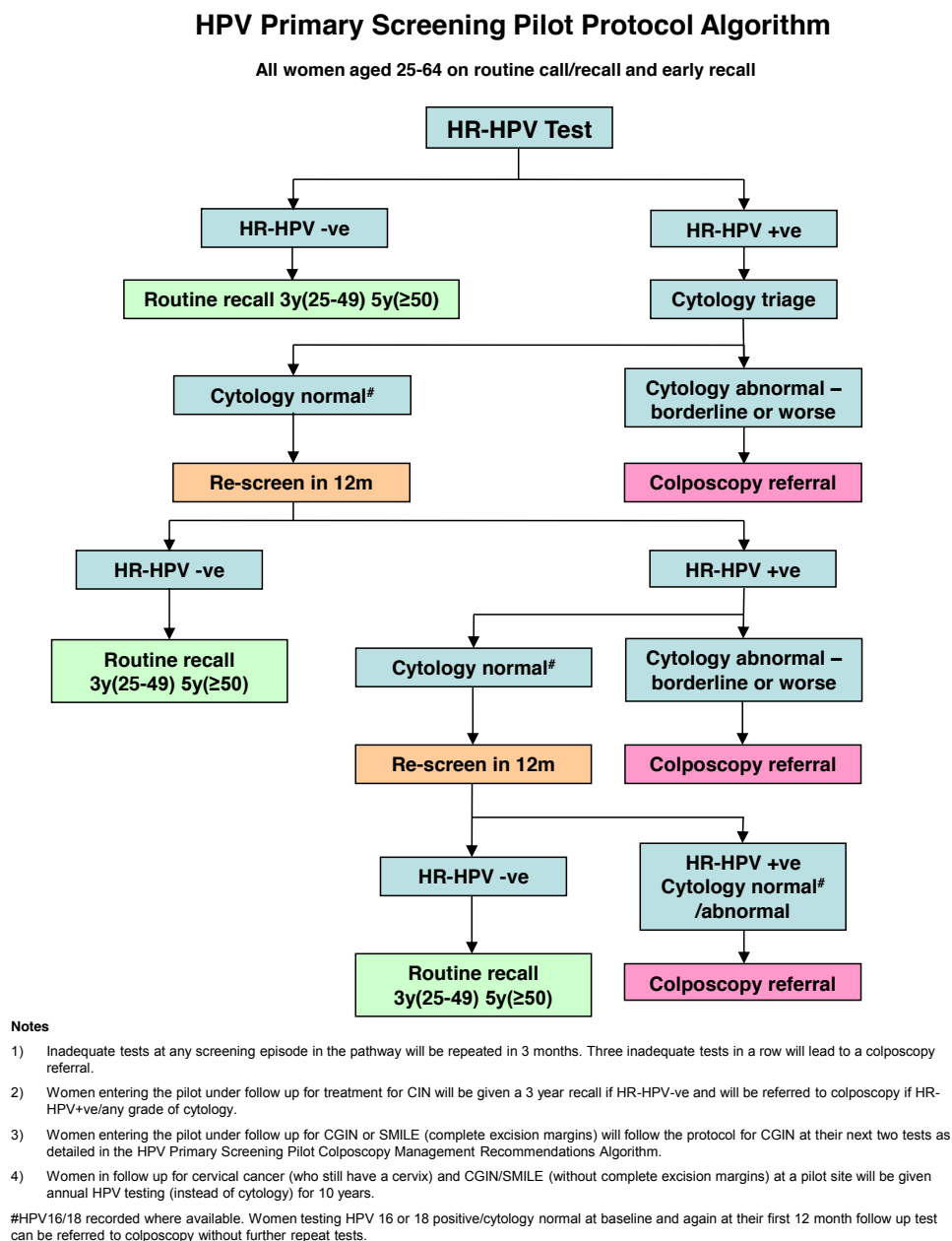


Figure 5. UKNHSCSP cervical screening algorithm with HPV primary screening and cytology triage introduced in 2019. Source: Public Health England 2016

5.2.3 Triage of HPV positive women

Reflex Cytology

While the best policy for triage of hrHPV-positive women remains unclear, the most evidence currently exists for a combination of hrHPV testing and cytological triage¹²⁶⁻¹²⁸. In many countries where hrHPV testing has been introduced, cytology is used as a triage test in part due to existing safety evidence but also due to pre-existing infrastructure (Figure 5). Although the sensitivity of cytology in detecting CIN3 or cancer may improve with the knowledge of hrHPV testing, accuracy is still modest at best, (sensitivity of 50-70%; specificity of 78-86%) and specificity may drop¹²⁹. Molecular triage markers have the potential to improve the accuracy, efficiency, and coverage of cervical screening. Current leading triage tests under investigation include hrHPV genotyping, hrHPV and human methylation, viral load and viral protein detection (e.g., E6 protein) and P16/Ki-67 immunostaining¹³⁰.

hrHPV genotyping

The risk of cervical cancer varies substantially by hrHPV type, with HPV16 and 18 causing the majority of cancers, followed by other types with some geographical variation. Currently in the ASCCP screening guidelines for the US, the triage test depends on the result of the primary hrHPV screen, where hrHPV-positive women receive HPV16/18 genotyping for triage. Resultantly any woman positive for HPV16/18 is referred immediately to colposcopy, while women with other hrHPV types have cervical cytology triage, and are referred to colposcopy if ASCUS or worse, or re-screened in 12 months if normal cytology. HPV16 prevalence in high-grade lesions is estimated at 50%. However, the majority of hrHPV infections are transient, and therefore the incidence of cervical lesions in women testing positive for HPV16 and 18 is low, with a PPV of 15-20%¹³¹. This increases referrals and causes challenges for colposcopy services, increasing both the number of women who need to be seen and the number of women without visible cervical lesions. Other difficulties include that the role of HPV16 decreases in older women and the less accurate performance of HPV18 as a diagnostic marker, due to its association with adenocarcinomas in the endocervix and their slower natural history. In the presence of increased vaccination coverage for HPV16 and 18, HPV16/18 genotyping may also become a less useful tool.

5.2.3.3 Host DNA methylation

DNA methylation is a regulatory chemical process, which occurs at specific methylation sites in the DNA, called methylation 5-cytosine-phosphate-guanine-3 (CpG) sites, to influence gene expression. Such sites occur where a cytosine is followed by a guanine nucleotide in the 5'-3' direction and are scattered throughout the human genome, although promoter and enhancer regions are particularly

enriched. The degree of methylation is reflected by the quantification of the percentage of cytosines with a covalently added methyl-group at individual CpG sites. Associations between DNA methylation change and cancer progression have been reported for multiple cancer types.¹³²⁻¹³⁴, and is thought to reflect ongoing carcinogenesis through processes such as loss of tumour suppressor gene function and other aberrant cell cycle and cellular growth controls, including genes related to cell cycle, apoptosis, development, cell adhesion and cellular signalling.

There is increasing evidence that changes in human (host) DNA methylation occurs during carcinogenesis, and that such changes may serve as a potential diagnostic marker. However the strength of the association, and most discriminatory epigenetic sites and diagnostic accuracy are yet to be determined¹³⁵.

The majority of studies point towards an overall gain in methylation associated with progression of cervical disease towards cancer. While some argue that distinctly different cellular pathways appear to be hypo or hyper-methylated, where hypomethylated genes were related to the immune system response, and hypermethylated genes were involved in the biological processes of cell development, cell differentiation, and transcriptional regulation. Additionally in a genome-wide methylation assay it was noted that hypermethylated CpG sites were predominantly located in CpG islands and hypomethylated CpG sites in the CGI shores¹³⁶.

Several previous targeted epigenetic studies have identified CpG sites as candidate markers of cervical disease progression such as *CADM1*, *EPB41L3*, *FAM19A4*, *PAX1*¹³⁷, and *SOX1* genes^{135,138-140}. To date limited epigenome-wide exploration has been performed in cervical cancer, Verlaet (2017) *et al*¹⁴¹ examined a limited number of samples (15 cell lines and 25 tissue samples) and detected 3 additional candidate genes (*GHSR*, *SST* and *ZIC1*; $P < 0.0005$) with validation.

Genome-wide studies have expanded the coverage of CpG sites. Two studies explored the association of CpG sites on the 450k arrays, while one study has used the newer 850k (EPIC) array¹⁴².

The majority of previous studies focussing on CIN only, and it is likely that the majority of methylation markers associated with high-grade cervical phenotypes remain unknown. Increased coverage could uncover a greater proportion of the epigenetic change ongoing in cervical carcinogenesis and identify novel markers for use in screening.

The study of DNA methylation has been performed across a wide range of sample types including tissue, vaginal or cervical swabs and LBC¹⁴³. For use as triage marker then ideally methylation testing would be performed on residual LBC samples used for primary hrHPV testing.

It is likely that increased coverage of the epigenome, including greater exploration of cancer samples, could allow for novel associations, as have been found in other cancers including breast and ovarian¹³²⁻¹³⁴. However, larger studies in cervical cancer are needed to detect novel associations at EWAS level significance which will withstand validation ($P < 5 \times 10^{-8}$).

To date, there are no prognostic biomarkers available for cervical cancer, it is thought that DNA methylation markers may allow for enhanced stratification of CIN2 lesions into those which may progress or regress. It is thought that methylation changes, in the presence of persistent hrHPV infection and cervical carcinogenesis, may occur in advance of visible cellular changes detectable at cytology or histology. Several longitudinal studies have demonstrated that a negative methylation test in hrHPV positive women provides a low risk of cervical cancer^{119,144}.

HPV DNA methylation

As well as host factors, a number of viral factors have been associated with hrHPV viral persistence versus clearance, and high-grade CIN or cancer, including hrHPV genotype and hrHPV viral load¹⁴⁵, but also genetic and epigenetic variation within HPV genotypes^{143,146}. Rapid developments in sequencing technologies have enhanced scientific ability to explore the hrHPV epigenome, together with its host interactions. Consequently, the number of studies investigating the role of hrHPV DNA methylation in disease progression has increased in recent years, the most comprehensive of which suggest an increase in methylation by disease grade in common high-risk HPV types^{139,147,148}.

Although the mechanism by which methylation is linked to cervical carcinogenesis is still incompletely understood, it is established that some viruses can find ways to adapt different tactics to regulate expression of their genes through modulation of DNA methylation. Potentially hrHPV may silence gene expression in order to allow for a persistent infection that evades the host immune defence¹⁴⁹.

Although results are encouraging, variation in measurement and reporting of methylation has made comparisons difficult. HPV is a dsDNA virus with a circular genome. CpG are scattered throughout HPV viral genomes (Figure 6), with the number of CpGs varies by HPV genotype with HPV16 containing 112 CpGs¹⁴⁶. Studies examining genome-wide HPV methylation have thus far been limited and methylation levels appear to vary by HPV genotype, gene (L1, L2, E2-4, URR) and even amongst adjacent CpG sites. One study reported that high compared to low viral methylation across several HPV

types, increased the risk of CIN3 by 50 times ($OR=52, 4.0-670$)¹⁵⁰. Several small longitudinal studies have also demonstrated that DNA methylation in pre-diagnostic samples, may predict in advance the development of high-grade CIN and cancer. One of the largest longitudinal cohorts from Guanacaste, Costa Rica¹⁵⁰, demonstrated that high methylation in L1/L2 of 72 pre-diagnostic specimens was associated with increased risk of future CIN2+, with an OR of up to 9.3 (2.3-45.1)^{150,151}. Niyazi et al¹⁵² found similar results for L1 methylation in 145 women infected with HPV16, with an OR of up to 9.9 (3.6-27.4). While DNA methylome analysis in other cancers has revealed methylation pan-cancer signals up to 5 years before diagnosis, suggesting potential applications to cancer surveillance¹⁵³.

To date, current evidence has not permitted analysis of the comparative performance of different hrHPV methylation sites¹⁴⁰. Differences in case definitions and endpoints (e.g. cytology vs. histopathology, CIN2 vs. CIN3) further complicate comparisons and interpretation of the literature. Further work is needed to determine the diagnostic accuracy of hrHPV methylation in detecting high-grade pre-invasive disease in hrHPV positive women and the potential of hrHPV methylation pre-diagnosis, to detect future high-grade CIN or cancer.

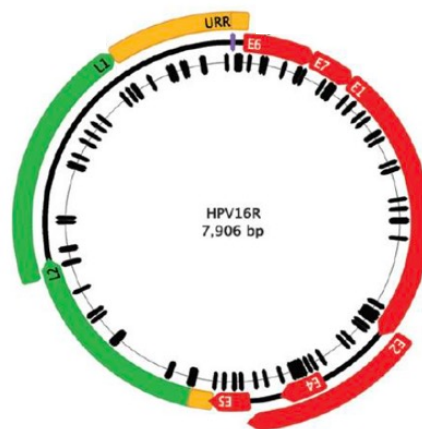


Figure 6. HPV16 genes (coloured bars) and CpG sites (black hash marks)

Biomolecular markers, such as DNA methylation testing, that go beyond the histological classification of the thirds of the epithelium occupied, could give a full molecular picture of cervical carcinogenesis which is a vital step towards personalised treatment.

Viral load

Viral load is a promising option for triage. Several studies demonstrate a strong association between viral load and high-grade disease, particularly for HPV16¹⁵⁴⁻¹⁵⁶. This field however requires further investigation, including studies to evaluate the viral load in the context of number of cells.

P16^{INK4a} immunostaining

P16/ki67 or p16INK4a protein immunostaining is the only molecular marker, other than hrHPV testing, currently used in clinical practice worldwide¹⁵⁷. Mostly this has been used as an adjunct to histology, as a negative predictor of high-grade disease in equivocal cases, especially to prevent misclassification between CIN1 and CIN2, in cases where morphology is uncertain. As high-grade histology samples are more likely to stain positive for P16/ki67 protein¹⁵⁸ this may be a useful triage tool in hrHPV positive women. One study showed that if only hrHPV and p16INK4a immunostaining positive women were referred to colposcopy, the referral rate would be similar to that of cytology triage with a 53% increase in detection of CIN2 or worse¹⁵⁹. More recently other studies have applied the p16INK4a/Ki-67 dual staining technology (CINtec plus, Ventana) and shown that dual staining is more sensitive than cytology in detecting CIN2 or worse in hrHPV-positive women^{160,161}. However, the additional training and costs of this testing needs to be taken into consideration¹⁶². While P16INK4a has been adopted in the US as an adjunct to histology, in the ASCCP guidelines it is stated that P16 should not be used to upgrade a histological lesion from CIN1 to CIN2 where P16 is positive, as this has the potential to overcall disease¹⁶³. In the UK, the current consensus is that P16INK4a should not be used in standard practice as an adjunct, more studies are needed to clarify its role as a triage test after hrHPV primary screening.

5.3 Treatment of CIN and reproductive consequences

Cervical cancer is preventable in the majority of cases through detection and local excision of the precursor, cervical intra-epithelial neoplasia (CIN). Prophylactic vaccination in prepubertal boys and girls is expected to substantially reduce the number of high-grade CIN cases and resultantly the number of cervical treatments required¹⁶⁴. However, numbers currently remain high. In the UK, over 5 million women are invited for screening every year. In England alone almost 23,000 local cervical treatments take place annually¹⁶⁵.

The majority of hrHPV associated disease is treated by local surgical excision. Local treatment for CIN removes or destroys a cone-shaped part of the cervix that contains the transformation zone (TZ), where abnormal cells reside, and has high success rates in reducing risk of cancer (Figure 1)¹⁶⁶. Excisional treatments include Large Loop Excision of the Transformation Zone (LLETZ), also known as Loop Electrosurgical Excisional Procedure (LEEP), needle excision or straight wire excision (NETZ) or

SWETZ), laser conisation (LC) or cold-knife conisation (CKC). Ablative techniques include laser ablation (LA), cold coagulation (CC) and cryotherapy (CT). be used as a triage for conservative management particularly in the under 30 age group¹⁶⁷. Choice of treatment varies in different countries. Excisional treatments are commonly used in preference to destructive techniques in most settings. Excision is preferred as it permits histological assessment of the entire transformation zone (TZ), allowing assessment of length of excision and the presence of residual disease at the excision margins. High-grade CIN is treated most commonly by Large Loop Excision of the Transformation Zone (LLETZ) in the UK, which is frequently carried out under local anaesthetic in colposcopy clinics.

The risk of progression to cancer must be balanced with the reproductive consequences. Local excision has been found to be associated with increased risk of reproductive morbidity in subsequent pregnancies^{168-171 168,172}. A meta-analysis of 15 studies by Kyrgiou *et al.* in 2014^{170,173}, examined fertility outcomes after treatment and concluded there was no evidence to suggest that local CIN treatment adversely impacts on the ability to conceive¹⁷⁴. However, the limited published data did not permit stratification of the risk according to the length of the cone and the number of treatments¹⁷⁰. The impact of repeat and large conisation on fertility outcomes remains unclear. The impact of treatment on miscarriage and pre-term birth however is more convincing. A systematic review and meta-analysis by Kyrgiou *et al.* in 2014¹⁷⁰, and subsequent Cochrane review¹⁷³ found no difference in the first trimester miscarriage rates (less than 12 weeks gestation) (four studies; 9.8% v 8.4%, RR 1.16, 95% CI 0.80-1.69) and overall (less than 24 weeks of gestation) (10 studies; 4.6% v 2.8%, RR 1.04, 95% CI 0.90-1.21) in treated versus untreated women (Figure 2). However, treatment significantly increased the risk of second trimester miscarriage, when compared to untreated reference populations (8 studies; 1.6% versus 0.4%; RR 2.60, 95% CI 1.45–4.67). The same meta-analysis¹⁷⁰ also reported higher rates of ectopic pregnancy (6 studies; 1.6% versus 0.8%; RR 1.89, 95% CI 1.50–2.39) and termination of pregnancy (12.2% versus 7.4%; RR 1.71, 95% CI 1.31–2.22) in treated women when compared to untreated controls.

The first meta-analysis linking an increased risk of preterm birth after excisional cervical treatment was published in 2006 and included 27 retrospective cohort studies¹⁶⁸. This study reported that treatment with excision increased preterm birth rates (defined as gestation less than 37 weeks of gestation) (CKC versus. no treatment: 14% versus 5%; RR 2.59, 95% CI 1.80–3.72; LLETZ versus no treatment: 11% versus 7%, RR 1.70, 95% CI 1.24–2.35). The risk of infants being born with low birthweight (defined as weight less than 2500g) and preterm prelabour rupture of membranes (pPROM) were also found to be increased after local excisional techniques. Laser ablation did not increase the risk, although the number of studies was small¹⁷⁵.

A subsequent meta-analysis of 20 studies was the first to report that the frequency and severity of these complications increased with methods that are known to remove large amounts of cervical tissue¹⁶⁹. After CKC, women were three times more likely to deliver before 32–34 weeks of gestation (severe prematurity), and more than five times more likely to deliver before 28–30 weeks of gestation (extreme prematurity) when compared to no treatment (4.6% versus 1.6%; RR 2.78, 95% CI 1.72–4.51; and 4.2% versus 0.8%; RR 5.33, 95% CI 1.63–17.40 respectively). Perinatal mortality was also significantly higher after CKC (4.3% versus 0.5%; RR 2.87, 95% CI 1.42–5.81). Severe adverse outcomes were not increased after LLETZ or laser ablation, where length of excision is generally less^{175,176}. The update of this meta-analysis published a decade later in 2016¹⁷² of 71 studies (6 338 982 women) and a Cochrane review published in 2017¹⁷¹ confirmed the above findings with very similar results; treated women were found to be at an increased risk of preterm birth when compared to untreated women (10.7% versus 5.4%, RR 1.78, 95% CI 1.60-1.98).

The depth of excisional treatment is important for the subsequent risk of preterm birth, cone depths exceeding 10 mm in length increased the risk of preterm (RR 2.61, 95% CI 1.28–5.34), while less deep excisions did not¹⁶⁸. More specifically, a relative increase in risk of preterm birth when compared to untreated of 1.54 (95% CI 1.09-2.18) for up to 10mm, 1.93 (95% CI 1.62-2.31) for more than 10mm, 2.77 (95% CI 1.95-3.93) for more than 15mm and 4.91 (95% CI 2.06-11.68) for more than 20mm¹⁷⁷. This study also reported that having CIN confers an increased risk of preterm birth, but treatment increases this further; the additional risk conferred by treatment in cones less than 10mm length was shown to be unclear.

Conservative management of CIN2

Traditionally, the diagnosis of CIN2 or worse on histological biopsy has been considered the cut-off to proceed to local excision of the transformation zone. This relied on data suggesting significant risk for malignant transformation, although concerns have been raised that this may lead to overtreatment in a substantial number of young women with CIN2 lesions that do not have carcinogenic potential and would otherwise regress without intervention. Given that CIN2 pre-invasive cervical lesions are not reliably recorded in cancer registries the true incidence of CIN2 is difficult to establish. It is estimated that around 2-3% women are diagnosed as having CIN2/3 in UK screening programmes annually with the incidence being highest among women aged between 25 and 29 years at 10%¹⁷⁸. In the UK the incidence of CIN3 is estimated at 75 per 100,000 women (2017)¹⁷⁹.

In a recent meta-analysis over 50% of CIN2 lesions regressed in young women below the age of 30¹⁸⁰.

Despite the equivocal nature of these lesions, the updated WHO 2014 histopathological classification graded these lesions as a single entity: high grade squamous intraepithelial lesion (HSIL)¹⁸¹. The American Society of Colposcopy and Cervical Pathology (ASCCP) also adopted these recommendations proposing p16 immunostaining as a marker to classify an equivocal CIN2 lesion in the low-grade or high-grade phenotype^{78,163}. These recommendations have not been universally adopted in many countries including in the UK. There is evidence that P16 CIN2 lesions have lower risk of progression compared to morphological CIN3 and therefore should be considered separately.

Although active surveillance of CIN2 lesions is practiced in selected cases in the UK, guidelines on this topic are lacking. The eligibility, frequency of surveillance and criteria for return to routine recall have not been previously described.

The number of CIN2 lesions which would have regressed without treatment is difficult to determine. Data from longitudinal studies on the natural history of CIN2 lesions have been limited due to the equivocal histological diagnosis and ethical dilemmas with most data coming from studies in young women and adolescence. The first report on the natural history of CIN was published in 1984 by McIndoe *et al*¹⁸² only reported on CIN3 progression to invasive cancer that was as high as 30%. The most cited report by Ostor *et al* (1993) documents a CIN2 40% rate of progression to CIN3, 5% rate of progression to invasive cancer and 40% rate of regression¹⁸³. This review included studies going back to 1951 when cytological classification differed to current practice and included small cohorts with different inclusion criteria. More recent studies reported high regression rates for CIN2 in young women. A prospective cohort study of 95 young women (aged 18-23) with CIN2 reported a regression rate of 63%, with only 15% showing progressing to CIN3 in three years²³. Another prospective cohort of 5052 women aged 18 to 62 reported that over 40% of CIN2 lesions regressed within 2 years¹⁸⁴.

A recent systematic review and meta-analysis conducted an extensive interrogation of reporting on histologically-confirmed untreated CIN2 managed with active surveillance and found that regression was as high as 68% in a cohort of young women²³. This included 36 studies with over 3160 eligible women providing the best level of evidence presented to date¹⁸⁰. This meta-analysis reported that 50% (95% confidence interval (CI) 43-67%) of CIN2 lesion will regress after 2 years, 32% (95%CI 23%-42%) will persist, and 18% (95%CI 11-27%) will progress. In a subgroup analysis including 1069 women under the age of 30, the rates of regression were even higher at 60% (95%CI 57-63%), 23% for persistence (95%CI 20-26%) and 11% for progression (95%CI 5-19%), respectively. In summary, approximately half of the CIN2 lesions will regress after two years and just under one fifth will progress.

Risk of high-grade disease recurrence after treatment is estimated at 5-10%, with risk of invasive cancer two to four times above population incidence rates. After treatment a test of cure hrHPV test is performed at 6 months – a negative hrHPV result greatly reduces risk of recurrence and allows discharge to routine screening.

The harms of immediate local excision should be balanced against the number of repeat visits, risk of non-compliance, rates of recurrence and delayed treatment. Most studies included in the meta-analysis provided only limited follow-up up to 2 months. Recurrence rates after clearance of disease should also be considered. Wilkinson *et al*¹⁸⁵ followed up 683 women under 25 with CIN2; 106 had spontaneous regression of CIN2. After a median follow-up of four years, 17% developed a new high-grade lesion compared to a 4% recurrence rate expected following treatment of CIN2. A recurrence rate for a high-grade lesion of 12% is expected in women after spontaneous regression of CIN1. Likelihood for a high adherence to follow-up is important in the shared decision making for active surveillance versus immediate treatment. Cost-effectiveness of this approach remains unknown.

6. HYPOTHESES AND AIMS

Hypotheses

We hypothesise that there are interacting genetic, environmental, epigenetic and viral factors which influence the of hrHPV infection to cervical cancer in only the minority of women that are infected with hrHPV.

Specifically, we hypothesise that:

- a) Proposed life course exposures for cervical cancer risk may be based upon weak and/or biased evidence, due to the observational study design of the majority of existing research.
- b) Genetic variation in CIN and Cancer has been inadequately explored to date, novel genetic signals associated with the risk of developing cervical cancer in the presence of hrHPV infection exist.
- c) Study of epigenetic associations related to CIN and cervical cancer could identify the strongest molecular markers, for potential use in improving the accuracy of detection of high-grade CIN and early invasive cervical cancer.

Aims

1. To identify and interrogate the current evidence regarding comorbidities, behavioural and environmental factors related to hrHPV infection, persistence, development of CIN and Cancer with an Umbrella review of the literature.
2. To conduct the largest genome-wide association study (GWAS) of cervical cancer to date, using UK Biobank data to identify genetic polymorphisms associated with CIN and cervical cancer.
3. To undertake an epigenome-wide association study to explore the association between DNA methylation in CIN and Cancer compared to normal controls.
4. To systematically review, and meta-analyse the literature published on hrHPV methylation to estimate the most accurate diagnostic markers in identifying high-grade cervical lesions.

Chapter 1: An Umbrella Review of environmental risk factors in the development of hrHPV persistence, CIN and Cervical Cancer

Acknowledgments:

Assistance was provided in computational analyses in these chapter, including for the umbrella meta-analyses and Mendelian randomisation, by Dr I Kalliala, Dr E Bouras and Dr G Markozannes.

1.1 BACKGROUND

Although persistent hrHPV infection is necessary for development of cervical cancer, it is not sufficient. HPV is the most common sexually transmitted infection with over 70% of women being infected during their lifetime; while most hrHPV infections are cleared by the immune response, 8-10% of women screened in the UK will have an abnormal smear at some point. However, the epidemiological and lifestyle factors leading to hrHPV persistence, and especially neoplastic progression, are not fully understood.

Over the last three decades, many epidemiological studies have investigated the risk factors associated with hrHPV persistence and development of cervical cancer. The most evidence exists for immunosuppression^{52,186-188}, concomitant sexual infections¹⁸⁹⁻¹⁹¹, risky sexual behaviour^{192,193} and tobacco smoking⁴⁷, all of which have plausible biological mechanisms. However, most environmental exposures are not suitable for investigation by randomised trial designs and may be subject to inherent bias. For some reported associations, a wide range in the magnitude of the effect size has been observed. Studies have also reported opposing directions of effect for the same exposure, such as for early age of first pregnancy^{194,195} or tobacco smoking^{186,196}. It can be difficult to determine the true strength of an association from P-values alone, and selective reporting of positive results can additionally lead to an overestimation of the strength of an association. Simply accepting a statistical significance of P value less than 0.05 and transferring this to clinical guidelines ignores potential biases and has multiple implications. Previous umbrella reviews have demonstrated that despite the numerous reported significant associations across differing scientific specialties, very few survive more rigorous analyses^{5,197-201}. Further, causal investigations relying on Mendelian randomisation techniques, has been limited in the field of cervical cancer so far. The aim of an umbrella review is to pool together all evidence on a given exposure or outcome and gauge the evidence, in order to enable readers, whether clinicians, academics or public health experts, to find in a single report an up-to-date summary and evaluation of all available evidence.

We performed an umbrella review of systematic reviews and meta-analysis of the association between all exposures and HPV infection, CIN and cervical cancer outcomes.

1.2 METHODS

The aim of this umbrella review is to synthesise all meta-analyses related to environmental and behavioural risk factors for HPV persistence (low or high-risk genotypes), cervical precancer and cancer, through a search with no prior restriction for type of exposure (except genetic factors), with the goal of producing a summary report of all the available evidence related to these outcomes. As well as identifying studies related to the outcome. The umbrella review uses set predefined criteria to evaluate in each meta-analysis, the strength of evidence and risk of bias. This requires a) identifying all relevant systematic reviews and meta-analyses for inclusion b) re-extracting the data and repeating the meta-analyses in random and fixed effect models c) using a series of statistical tests to evaluate the potential biases of each meta-analysis e.g. size of the meta-analysis, size of the largest included study, observed versus expected effect sizes (based on study sample size) and statistical significance, small study effects, excess significance d) exploring duplicate studies for potential discrepancies e) comparing cohort data to case-control data in sensitivity analyses f) performing an overall grading of the strength of evidence against set criteria g) evaluating methodological quality using the AMSTAR-2 criteria for umbrella reviews. The detailed methods were as follows:

Literature search and eligibility criteria: We first designed a protocol for this study which was made available on PROSPERO (CRD42020189995). We searched PubMed, Ovid MEDLINE and Embase Classic, and the Cochrane database for systematic reviews and meta-analyses that investigated the association between non-genetic risk factors and incidence or prevalence of HPV, CIN or cervical cancer. All articles were screened at least in duplicate using pre-defined search terms (Appendix 1). We further hand-searched the reference lists of included papers and the proceedings of relevant conferences for unpublished data.

Inclusion and exclusion criteria: We included all systematic reviews and meta-analyses of observational or interventional studies on any modifiable or environmental risk factors affecting HPV, CIN or cervical cancer incidence or mortality through an unrestricted search; including outcomes concerning disease regression and progression. We will include studies examining both low and high-risk HPV genotypes for the HPV phenotype. We excluded genetic studies as this study focussed on environmental and modifiable risk factors. We also excluded meta-analyses that did not report the necessary study-specific data including the relative risk (RR), 95% confidence intervals (CI), the number of cases/controls or total population (where we could not retrieve data from original studies). Where more than one meta-analysis examined the same exposure-outcome pair, we chose the meta-

analysis containing the most cohort studies. If a similar number of cohort studies were included, we took the more recent study. Duplicate studies were then analysed separately for later comparison.

Data extraction: We extracted the individual study level data for each study within each meta-analysis including number of cases and controls or total population and the maximally adjusted relative risk. All the data extraction was performed at least in duplicate, with any discrepancies resolved by discussion with a third investigator. In detail the extraction was as follows:

We extracted the name of the first author and the year of publication, the modifiable exposure and outcome studied, and the summary effect (relative risk, odds risk, hazard ratio, standard incidence ratio, standard mortality ratio), and 95% confidence intervals (CI) or standard error (SE) from each eligible meta-analysis. From each individual study in a meta-analysis, we then extracted the first author and the publication year, epidemiological design (cohort, case control), number of cancer cases and controls in case- control studies or the number of cases and total population or person years in cohort studies, maximally adjusted relative risk (odds ratio in case-control studies, risk ratio or hazard ratio in cohort studies) and the 95% confidence intervals.

Evaluating the strength of evidence by grading criteria

Assessment of summary effect, heterogeneity and prediction intervals

For each exposure, we calculated the summary effect and 95% confidence interval using both fixed and inverse variance weighted random effects methods²⁰². We used the Cochran Q test²⁰³ and the I^2 metric of inconsistency²⁰⁴ including its 95% confidence intervals²⁰⁵ to evaluate for inter-study heterogeneity. The I^2 metric ranges between 0% and 100% and could reflect either genuine diversity across the studies, chance or bias. A further assessment of inter-study heterogeneity was performed by calculating the 95% prediction intervals (PI) for the summary random effect estimates. The PI enables direct comparison with future clinically relevant effect estimates as it provides a range within which the effect estimate is likely to fall^{206,207}.

Assessment of small study effects

To evaluate whether the smaller studies in a meta-analysis were creating an exaggerated risk estimate relative to the larger studies, we used Egger's test at $P < 0.10$ ²⁰⁸ with the random-effects summary estimate being further away from the null-value compared to the point estimate of the largest study in a meta-analysis (i.e. the study with the smallest standard error). Small study effects can occur due to publication or reporting bias, lack of statistical power and true heterogeneity or chance²⁰⁹.

Evaluation of excess statistical significance bias

The test for excess significance aims to evaluate whether the observed (O) number of statistically significant results ($P < 0.05$) in the studies included in a meta-analysis is too large compared to the expected (E) number²⁰⁵. The number of significant associations that were expected in each meta-analysis was calculated using the sum of the statistical power estimates for each included study using non-central t -distribution^{205,210}. The effect of the largest study (i.e. has the smallest standard error) was used as the plausible effect size as the true effect size is not known. Sensitivity analyses were performed using the summary fixed and random effect estimates as alternative plausible effect sizes. Excess significance for each individual meta-analysis was defined as two-sided $P < 0.10$.

We used credibility ceilings, a sensitivity analysis tool, to account for potential methodological limitations of observational studies that might lead to spurious precision of combined effect estimates. Because of the large amount of data synthesised in each meta-analyses, the CI can become spuriously narrow and underestimate biases that may exist when combining non-randomised studies. The main assumption of this method is that every observational study has a probability c (credibility ceiling) that the true effect size is in a different direction from the one suggested by the point estimate. This was measured by the last credibility ceiling at which the summary effect size was still statistically significant²¹¹.

For grading of included meta-analyses, a strong association is claimed when the P value of the random effects meta-analysis is smaller than 10^{-6} , if the meta-analysis has more than 1000 cases, the largest study in the meta-analysis is nominally statistically significant ($P < 0.05$), the I^2 statistic of heterogeneity between studies is smaller than 50%, the 95% prediction intervals excludes the null value, and small study effects or excess significance bias are not indicated (Table 5).

Table 5: Statistical criteria for grading of the evidence in an Umbrella Review⁵

Evidence grading criteria
For strong evidence:
$P < 10^{-6}$
>1,000 cases
$P < 0.05$ of the largest study in a meta-analysis
$I^2 < 50\%$
no small study effect*
prediction interval excludes the null value
no excess significance bias**
For highly suggestive evidence:
$P < 10^{-6}$
>1,000 cases
$P < 0.05$ of the largest study in a meta-analysis
For suggestive evidence:
$P < 10^{-3}$
>1,000 cases
For weak evidence:
$P < 0.05$

Abbreviations: P = P -value of random effects model

* Egger's $P < 0.10$ signifies no small study effect

** when comparing observed and expected number of studies

Evaluation of the quality of included meta-analyses

We used the AMSTAR-2 criteria²¹² to assess the quality of the evidence. Evidence was graded based on the strength and validity as according to previous published umbrella reviews^{5,197}. The AMSTAR-2 tool uses 16 measures to appraise the methodological quality of systematic reviews and ranks them into high, moderate, low or critically low quality. Seven of the sixteen measures are classed as major components, with unsatisfactory answers to any of these being regarded as a critical flaw. Assessed criteria includes a published protocol, a comprehensive literature search, a list of excluded studies, satisfactory risk of bias assessment for each included study, appropriate statistical analysis, adequate discussion regarding any bias detected and an appropriate investigation of publication bias ²¹². By assessing these criteria four grades are reached: ‘high’ to ‘critically low’ with ‘high’ having zero or one non-critical weakness in the study and ‘critically low’ having more than one critical flaw with or without non-critical weaknesses. As the AMSTAR 2 tool aims to evaluate systematic reviews of randomised and non-randomised trials, some of the criteria were not applicable to umbrella reviews.

The four grades range from ‘high’ to ‘critically low’, with ‘high’ evidence having zero or one non-critical weakness in the study, and ‘critically low’ having more than one critical flaw, with or without a non-critical weakness.

Sensitivity Analyses

The main analysis was restricted to cohort studies only. We conducted a sensitivity analysis which included all eligible observational studies i.e. meta-analyses of both cohort and case-control studies. These were then graded using the same criteria as for the main analyses.

In the event of multiple meta-analyses reporting on the same exposure-outcome associations, we selected the one with the largest number of studies to prevent duplication of the original studies. The concordance between the included and duplicate meta-analyses was explored in a sensitivity analysis.

1.3 RESULTS

The literature search was performed in November 2020 and identified 4722 papers. Following sequential title, abstract and full-text screening, 56 studies met the inclusion criteria. After exclusion of duplicate studies, 50 studies remained^{16,17,47,48,51,186,189,192,195,213-253}, which included 171 meta-analyses of 1513 individual study estimates, 447 studies were cohort studies (prospective or retrospective) and 1056 were from case-control design (Figure 1); no randomised-control trials were identified.

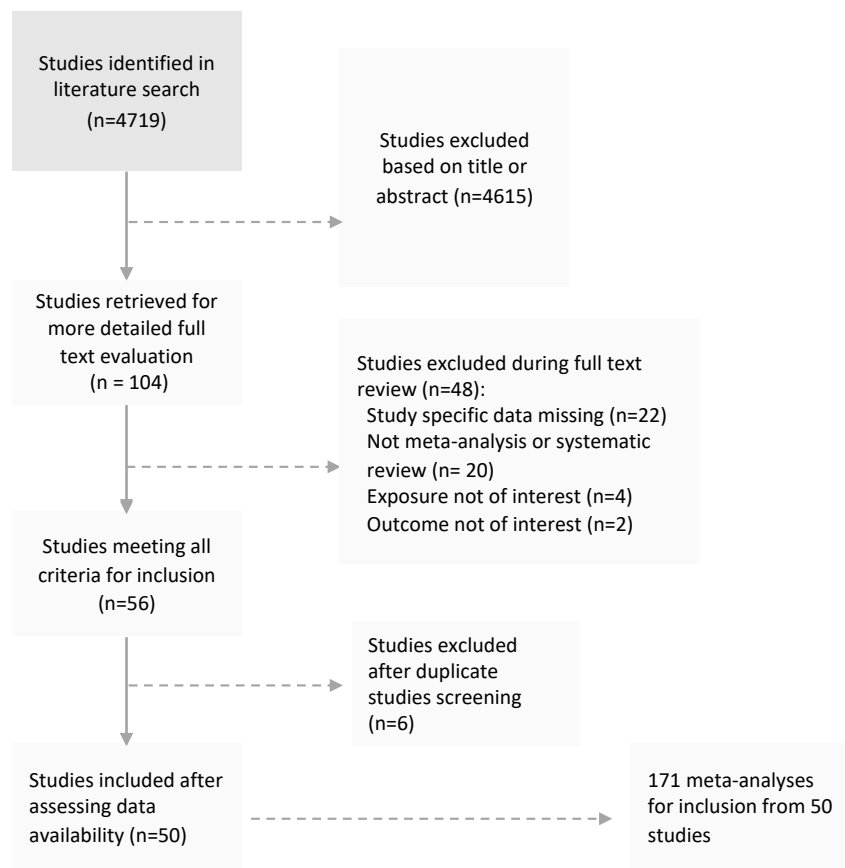


Figure 7. PRISMA flow chart of studies for cervical cancer umbrella review.

Characteristics of the meta-analyses

A total of 11 outcomes were identified, which related to HPV infection, CIN or cervical cancer; these can be sub-categorised according to cervical disease regression (HPV clearance; CIN regression), disease incidence and prevalence (HPV incidence; HPV prevalence; CIN incidence; CIN prevalence; cancer incidence; cancer prevalence), and disease progression (HPV persistence; CIN persistence; CIN progression; cancer mortality).

Fifty exposures were identified, which belonged to eight broad categories: immunocompromise (HIV+, inflammatory bowel disease on medication, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), transplant recipients); co-infection and vaginal microbiome (Epstein-Barr virus (EBV) infection, *Chlamydia trachomatis*, *Trichomonas*, *U.urealyticum*, Mycoplasmas, *Candida Albicans*, vaginal *Lactobacillus* spp., herpes simplex virus (HSV) infection,); anthropometric measures (body mass index (BMI), height); lifestyle and/or behavioural factors (smoking, alcohol intake, number of sexual partners); medical co-morbidities and/or medication use (retinoid use); gynaecological and obstetric

factors (parity, age of first pregnancy, pregnancy, in-vitro fertilisation (IVF), gestational diabetes mellitus (GDM), vaginal douching); hormonal medication use (combined oral contraceptive pill (COCP), intrauterine devices (IUD), injectable contraception); dietary, vitamin or antioxidant intake (vitamin A, E, C, lycopene, folate and carotenoids, vegetable/fruit, zinc, copper and selenium).

Quality assessment

Twelve percent of all the included papers were graded as high quality (1/50)²²⁷ or moderate quality (5/50)^{189,214,215,233,235}. Helm *et al*²²⁷ being the only study that adequately fulfilled all the major components of the AMSTAR2 questionnaire. On the contrary, 14% (7/50) and 74% (37/50) of studies were graded as low or critically low quality, respectively (Appendix 2). Papers characterised as of ‘low’ or ‘critically low’ quality failed to meet criteria related to protocol, literature search, description of excluded studies and risk of bias assessment. Specifically, only 18% (9/50) of included meta-analyses listed the excluded studies and reason for exclusion. Additionally, 38% of the studies (19/50) did not use a pre-registered protocol, while 20% of the studies (10/50) did not screen the literature adequately. Study selection and data extraction were performed in duplicate in 40% (20/50) and 42% (21/50) of the included meta-analyses. Most meta-analyses provided satisfactory methods according to AMSTAR-2 criteria for the risk of bias assessment (80%, 40/50), statistical analysis (76%, 38/50), interpretation of the main results (70%, 35/50) and investigations of small study effects (66%, 33/50) (Appendix 2).

Main analysis

Characteristics of the studies

The primary analysis focussed on cohort studies, whilst sensitivity analyses of studies including case-control studies were also performed. Of the 171 meta-analyses, 87 included two or more cohort studies and were included in the main analysis (Table 6).

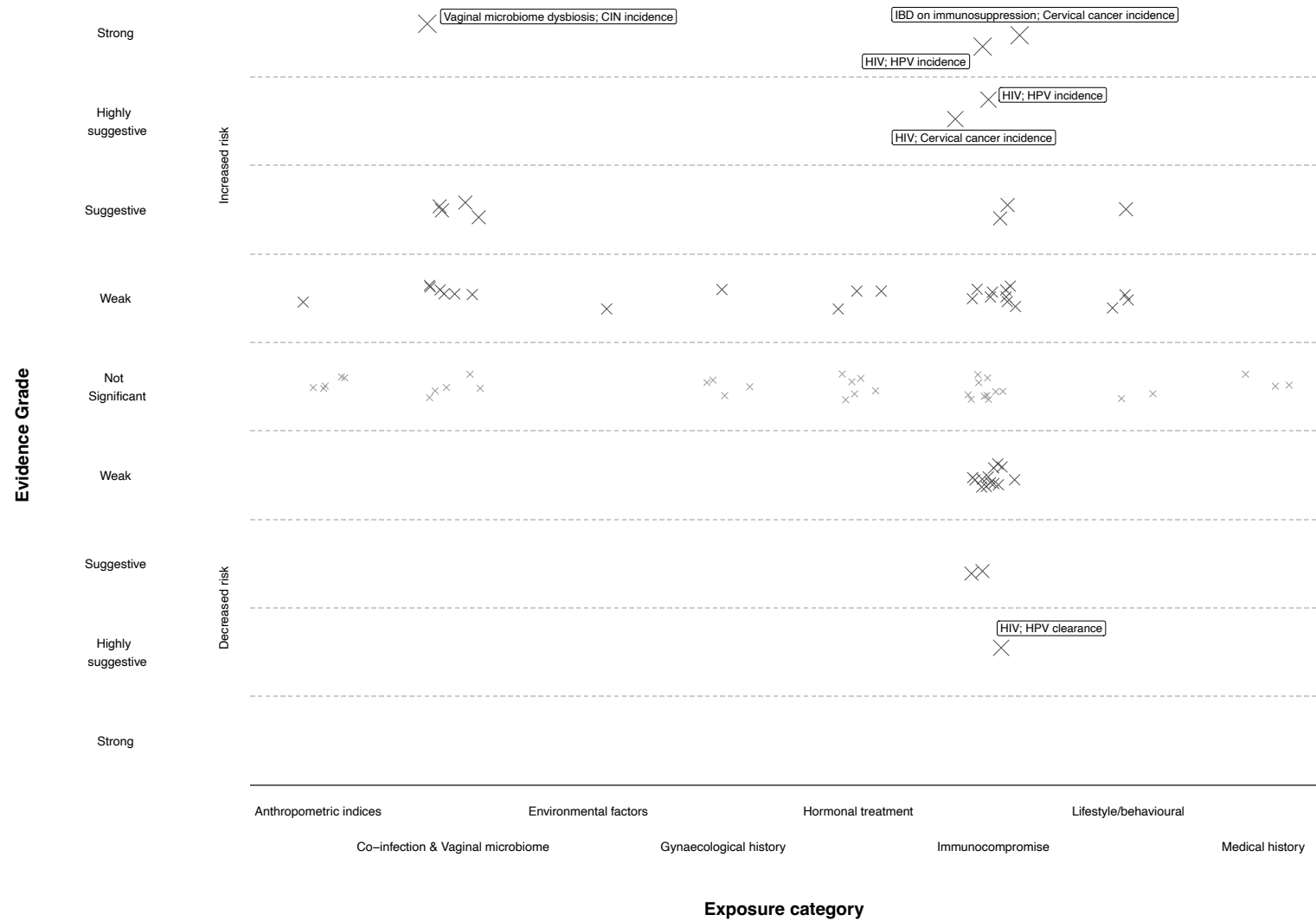
The meta-analyses assessed a total of 39 exposures across the eight categories (anthropometric indices, co-infection and vaginal microbiome, environmental factors, gynaecological history, hormonal treatment, immunocompromise, lifestyle/behavioural and medical history), while outcomes included HPV incidence, HPV prevalence, HPV clearance and HPV persistence, CIN incidence, CIN prevalence, CIN persistence, CIN regression and CIN progression, cervical cancer incidence, cervical cancer prevalence and cervical cancer mortality (Figure 8).

Table 6: Summary of evidence grading for meta-analysis of risk factors associated with all outcomes and studies reaching a grading of weak evidence or above.

Evidence	HPV related outcomes	CIN and Cervical Cancer related outcomes
	Increased risk	Increased risk
Strong	HIV: positive vs negative (hrHPV incidence)	IBD on immunosuppression vs healthy controls (CC incidence); VMB:: dysbiosis vs no (progression to dysplasia and CIN)
Highly Suggestive	HIV: positive with CD4>200 vs negative (HPV incidence) HIV: positive vs negative (HPV persistence)	HIV: positive vs negative (CC incidence)
Suggestive	HIV: positive vs negative (HPV incidence); HIV: positive vs negative (hrHPV persistence) VMB: LL vs HL (HPV incidence); VMB: dysbiosis vs no (HPV incidence); Chlamydia tr: yes vs no (HPV incidence); Smoking: yes vs no (HPV incidence)	HIV positive: on ART vs not on ART (CIN regression); HIV: positive vs negative (CIN persistence); Bacterial vaginosis: yes vs no (CIN prevalence)
Weak	Smoking: yes vs no (HPV prevalence); VMB: dysbiosis vs no (HPV persistence); Bacterial vaginosis: yes vs no (HPV prevalence); Chlamydia tr: yes vs no (HR HPV incidence); Pregnant: yes vs no (HPV incidence); HIV: positive vs negative (HPV incidence, HR HPV incidence, HPV 16 incidence, HPV 18 incidence); HIV: positive with CD4<=200 vs negative (HPV incidence); HIV: positive with CD4>200 vs negative (hrHPV incidence); HIV: positive vs negative (prevalent and newly detected hrHPV, HPV 16, HPV 18 , HPV-any type persistence); HIV: positive vs negative (HPV 16 persistence); HIV+ve: CD4<200 vs CD4>500 (HPV persistence); HIV+ve: CD4 200-500 vs CD4>500 (HPV persistence)	COCP: <5 years of use vs never (ICC incidence); COCP use: 5-9 years of use vs never (ICC incidence); COCP: >10 years of use vs never (ICC incidence); Smoking: current smoker vs never (CC incidence); Smoking: previous smoker vs never (CC incidence); Environmental tobacco smoke exposure: increased vs lower (CC incidence); HIV: positive vs negative (CIN incidence, CIN persistence); HIV positive: on ART vs not on ART (CIN incidence*, CIN progression*, ICC incidence*); Transplant recipient: yes vs no (CC incidence); Rheumatoid arthritis: yes vs no (CC incidence); BMI: highest vs lowest levels (CC mortality); Chlamydia tr: yes vs no (CC incidence); Co-infection of Chlamydia tr and HPV: yes vs no (CC incidence)

Abbreviations: HPV: human papilloma virus, hrHPV: high-risk HPV; IBD: inflammatory bowel disease; HIV: human immunodeficiency virus; VMB: vaginal microbiota; ART: antiretroviral treatment; CIN: cervical intraepithelial disease, BV: bacterial vaginosis; COCP: combined oral contraceptive pill; BMI: body mass index; CC: cervical cancer

Figure 8. Distribution of studies across evidence grade for all exposures of either increased or decreased risk (y-axis) by exposure category (X-axis) from the main analysis (summary random effects for cohort studies only)



Quality assessment

Two of the studies^{214,215} that met criteria for strong evidence were of moderate quality on AMSTAR assessment while the third study rated strong²³⁶ was evaluated as critically low quality. Most of the studies that graded as highly suggestive evidence were graded as low, or critically low quality on AMSTAR assessment.

Summary effect size

Applying $P < 0.05$ as a threshold for the level of significance, the summary fixed effects estimates were significant in 62% of cohort studies (54/87) and the summary random effects in 60% (52/87) of the meta-analyses (appendix 3a). When using $P < 0.001$ as a cut-off, 53% (45/87) and 39% (34/87) of the studies presented significant summary fixed and random effects estimates, respectively. Where a cut-off of $P < 10^{-6}$ was applied, 32% (28/87) and 19% (16/87) of the meta-analyses produced significant summary results in the fixed and random effects model, respectively. Out of the 16 studies with a random effects model $P < 10^{-6}$, 14 exposures were associated with an increased risk of either HPV incidence or persistence, or the increased risk of CIN or cervical cancer these included: vaginal dysbiosis, HIV+, IBD on immunosuppression, *Chlamydia trachomatis* infection and co-infection with HPV, smoking and rheumatoid arthritis, while two exposures were associated with a decreased risk for HPV clearance (HIV+; HIV+ with low CD4+ cell count).

Heterogeneity between studies

The Cochrane's Q test for heterogeneity was significant at $P \leq 0.10$ in 27 out of the 87 meta-analyses (31%). Twenty-five studies (29%) presented a high heterogeneity ($I^2 = 50-75\%$) and nine (10%) very high heterogeneity ($I^2 > 75\%$), which included 5 different exposures (HIV+; HIV+ on treatment; bacterial vaginosis; *Chlamydia trachomatis*; pregnancy). When calculating the 95% prediction intervals, the null hypothesis was excluded for nine associations (IBD on immunosuppression - cervical cancer incidence; vaginal dysbiosis – HPV incidence; vaginal dysbiosis - CIN incidence; HIV+ - HPV incidence; HIV+ - CIN2+ treatment failure; HIV+ - CIN1+ treatment failure; smoking - HPV incidence; smoking – cervical cancer incidence; COCP - cervical cancer incidence) (appendix 3b).

Small study effects

In three studies there was evidence of small study effects (Egger's test of $P < 0.10$ and detection of more conservative effects in the largest study of a meta-analysis compared with the summary random effects estimate): *Chlamydia trachomatis* infection - HPV incidence, HIV+ - CIN1+ treatment failure recurrence and gestational diabetes mellitus – cervical cancer incidence) (appendix 3b).

Excess significance

When using the largest study estimate as the plausible effect size, evidence of excess significance was observed in eight studies (9%), of varying exposures including bacterial vaginosis, *Chlamydia trachomatis* infection, HIV positivity, rheumatoid arthritis, BMI, and current pregnancy. Using the fixed or random effect estimate as the plausible effect sizes, five and four studies presented excess significance bias respectively.

Credibility ceilings

Of the 87 meta-analyses, 37 (43%) retained nominal statistical significance ($P < 0.05$) with a credibility ceiling of 5%. With ceilings of 10%, 15% and 20%, twenty-two (25%), fourteen (16%) and four (5%) studies remained significant respectively (appendix 3c).

Grading the evidence

Of the 87 meta-analyses included in the main analysis, three met criteria to be graded as strong evidence, including meta-analyses which examined a) the association between HIV+ and increased risk for HPV infection ($N=2323$, $RR\ 2.20$, 95% $CI\ 1.89-2.54$, $P=3.01 \times 10^{-26}$, $I^2=22\%$), b) vaginal dysbiosis and increased risk for CIN incidence ($N=27405$, $RR=1.6$, 95% $CI=1.4-1.81$, $P=5.34 \times 10^{-12}$, $I^2=26\%$) and c) people with inflammatory bowel disease using immunosuppressive medications and increased risk of cervical cancer incidence ($N=10829$, $RR=1.33$, 95% $CI=1.89-2.54$, $P=7.78 \times 10^{-37}$, $I^2=0\%$). A further three meta-analyses met criteria for highly suggestive evidence, all three studies examined HIV positivity as the exposure which was associated with three outcomes: an increased risk of HPV incidence ($N=1151$, $RR\ 3.1$, 95% $CI=2.17-4.4$, random $P=3.75 \times 10^{-10}$, $I^2=82\%$), a decreased risk of HPV clearance ($N=2977$, $RR\ 0.53$, 95% $CI=0.43-0.65$, $P=4.32 \times 10^{-10}$, $I^2=73\%$) and an increased risk of cervical cancer incidence ($N=1160$, $RR=5.82$, 95% $CI=2.98-11.34$, $P=2.34 \times 10^{-07}$, $I^2=86\%$) (Figure 9).

Nine studies met criteria for suggestive evidence, while thirty-seven studies were of weak evidence; the remaining studies did not meet any criteria (appendix 3d).

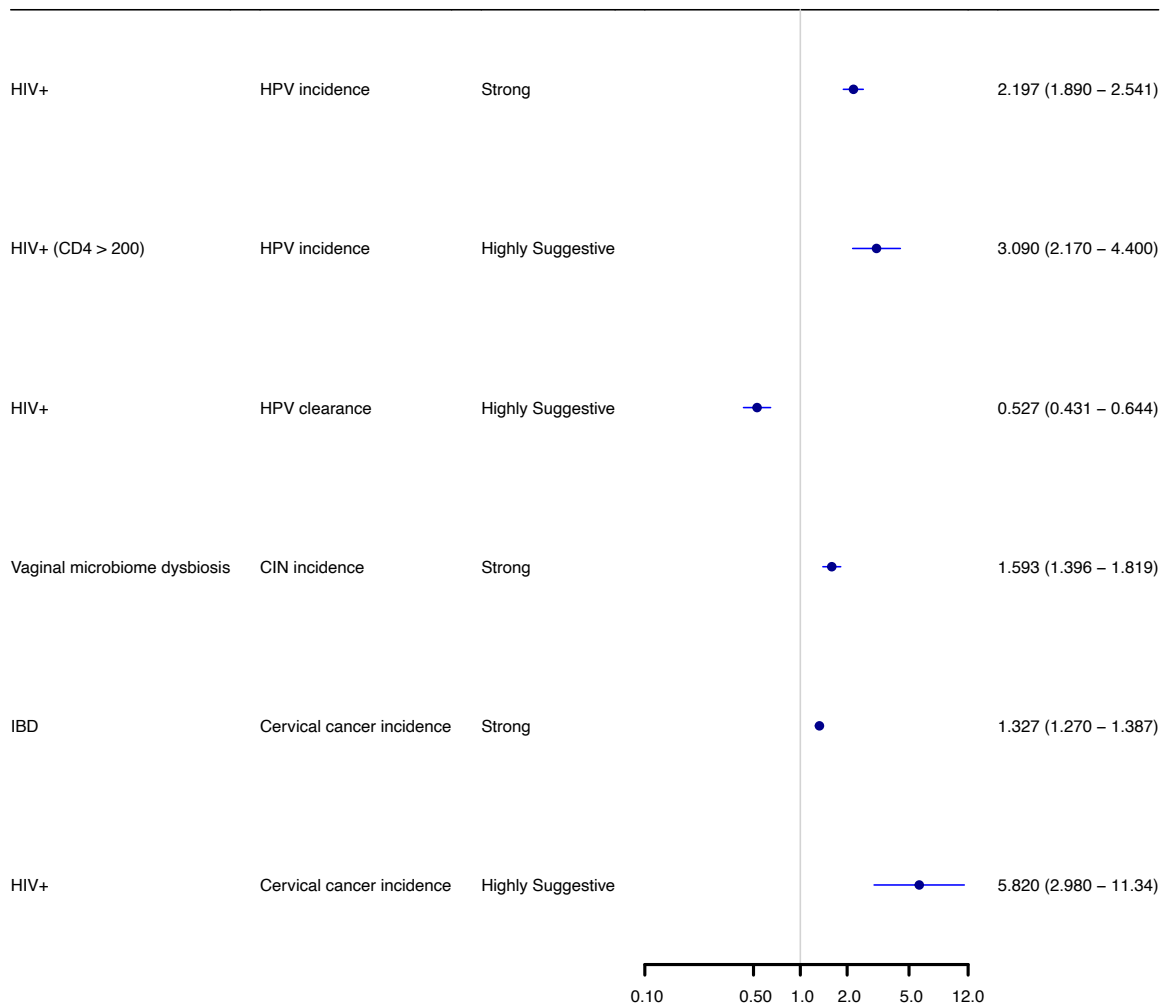


Figure 9. Forest plot of effect estimates and 95% confidence intervals for all exposure-outcome pairs for all outcomes (HPV, CIN and cervical cancer) in the main analysis (summary random effects for cohort studies only), which graded as strong or highly suggestive evidence (n=6).

Sensitivity analyses

In the sensitivity analysis including case-control studies, of 171 meta-analyses, 11 meta-analyses met criteria for strong evidence, including eight additional exposure-outcome pairs. *Chlamydia trachomatis* infection was linked to an increased risk of HPV incidence (N=5049, RR=2.32, 95% CI=2.02-2.65, $P=3.45 \times 10^{-34}$, $I^2=0\%$), cervical cancer incidence (N=3392, RR=2.19, 95% CI= 1.74-2.74, $P=1.03 \times 10^{-11}$, $I^2=47\%$) and in a subgroup analysis, squamous cell cervical cancer incidence (N=3198, RR= 2.09, 95% CI=1.79-2.44, $P=6.87 \times 10^{-21}$, $I^2=32\%$). Meanwhile, co-infection of HPV with *Chlamydia trachomatis* increased the risk of cervical cancer by more than four times (N=1086, RR=4.37, 95% CI=2.75-6.96, $P=4.59 \times 10^{-10}$, $I^2=44\%$). *Trichomonas Vaginallis* infection was associated with an increased risk of cervical cancer incidence (N=7715, RR=2.09, 95%CI=1.69-2.6, $P=2.33 \times 10^{-11}$, $I^2=34\%$) (evidence for adenocarcinoma was weak only). Multiple sexual partners almost doubled the

risk of CIN (N=5638, RR=1.97, 95% CI=1.8-2.15, $P < 1 \times 10^{-100}$, $I^2=0\%$), while oral contraception was associated with an increased risk for cervical cancer incidence (N=5839, RR=2.13, 95% CI=1.87-2.42, $P=4.37 \times 10^{-31}$, $I^2=1.4\%$). Of the eight additional associations that met strong criteria in the sensitivity analysis, five were graded as weak or non-significant evidence in the main analysis when cohort studies only were included. The remaining three included only case-control studies and were not assessed in the main analysis. All three ‘strong’ associations from the main analysis remained as ‘strong’ when case-control studies were included as well. 14 studies met criteria for highly suggestive evidence, 27 met criteria for suggestive and 69 studies were classified as weak evidence only; the remaining 51 meta-analyses did not reach criteria for weak evidence.

We identified duplicate meta-analyses meeting inclusion criteria for six exposure-outcome pairs. For all duplicates the same direction of effect was observed in both the magnitude and significance of the summary associations, between included and excluded studies (appendix 3e).

1.4 DISCUSSION

We included 87 studies from 50 meta-analyses of observational studies that evaluated the current evidence on the association between different environmental and behavioural exposures with persistent hrHPV infection, CIN or cervical cancer outcomes. No randomised trials were identified. Three meta-analyses of observational studies examining three outcomes met criteria for strong associations. These examined the associations between HIV, vaginal microbiome and immunosuppressive medications in women with inflammatory bowel disease and hrHPV, progression from normal cytology to ASCUS, LSIL or HSIL and cervical cancer. We observed highly suggestive evidence for three associations linking HIV with hrHPV, preinvasive and invasive cervical disease indices, and additionally included new outcomes of HPV clearance. We did not identify any randomised or interventional meta-analyses.

HIV and cervical cancer

We identified studies with strong, highly suggestive, and suggestive evidence for an increased risk of hrHPV and cervical cancer incidence in HIV positive women, when compared to general population. There was also highly suggestive evidence that HIV positivity reduces the risk of hrHPV clearance, whereas antiretroviral treatment increases CIN regression rates. Our findings are in agreement with a recent study published jointly by the world health organization (WHO) and the International Agency for Research on Cancer (IARC), which reported that women living with HIV have a six-fold higher risk of developing cervical cancer¹⁸⁸. The largest of the included meta-analyses on HIV²⁵⁴ (N=122692)

showed highly suggestive evidence that HIV positivity significantly increases the risk of cervical cancer incidence (RR=5.2, 95%CI=3.81-6.93). A meta-analysis provided strong evidence (8668 individuals) and demonstrated that the risk for hrHPV incidence is doubled in HIV positive people, while the rate of hrHPV clearance is almost halved²³⁶. We also found suggestive evidence that hrHPV clearance decreases as CD4 T cell level declines²³⁴, this is supported by another study correlating CD4 levels with HPV infection²⁵⁵. Immunosuppression is considered the primary mechanism of many HIV related diseases, and of HPV persistence, it is therefore plausible that early ART initiation seems to significantly improve HPV clearance and CIN regression through maintain high levels of CD4+ T cells²³¹. HPV infection is cleared through a cell-mediated immune response involving T cell (CD4+ and CD8+) and macrophages activation leading to a release of proinflammatory cytokines. Immunocompromised HIV infected people are unable to upregulate the immune response leading to persistent HPV infection²⁵⁶. While disruption to the *HLA* coding region responsible for MHC class 2 cell production and T cell activation have been identified in genome-wide association studies²⁵⁷. Our findings support the concepts that early ART initiation for individuals living with HIV has a multitude of benefits, and maintenance of more frequent screening intervals for HIV positive women, as well as screening awareness campaigns specifically targeted to these individuals may be beneficial. It also raises the importance of HPV vaccination for girls and boys in geographical areas of high concomitant HIV prevalence such as areas of sub-Saharan Africa²⁵⁸.

Non-HIV immunosuppression and cervical cancer

The data for women with other forms of chronic immunosuppression was more limited. In our umbrella review we found strong evidence that women with inflammatory bowel disease (IBD) on immunosuppression are at increased risk for high grade cervical dysplasia and cervical cancer compared to the general population²¹⁴. Other studies in this area have been small but largely support these results. In a study of 40 individuals²⁵⁹, published in 2008, women living with IBD on immunosuppressant medication had a higher risk of high-grade CIN or ICC when compared to those not on immunosuppressive medications; they also report that women with IBD, regardless of whether they are taking medication, are at an increased risk of high-risk cervical abnormalities when compared to healthy controls. While Hutfless *et al* (2008, total N=13289) found that people with IBD were 45% more likely to be diagnosed with cervical cancer, even though it did not reach statistical significance²⁶⁰. Bhatia *et al* (2006, total N=232) identified an increased risk of abnormal cytology in women with IBD compared to healthy controls, but found no association with type of IBD or exposure to immunosuppression²⁶¹.

We found weak evidence for an increased risk of cervical cancer for other autoimmune diseases. It has been demonstrated in previous studies that there is an increased risk of cervical disease in systemic lupus erythematosus (SLE) patients compared to healthy controls²⁶²⁻²⁶⁷. SLE patients are thought to be at increased risk for hrHPV acquisition²⁶⁷, after adjusting for known social, behavioural, and biologic risk factors (including hrHPV status). Multiple studies have suggested that the use of immunosuppressants in SLE patients is associated with an increased incidence of cervical dysplasia²⁶⁶.

The risk associated with rheumatoid arthritis (RA) was also weak²⁴⁰; in another meta-analysis, Simon *et al* pooled 17 studies, and identified no increased risk for cervical cancer among patients with RA compared to the general population²⁴⁰. While, Kim *et al* at their large cohort study of over 666,000 participants, noted an increased risk of high-grade cervical dysplasia in women with RA²⁶⁹. People living with rheumatoid arthritis have been found to be at significantly higher risk of other cancers including lymphoma and lung cancer, compared to the general population^{240,270}. Rheumatoid arthritis is often treated with steroids, disease-modifying antirheumatic drugs (DMARDs) such as methotrexate and TNF inhibitors, which can decrease the number and function of T-suppressor lymphocytes²⁷¹. As well as the immune dysfunction induced by RA itself, it is likely that the use of immunosuppressive medications play a role in the risk of cancer.

The risk of cervical cancer in organ transplant recipients was similar to HIV positive women²²⁵ but only supported by weak evidence, this study was smaller than HIV studies however, with 188,000 participants. In our search, there was no evidence to support an increased risk of HPV persistence, CIN or cervical cancer in any other immunosuppressive disorder. Whilst there was only specific evidence for SLE, RA and transplant recipients, our findings suggest that all women with chronic immunosuppression, especially where affecting T Cell function, would potentially benefit from shorter screening intervals, especially in the context of longer screening intervals in future vaccinated populations.

Vaginal dysbiosis, cervicovaginal infection and cervical cancer

Strong evidence was provided by a large MA including 9 cohort studies and 460746 women which supported an association between vaginal dysbiosis and progression to LSIL or HSIL²¹⁵. Vaginal dysbiosis was defined as a *Lactobacillus* spp. deplete microbiome. Two smaller meta-analyses,^{215,242} each including 4 cohort studies and 5280 and 6995 individuals respectively, provided suggestive evidence that vaginal microbiome low in *Lactobacillus* is associated with an increased risk of HPV incidence. Some evidence exists that increased vaginal microbiome diversity is associated with

advancing CIN disease severity²⁷². One definition of vaginal dysbiosis is depletion of the *Lactobacillus* spp. however this may be an oversimplification. The vaginal microbiome is difficult to characterise as it is known to be highly complex and dynamic, including a common shift in community state types of bacteria during the menstrual cycle. Bacterial Vaginosis is acknowledged to be a form of vaginal dysbiosis where the microbial community is dominated by anaerobic bacteria, particularly *Gardnerella Prevotella* and *Peptostreptococcus* which results in a particular offensive odour and grey-white vaginal discharge. A meta-analysis of bacterial vaginosis specifically, provided suggestive evidence that bacterial vaginosis was associated with CIN prevalence and weak evidence for an association with increased HPV prevalence and persistence. *Lactobacillus* spp. maintain a low pH and the acidic environment is essential for the function of the cervical epithelial barrier²⁷³⁻²⁷⁵. In the presence of *Lactobacillus* depletion, anaerobic bacteria can dominate, altering the mucosal barrier and increasing HPV entry to the basal epithelium²⁷⁶. A disruption to the cervicovaginal ecosystem, such as a low *Lactobacillus* spp. has also been shown to be a risk factor for sexually transmitted infections (STIs)^{242,277,278}. A healthy vaginal microbiome rich in *Lactobacillus* spp. has been shown to be protective against the development of cervical dysplasia^{52 279}. There is also evidence that a *Lactobacillus* rich environment may be associated with CIN regression – in a recent longitudinal study of 87 women with histologically confirmed CIN2, Mitra *et al* showed that a *Lactobacillus* spp. depleted vaginal microbiome was associated with a significantly lower chance of regression at 12- and 24-month follow up²⁸⁰. It is possible that the interaction between hrHPV, the vaginal microbiome and other sexually transmitted infections are important factors in hrHPV persistence and cervical metaplasia.

There was additional highly suggestive and suggestive evidence that cervicovaginal infections (including *Chlamydia trachomatis* and *Trichomonas*) increase the risk of hrHPV acquisition, CIN incidence and cancer incidence and progression from a normal cervix to a low (LSIL) or high grade squamous intraepithelial lesion (HSIL). We found suggestive evidence that *Chlamydia* infection increases the risk of hrHPV incidence in the form of a meta-analysis including 6 cohort studies and 7426 individuals¹⁷. When also including case-control studies, there was strong evidence associating *Chlamydia*¹⁸⁹, co-infection of *Chlamydia* and hrHPV¹⁸⁹, and *trichomonas* infection²⁴⁷, with cervical cancer incidence. A large meta-analysis by Zhu *et al.*¹⁸⁹ of 22 studies including 4291 cases and 7628 controls, demonstrated that *Chlamydia* infection increases the risk of cervical cancer. This has also been observed systematic reviews²⁸¹ and in large population-based studies^{191,282,283}. In a 2020 longitudinal study of 310,545 females, positive *Trichomonas* vaginitis was correlated with hrHPV and higher odds of CIN1-3¹⁹¹. There is also evidence that *Chlamydia* infection increases the risk of hrHPV persistence^{284,285}, although the exact mechanism is unclear, it is thought that inflammation can disrupt the epithelium allowing more viral entry to cells. Also the natural vaginal microbiome is altered in the

presence of bacterial infection.^{274,286-288} In addition, the inflammatory response caused by Chlamydia and other infections can lead to oxidative stress, which causes DNA instability and carcinogenesis^{289,290}. Some studies support the finding that Chlamydia and hrHPV may have a synergistic relationship in increasing the risk of CIN^{291 292} – in a large prospective cohort study including 1390 participants²⁹², and 19-years of follow up, Chlamydia infection increased the risk of CIN in women with hrHPV infection.

Our analysis suggests strong evidence that the presence of Chlamydia and Trichomonas infections act as an independent risk factor for the development of cervical cancer in the presence of hrHPV and should be treated as a preventative strategy^{293,294}. Prompt treatment of sexually transmitted infections has been suggested as an important strategy for the management of low-grade CIN, and also for conservative management of young women with CIN2 and should be considered in national guidelines^{295,296}. Although strong evidence is suggested for a link between a Lactobacillus Spp. deplete vaginal microbiome and hrHPV persistence or CIN, the vaginal microbiome is highly dynamic, and it is still unclear whether monitoring the vaginal microbiome, or even manipulation, can be used as a preventative strategy for cervical cancer.

Smoking and cervical cancer

Although smoking is widely considered to be a strong risk factor for cervical cancer, in our umbrella there was only suggestive evidence linking smoking with HPV incidence and only weak evidence for an association between active smoking and cervical cancer; two included meta-analyses examined the relationship between smoking and cervical cancer specifically, while one study examined smoking and HPV incidence and prevalence. In studies examining cervical cancer as an outcome, the sample size was limited to less than 1000 cases and therefore they could not be graded as more than weak evidence. The meta-analysis looking into HPV incidence provided suggestive evidence and included 9442 cases across 10 cohorts. While the study examining HPV prevalence as an outcome included 4451 cases across 5 cohorts, the random P-value did not reach statistical significance at a level to be graded more than weak evidence. When we examined the sensitivity analysis including case-control studies also, smoking was graded as highly suggestive evidence, meaning there were more than 1000 cases, effect size random P was $<10^{-6}$ and the largest study effect size was statistically significant at an alpha of <0.05 . There was also weak evidence observed for a link between exposure to environmental tobacco smoke and CIN2 or more, this meta-analysis included seven cohort studies and 65031 women.

Cigarette smoking has long been suspected to be a risk factor for cervical cancer, most likely as a co-factor in the relationship between HPV and cervical cancer. However, most studies examining smoking

have been case-control studies by design and few have controlled for HPV infection. A series of case-control studies conducted by IARC between 1985-1997 did include HPV data and were grouped into a pooled analysis by Plummer et al in 2003 (the data was deemed too heterogeneous for meta-analysis). Plummer *et al* demonstrated an increased risk of cervical cancer in HPV positive women. However, the non-randomised and retrospective study design of original case-control studies is graded as weak in most risk of bias assessments and did not meet criteria for inclusion in the main analysis of this umbrella review. Our sensitivity analyses suggest a strong association between smoking and cervical cancer; however we are unable to completely exclude confounding and biases such as selection and information bias. We explore this relationship between smoking and cervical cancer further in chapter 2 as part of a Mendelian randomisation analysis where genetic instruments are used to control for potential unknown confounding.

Parity, sexual behaviour, and cervical cancer

Pregnancy was found to have a weak association with HPV incidence in a study of 9518 women which could be related to the altered immune response during pregnancy^{297,298}. There were no meta-analyses based on cohort studies for the evaluation of the number of sexual partners and parity on cervical cancer. In our sensitivity analysis however, there were six meta-analyses which examined sexual and reproductive history in relation to cervical cancer. There was one study suggesting a strong association for an increased risk of CIN with multiple sexual partners and three highly suggestive associations demonstrating increased risk of cervical cancer with exposure to multiple sexual partners, early age at first pregnancy and increased number of full-term pregnancies.

While an increased number of sexual partners is thought to increase hrHPV exposure, particularly at an early age, the mechanism regarding parity and early age of pregnancy is less well understood. It is possible that an early age of first pregnancy affects the transformation zone, increasing its vulnerability to infection^{299,300}. However, high parity and early age of first pregnancy may be surrogate markers for increased HPV exposure at an earlier age. We explore this in chapter 2.

Additional risk and protective factors

While BMI was linked to increased cervical cancer mortality, there was no link to any other HPV or cervical cancer outcome. This suggests that the increased mortality risk is linked to other obesity-related health conditions in these women³⁰¹, or possibly for those women who receive surgical treatments, a higher incidence of intraoperative and postoperative complications³⁰².

Our study provided only weak evidence that the use of combined oral contraceptives increases the risk of invasive cervical cancer, although the evidence became strong when case-control studies were included as well³⁰³. Possible mechanisms include the association of oral contraception with increased risk of HPV persistence³⁰⁴ as well as the synergistic action of oncogenic HPV-16 and chronic oestrogen exposure which modulates squamous cell carcinogenesis³⁰⁵.

Strengths and weaknesses

To our knowledge, this cervical umbrella provides the largest summary of the evidence for comorbidities, behavioural and environmental risk factors on HPV, CIN and ICC risk to date. We used a well-established methodology for the analysis, exploration, and quality assessment of meta-analytical studies. A wide range of statistical tests and sensitivity analyses were performed to categorise the evidence into clearly defined levels of evidence. This can be used to reinforce the findings of high-quality evidence. Whilst a lack of evidence does not infer the absence of an association, where a weak or no evidence grade was assigned, this may suggest the need for further exploration, especially in the context of associations that are widely thought to be causal. Overall, this evidence highlights the importance of preventative strategies for sexually transmitted infections, including HIV, and early treatment. This strengthens the call for more evidence regarding the importance of a healthy vaginal microbiome in preventing HPV persistence and cervical cancer. Additionally, we identify the potential high-risk groups for targeted screening in the era of HPV vaccination where screening intervals are anticipated to increase.

Possible limitations should be considered in the interpretation of our findings. This review relies on the previously published meta-analyses and literatures searches performed by the authors of those studies. No randomised studies were identified and all literature were observational studies; inherent confounding of the association between exposure and outcome could not be accounted for. Whilst the most recent meta-analysis was taken wherever possible, the majority of included studies did not comment on vaccination status of participants, and we were unable to discern the effect modification of HPV vaccination on the exposure-outcome pairs. It is possible that certain studies may have been missed in strategic searches; however assessment of duplicate analyses did not highlight any discordant results which minimises this risk. Additionally, we studied exposures against several outcomes linked to the development of cervical cancer, these results were also consistent across meta-analyses. Although the overall number of studies included were large, for some associations the number of studies and participants were small, limiting our ability to assess for the presence of small study effects and excess significance due to low power. It is likely that this would results in a more conservative estimate and the true association may be more severe. In this umbrella we were unable to perform a sub-analysis on

squamous carcinoma versus adenocarcinoma, the majority of CIN and ICC are squamous type, it is possible that different exposures are associated with adenocarcinoma. Although we assessed for risk of bias, the statistical tests are unable to explain the definitive presence or the likely source of bias.

1.5 CONCLUSIONS

This umbrella review provides a comprehensive summary and analysis for the published studied risk and protective factors related to hrHPV infection, CIN and cervical cancer incidence and cervical cancer mortality. There is consistently strong and highly suggestive evidence that HIV positivity reduces HPV clearance rates (Relative Risk (RR) = 0.53, 95%CI 0.43 to 0.65) and increases the risk of HPV infection (2.20, 95%CI 1.89 to 2.54) and cervical cancer development (RR = 5.82, 95%CI 2.98 to 11.34). Vaginal dysbiosis and immunosuppressive medications for women with inflammatory bowel disease are also strongly associated with increased risk of progression from normal to LSIL or HSIL and cervical cancer. While for smoking we found highly suggestive evidence in the sensitivity analyses only, which is likely due to a lack of high-quality research in smokers and difficulty in accurate reporting of smoking in observational studies. Through the identification of exposures which put women at high risk of hrHPV persistence, CIN progression and cervical cancer, we can target and personalise cervical screening programmes and public health education messages. Our research highlights that providing treatment for HIV, as well as other sexually transmitted infections, including bacterial vaginosis, can reduce the risk of cervical cancer. Since currently the highest incidence of cervical cancer is in countries with high rates of HIV and STIs, provision of sexual health services alongside cervical screening should be prioritised.

In addition to the environmental component, family linkage studies suggest a significant genetic contribution to the risk of cervical cancer; to date this has been incompletely investigated, further study could offer new insights into prevention and treatment of cervical preinvasive and invasive disease.

Chapter 2. A genome-wide association study (GWAS) of CIN and Cervical Cancer in the UK Biobank

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2.1 BACKGROUND

Despite the advent of screening and vaccination, invasive cervical cancer remains one of the most common female malignancies globally³⁰⁶. Persistent infection with oncogenic high-risk human papillomavirus (hrHPV) subtypes is necessary for the development of cervical cancer and its precursor cervical intra-epithelial neoplasia (CIN)⁴³. HPV infection is common, with a lifetime incidence over 70%⁵⁵. Most infections are transient and cleared through an incompletely understood immune response; only a fraction of infected women develop HPV persistence, which ultimately progresses to CIN or invasive cervical cancer (ICC)²².

A number of factors, both host and viral, have been previously reported to affect HPV clearance and the risk of cervical disease progression. HPV genotype, HPV genetic and epigenetic variation¹⁴³ and viral load¹⁴⁵ have been shown to affect disease outcome. Collaborative large pooled analyses have also reported on host behavioural and environmental factors which may influence exposure to HPV or immune response to infection; these include tobacco smoking¹⁸⁶, hormonal contraceptives³⁰⁷, socioeconomic, reproductive¹⁹⁵ and sexual factors³⁰⁸. The role of genetic variation in the risk of cervical preinvasive and invasive disease is more complex. Swedish family-based studies have reported family aggregation of cervical cancer but were not able to discriminate between the effect of shared genes and shared environmental factors^{58,60}. The first study to provide strong evidence for a significant contribution of genetic factors in the development of cervical tumours⁵⁷, estimated genetic variation accounted for 27% of total phenotypic variance. A second study reported common variant based heritability at 36%⁵⁹.

A number of candidate gene studies have explored genetic regions related to plausible immunological and carcinogenic pathways (including the human leukocyte antigen (*HLA*) region⁶¹), but have shown inconsistent results that frequently failed to replicate in larger cohorts. The advent of agnostic genome-wide association studies (GWAS) has greatly expanded the ability to explore complex traits. To date, seven studies have examined genetic variation in cervical precancer or cancer; three in European populations^{59,70,309}, one in Chinese³¹⁰ and three in Japanese populations⁷²⁻⁷⁴. These studies varied greatly in size and reported a total of 112 SNPs associated, at genome-wide significance level, with cervical disease. The most consistently identified allelic variation was at the 6p21.3 locus, within the *HLA* region, where 44 variants have been reported across six of these studies^{59,70,74,309,310} (with both protective and risk haplotypes described), followed by the major histocompatibility complex (MHC) class I polypeptide-related sequence A gene (*MICA*) gene in three of these studies^{59,70,309}.

Cervical cancer is a multi-factorial disease trait characterised by a complex interplay between the host, virus and vaginal microenvironment, which promotes disease progression in some but not others. Identifying genetic factors that influence the individual susceptibility to the disease and its progression could have major clinical applications.

Our study aimed firstly to perform a genome-wide exploration of genetic loci that are associated with invasive cervical cancer or high-grade preinvasive lesions (CIN3) in the UK Biobank (UKB) population; and to validate candidate associations in a large independent dataset (FinnGen). Using a two sample Mendelian Randomisation approach we explored the role of the previously reported life course exposures in the risk of developing cervical cancer.

2.2 METHODS

Study setup

An application (ID: 21146) was made to the UK Biobank in April 2016, approved in September 2016, and data access granted in September 2017; full genetic and phenotypic data were downloaded to the Imperial College high performance computer (HPC) network in June 2018.

Study Population

The UKB is a population-based cohort of ~500,000 volunteers (N=273,377 women), aged 40 to 69 at recruitment between the 13th March 2006 and 1st October 2010, ascertained in a clustering approach through NHS registers³¹¹. Participants who consented to enrolment in the study were asked to attend for a baseline interview with a registered nurse and required to complete a standardised questionnaire with characteristics, life course exposures, medical history, treatments and family history. They consented to lifelong linkage to health data including cancer registries and Hospital Episode Statistics and cause of death data. Patients also underwent standardised anthropometric physical measurements such as BMI and blood pressure at enrolment and donated urine and serum plasma.

Definition of the Phenotype

Cases and controls were identified from the UKB release for our project, which was coded in a series of fields.. To identify cases, we used ICD9 and 10 codes for cervical cancer and carcinoma in situ, and searched UKB fields linking to UK cancer registries and hospital episode statistics (HES). To define controls, we identified women who were not cases but also with no history of any cervical abnormality, including mild or moderate dysplasia according to participant UKB questionnaires, UKB interviews, and HES (appendix 4).

We restricted the main analysis to European ancestry, based on genetic ancestry provided by UK Biobank. A high degree of relatedness has been noted in the UK biobank population and therefore to reduce confounding we excluded related individuals. We used a graph representation of the kinship between participants to define a set of unrelated individuals, as described elsewhere⁷⁵. In this graph, women participants retained for analysis are the vertices and there is an edge between them if they are 3rd degree relatives or closer. Families correspond to connected components, i.e., sets of nodes that are inter-connected but isolated from the rest of the graph. For each family, we used the algorithm implemented in the igraph package in R to identify the largest set of unrelated individuals³¹², i.e. vertices that are not connected to any other vertex. These sets are combined to form the largest possible set of unrelated participants in the population under study. To adjust for covert relatedness, we adjusted for ten principal components

UK Biobank genotyping and imputation

Serum plasma was genotyped using a custom Affymetrix UKB Axiom Array Chip (820,967 variants) and the UK BiLEVE array (807,411 variants), which share 95% variant content³¹³. Imputation was undertaken centrally by the UKB in a merging of three reference panels, namely the UK10K, 1000 genomes Phase 3 panel and the Haplotype reference consortium (HRC)⁷⁵. This resulted in 9,600,464 SNPS with a minor allele frequency (MAF) >1% available for GWAS. Unexpected duplicates were later removed in R v4.02.

Samples which failed genotyping quality control (QC), had high heterozygosity for autosomal chromosomes or missingness (samples identified as outliers in UKB data field 22027), genetic sex discordance, high guanine-cytosine (GC) content, missing covariates and duplicates were excluded from the present analysis. Resultantly N=235,716 samples were retained for further analysis.

To reduce bias due to high degree of relatedness within the UKB, we used a graph representation of the kinship between participants from UKB kinship coefficient data, to define a set of (N=180,224) unrelated individuals of all ancestries.

Case numbers in different ethnicities were included, however the number of non-European cases were too small (N=57, 45, 33, and 17 cases in participants with Black, Mixed, Asian and Chinese ethnic backgrounds, respectively) (Table 7) to perform analyses stratified on each group separately. To avoid bias from trans-ethnic linkage disequilibrium (LD) pattern heterogeneity, data was restricted the largest ethnic group, which was European ancestry (N=150,314). Ancestry was identified by genetic information, as extracted from UKB field 22006.

Table 7. Individual characteristics of the 180,224 unrelated UK Biobank participants of all ethnicities, as identified by UKB genotyping data

	Controls (N=174,717)		CIN3 cases (N=4,620)		Cancer cases (N=887)	
	N	Mean (s.d.) / Proportion	N	Mean (s.d.) / Proportion	N	Mean (s.d.) / Proportion
Age at recruitment	174,717	55.83 (8.00)	4,620	53.05 (7.69)	887	56.16 (7.26)
Ethnic background						
European*	145,545	83.30%	4,005	86.69%	764	86.13%
<i>White (Other)</i>	19,987	11.44%	487	10.54%	99	11.16%
<i>Asian</i>	3,517	2.01%	22	0.48%	11	1.24%
<i>Black</i>	3,516	2.01%	51	1.10%	6	0.68%
<i>Mixed</i>	1,353	0.77%	40	0.87%	5	0.56%
<i>Chinese</i>	799	0.46%	15	0.32%	2	0.23%
Smoking status						
<i>Never</i>	106,244	60.81%	1,981	42.88%	411	46.34%
<i>Former</i>	53,492	30.62%	1,746	37.79%	330	37.20%
<i>Current</i>	14,981	8.57%	893	19.33%	146	16.46%
Townsend score**	174,717	-1.38 (3.02)	4,620	-0.79 (3.17)	887	-0.91 (3.24)

*Main analysis restricted to European ancestry as largest ethnic group

**Townsend score is a deprivation index used to measure material deprivation based on four variables: unemployment, non-car ownership, non-home ownership, household overcrowding, where increasing score implies increased deprivation.

Abbreviations. N: Number of participants; s.d: standard deviation.

The primary disease traits studied consisted of diagnoses of both cervical cancer and carcinoma *in situ* (cervical intraepithelial neoplasia stage 3 (CIN3)). Cervical cancer cases (N=4,769) were identified as either CIN3 or ICC using diagnoses from a series of predefined ICD (international classification of diseases) codes via linkage to UK cancer registries (follow-up until 14 December, 2016) and hospital episode statistics (HES, up to 31 March, 2017) (appendix P 10 and 11). Controls (N=145,545) were identified as women with no record or reported history of any cervical abnormality from nurse-administered questionnaires at recruitment and linkage to HES data.

Quality control

Several Quality Control (QC) steps were undertaken centrally by UKB prior to data release including examination for deviation from Hardy-Weinberg Equilibrium (HWE), batch and plate effects, sex effects, array effects and sex chromosome discordance across control replicates. These SNPs were set to missing for the corresponding batch in the genetic data files provided by UKB. Samples were tested for heterozygosity and missing rates resulting in genotyping data for 488,377 individuals after QC. We removed all individuals with high missingness, sex discordance or high heterozygosity for autosomal chromosomes. We excluded women that withdrew consent. Further information on the full QC performed by the UKB are available^{75,314}.

Analysis Model

The association study was performed under an additive univariate logistic model regressing the cervical cancer status (including N=4,005 CIN3 cases, and/or N=764 ICC cases) as the outcome against each of the imputed SNPs using PLINK v1.9b3.3.

To maximise statistical power, we adopted in our primary analyses a broad definition of cervical cancer including both CIN3 (carcinoma in situ, N=4,005) and invasive cervical cancer (ICC, N=764) outcomes (CIN3/ICC, N=4,769). To account for disease heterogeneity and progression, we subsequently analysed cases from each subtype separately.

Regression models were adjusted for established confounders including age, smoking status (classified as never/former/current, as obtained from UKB field 20116), social deprivation score (UKB field 189), and to model technically induced nuisance variation, the analytical batch (UKB field 22000). Effect size estimates were expressed as odds ratio (OR) and 95% confidence interval (CI) measuring risk change per copy of the minor allele. To control for population structure and covert relatedness, we adjusted our analyses for the first ten principal components as estimated by UKB and capturing the

study population latent genetic structure. We only considered SNPs with a minor allele frequency (MAF) $>1\%$ ($N=656,284$ genotyped; $9,600,464$ imputed). To control for multiple testing, we considered the established genome-wide significance level of 5×10^{-8} .

We also conducted a sensitivity analysis only considering genotyped variants (i.e., discarding imputed genotypes).

Derivation of independent loci

We performed a series of conditional analyses to identify a set of genetic variants that are independently associated with the cervical cancer outcomes and complementarily contribute to the outcome explanation. For each chromosome separately, an iterative procedure was performed which involved identifying the SNP with the strongest association and conditioning the logistic regression model on that SNP. This begins a stepwise and conditional process, where the next strongest SNP is added to the logistic regression model. Iterations of the analysis were continued until there were no remaining SNPs reaching genome-wide significance level ($P \leq 5 \times 10^{-8}$). Conditional P-values (P^{cond}) are defined for each SNP as the P-value for the model adjusted for all other associated SNP in the chromosome. We evaluated the novelty of our findings based on reported SNPs from the GWAS Catalog³¹⁵ [accessed 2020-05-20] and accounted for a 1Mb window. LocusZoom software and visual inspection of heatmaps (generated in LDlink based on 1k genomes project) were used to explore linkage disequilibrium (LD) blocks of SNPs identified in conditional analyses.

Replication dataset

We performed an independent lookup of the conditional SNPs identified in our discovery dataset using the FinnGen release 5 dataset. Replication in FinnGen r5 was sought for the SNPs and INDELS found genome-wide significant ($P \leq 5 \times 10^{-8}$) with a $\text{MAF} \geq 1\%$ following conditional analysis in our discovery set, using a nominal significance level of $P=0.05$.

Population: Patients and control subjects in FinnGen provided informed consent for biobank research, based on the Finnish Biobank Act. Alternatively, older research cohorts, collected prior the start of FinnGen (in August 2017), were collected based on study-specific consents and later transferred to the Finnish biobanks after approval by Fimea, the National Supervisory Authority for Welfare and Health. Recruitment protocols followed the biobank protocols approved by Fimea. The Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (HUS) approved the FinnGen study protocol Nr HUS/990/2017.

Genotyping: Genotyping was performed for ~600,000 samples on the FinnGen ThermoFisher Axiom custom array through the ThermoFisher genotyping service in San Diego. The array consists of 736,145 probes for 655,973 markers. In addition to the core GWAS markers it contains 116,402 coding markers enriched in Finland, specific markers for the *HLA* region, ClinVar markers, pharmacogenomic markers and selected markers that were of special interest for partners. Legacy samples from other biobanks have previously been genotyped over the years using various generations of Illumina GWAS arrays. All GWAS data was imputed against a Finnish population specific whole genome sequence (WGS) backbone. More details can be found at: <https://www.finnngen.fi/en/researchers/genotyping>.

Phenotyping: Cervical cancer phenotypes cases were identified from Finnish Hospital Discharge register, Finnish Cancer Registry, and Cause of Death registry according to ICD10, ICD9 and ICD8 codes: “cervical dysplasia”, including cases of CIN 1-3 (ICD10: N87.0-9; ICD8: 621), “cervical carcinoma in situ”, including both endo- and ectocervical carcinoma in situ / CIN3 cases (ICD-10: D06), and malignant neoplasm of the uterine cervix (ICD10: C53; ICD9 & ICD8: 180). CIN3 cases are in Finland invariably coded either as D06 or N87.2, but most of CIN3 cases can be assumed to be included in the “cervical dysplasia” phenotype. All other women were included as controls. Covariates such as smoking and social deprivation score were not available. In total we included 4,246 cases from three phenotypes (Table 8).

Table 8. Description for phenotypes available in the FinnGen database (available at: https://risteys.finnngen.fi/phenocode/N14_DYSPLACERVUT#endpoint-definition).

FinnGen Phenotype	Cases/Controls
Malignant neoplasm of cervix uteri (ICC)	1648/121931
Carcinoma in situ of cervix uteri (CIN3)	298/123281
Dysplasia of cervix uteri (cervical dysplasia CIN1-3)	4246/68969

Abbreviations: CIN: cervical intraepithelial neoplasia; ICC: invasive cervical cancer.

Functional analyses of variants and genes

To explore the phenotypic and functional effects of detected genetic loci, We used publicly available databases to annotate variants which included Phenoscanner³¹⁶, UCSC genome browser³¹⁷, dbSNP³¹⁸ and ENCODE³¹⁹. We searched for previously reported associations with other disease traits in the GWAS Catalog³¹⁵. We subsequently used publicly available datasets to explore potential carcinogenic pathways through the examination of gene expression in cervical cancer tissue, normal cervical tissue,

and other tumour tissues (including eQTL ratios in the PanCanQTL database³²⁰ and the GTEx Portal³²¹). We subsequently used publicly available datasets to explore potential carcinogenic pathways through the examination of gene expression in cervical cancer tissue, normal cervical tissue, and other tumour tissues (including eQTL ratios in the PanCanQTL database³²⁰ and the GTEx Portal³²¹). To establish if any gene already has a known role in cancer, we further searched somatic mutations (COSMIC³²²) in cervical, and other cancers. To explore the role of intronic variants, we investigated possible indirect effects on gene expression through transcription regulation including histone marks and motifs in HaploReg v4.1³²³.

Mendelian randomisation

To investigate the potential causal effects of proposed environmental risk factors on cervical cancer, we conducted a two-sample Mendelian randomisation (MR) analysis, using independent ($r^2 < 0.001$) genetic variants with known effects on the risk factors, as instruments for the exposure (at genome-wide significance)^{324,325}. From our umbrella review in chapter one we identified all exposures of interest (appendix 5). For the exposures of interest, we searched the GWAS Catalog³¹⁵ to identify relevant GWAS studies providing female-specific summary-level genetic data. We used data from this genome-wide association study to obtain associations of SNPs with risks of cervical cancer²⁵⁷.

We used inverse variance weighted (IVW) MR as the main analysis to estimate the causal effect on risk for cervical cancer³²⁶. For exposures with significant effects in the main IVW MR analysis (multiple testing adjusted using Benjamini and Hochberg's false discovery rate³²⁷), we performed sensitivity analyses using a range of robust MR approaches to circumvent possible violations of the instrumental variable assumptions (weighted median³²⁸ and Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO)³²⁹). We subsequently performed two multivariable MR models (appendix P 5) to account for potential pleiotropy and attenuation when adjusting for (i) age of first pregnancy or (ii) risky behaviours (lifetime smoking index, number of sexual partners). For multivariable MR, we used genetic variants associated with either of the two exposures as instrumental variables at genome-wide significance.

2.3 RESULTS

From the original 235,716 female participants with genotyping and phenotypic data available in UKB (after quality control), 85,402 were excluded (Figure 10). 150,314 women were included in subsequent analyses (4,769 cases (764 ICC and 4005 CIN3), and 145,545 controls). Characteristics of the participants are described in Table 9.

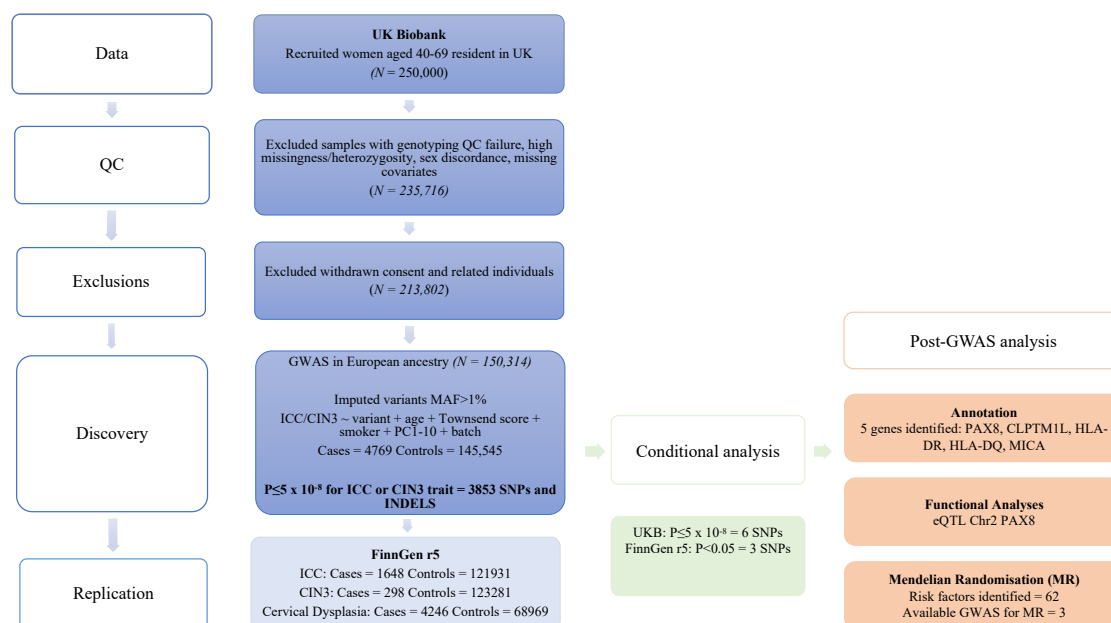


Figure 10. Flowchart describing the exclusion criteria and the analytical plan for the discovery (UK Biobank) and replication (FinnGen r5) sets. *Abbreviations: ICC: invasive cervical cancer; CIN3: cervical intraepithelial neoplasia grade 3; LD: linkage disequilibrium; P: P-value; QC: quality control; SNP: single nucleotide polymorphism.*

Table 9. Individual characteristics of the included 150,314 unrelated UK Biobank participants.

	Controls (N=145,545)		CIN3 cases (N=4,005)		ICC cases (N=764)	
	N	Median (LQ/UQ)/ Proportion	N	Median (LQ/UQ)/ Proportion	N	Median (LQ/UQ) / Proportion
Age at recruitment	145,545	57 (50/63)	4,005	53 (47/59)	764	57 (51/62)
Smoking status						
<i>Never</i>	88,213	60.61	1,729	43.17	348	45.55
<i>Former</i>	45,214	31.07	1,505	37.58	287	37.57
<i>Current</i>	12,118	8.33	771	19.25	129	16.88
Townsend score ⁺	145,545	-2.37 (-3.75/-0.04)	4,005	-1.65 (-3.37/1.20)	764	-1.86 (-3.47/1.00)

⁺Townsend score is a deprivation index used to measure material deprivation based on four variables: unemployment, non-car ownership, non-home ownership, household overcrowding, where increasing score implies increased deprivation.

Abbreviations: LQ: Lower quartile; N: Number of participants; s.d: standard deviation; UQ: Upper quartile; CIN3: cervical intraepithelial neoplasia grade 3; I CC: invasive cervical cancer

Our main GWAS identified 3,853 SNPs and INDELS associated with the risk of ICC or CIN3 (CIN3/ICC). These associations targeted three genetic regions: (i) the *HLA* region in which 3,815 of the 3,853 detected associations were located (chr6: base pairs (bp) 31324647–32623713), (ii) *PAX8* (2q14.1; lead SNP rs10175462, (OR=0.87 (95%CI=0.84–0.91); $P=1.07 \times 10^{-9}$), and (iii) *CLPTMIL* (5p15.33; lead SNP rs27069, (OR=0.88 (95%CI=0.84–0.92); $P=2.51 \times 10^{-9}$) (Figure 11). Conditional analyses identified six loci independently associated with the CIN3/ICC (Table 10). This included one signal in chromosome 2 (driving SNP rs10175462 and INDEL 113992800_TCC_T), one in chromosome 5 (driving SNP rs27069), and four independent signals in the *HLA* region: three common non-coding variants (rs9272050 (OR=1.27 (95%CI=1.21-1.32); $P=2.51 \times 10^{-28}$), rs6938453 (OR=0.79 (95%CI=0.75-0.83); $P=1.97 \times 10^{-17}$), rs55986091 (OR=0.66 (95%CI=0.60-0.72); $P=6.42 \times 10^{-22}$)) and one low-frequency missense variant in the *HLA-B* gene (rs9266183, (OR=0.73 (95%CI 0.64-0.83); $P=1.53 \times 10^{-6}$; OR^(cond)=0.68 (95%CI=0.59-0.77); $P^{(cond)}=6.20 \times 10^{-9}$); minor allele frequency (MAF) 0.04 in Europeans). Conditional analyses identified rs117960705 (chromosome 12) as the only variant independently associated with the ICC outcome, and rs150406145 (chromosome 2) and rs9272245 (chromosome 6, *HLA* region) as independent markers of CIN3 (Table 10). There was minimal evidence of genomic inflation, which reduced when examining the *HLA* region alone (Figure 12).

Table 10. Results from the logistic models predicting from each SNP the CIN3/ICC, CIN3, or ICC case/control status. Models are adjusted on age, smoking status, Townsend index, batch and the first 10 PCs. Results are reported for GWAS significant SNPs following conditional analysis ($P^{\text{cond}} < 5 \times 10^{-8}$) for CIN3/ICC (A), CIN3 only (B), and ICC only (C).

A. CIN3/ICC

RSID	CHR	BP	A1	MAF	Info	Variant Type	Nearest Gene	N	CIN3/ICC				CIN3				ICC			
									OR	95%CI	P	<i>P</i> ^{cond}	N	OR	95%CI	P	N	OR	95%CI	P
rs10175462	2	113988492	A	0.362	1.000	intron	PAX8	150,314	0.87	0.84-0.91	1.07E-09	1.07E-09	146,313	0.88	0.79-0.98	1.55E-2	146,309	0.91	0.82-1.02	9.26E-2
rs27069	5	1347128	T	0.431	0.986	intergenic	CLPTMIL	146,530	0.88	0.84-0.92	2.51E-09	2.51E-09	142,644	0.86	0.77-0.95	4.64E-3	142,641	0.86	0.77-0.95	3.61E-3
rs9266183	6	31324647	C	0.037	1.000	missense	HLA-B	150,314	0.73	0.64-0.83	1.53E-06	6.20E-09	146,313	0.75	0.55-1.03	7.73E-2	146,309	0.63	0.45-0.89	7.88E-3
rs6938453	6	31377793	A	0.228	0.982	intron	MICA	146,033	0.79	0.75-0.83	1.97E-17	1.35E-15	142,125	0.79	0.69-0.91	7.42E-4	142,121	0.76	0.66-0.87	7.78E-5
rs9272050	6	32599071	G	0.385	0.999	intron	HLA-DQA1	150,211	1.27	1.21 -1.32	2.51E-28	2.51E-28	146,212	1.42	1.29-1.58	9.56E-12	146,209	1.20	1.08-1.33	6.69E-4
rs55986091	6	32623713	A	0.091	0.995	intergenic	HLA-DQB1	149,757	0.66	0.60-0.72	6.42E-22	1.02E-11	145,773	0.68	0.56-0.84	3.22E-4	145,771	0.72	0.59-0.89	1.84E-3

B. CIN3

RSID	CHR	BP	A1	MAF	Info	Variant Type	Nearest Gene	N	CIN3/ICC				N	CIN3				N	ICC		
									OR	95%CI	P			OR	95%CI	P	<i>P</i> ^{cond}		OR	95%CI	P
rs150406145	2	6371190	A	0.012	0.929	Intron	PAX8	149,360	1.33	1.12-1.56	8.54E-4	145,385	2.37	1.74-3.22	4.14E-8	4.14E-8	145,380	1.05	0.66-1.65	8.51E-1	
rs9272245	6	32602872	C	0.364	-	intergenic	HLA-DQA1	149,283	1.26	1.21-1.31	1.59E-26	145,318	1.44	1.30-1.60	4.60E-12	4.60E-12	145,323	1.21	1.09-1.34	4.23E-4	

C. ICC

RSID	CHR	BP	A1	MAF	Info	Variant Type	Nearest Gene	N	CIN3/ICC				N	CIN3				N	ICC			
									OR	95%CI	P			OR	95%CI	P			OR	95%CI	P	
rs138446575	1	2811427	T	0.013	0.953	intergenic	TTC34	149,651	1.24	1.04-1.48	1.66E-2	145,674	1.03	0.64-1.64	9.12E-1	145,671	2.39	1.75-3.27	4.97E-8	4.97E-8		
rs117960705	12	109645816	G	0.090	1.000	intron	ACACB	150,314	1.22	1.04-1.44	1.71E-2	146,313	0.78	0.47-1.28	3.21E-1	146,309	2.41	1.81-3.23	2.64E-9	2.64E-9		

Abbreviations. A1: allele 1; BP: base pairs; CHR: chromosome; CIN3: cervical intraepithelial neoplasia grade 3; ICC: invasive cervical cancer; MAF: minor allele frequency; OR: odds ratio; P: P-value; P^{cond} : P-value after conditional analysis; RSID: genomic variant id number; 95%CI: 95% confidence interval

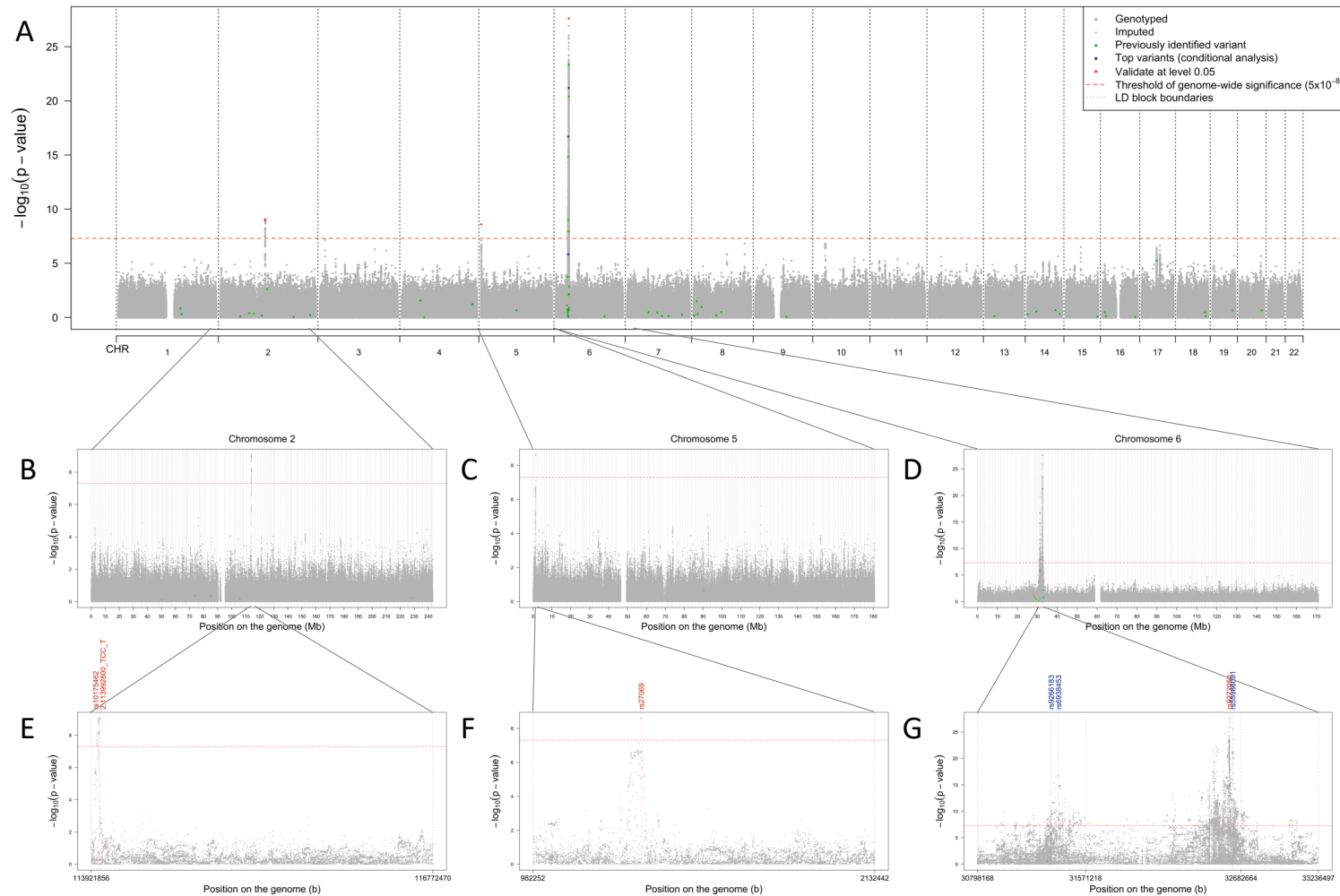


Figure 11. Manhattan plots showing the associations between genetic variants and CIN3/ICC in the European unrelated population (N=4,797 cases vs N=146,178 controls). Genetic variants are ordered by their position on the genome (X-axis). The P-values, measuring the strength of association, are derived from additive logistic models and are represented on the $-\log_{10}$ scale (Y-axis). The genome-wide significance level is set to 5×10^{-8} (horizontal red line). Results are presented for all the (N=9,600,464) assayed and imputed variants (A), for chromosome 2 (N=798,812 variants, B), 5 (N=616,800 variants, C), and 6 (N=651,104 variants, D). Within each chromosome, the regions containing significant associations are represented in more detail (E, F, G, respectively). Lead SNPs identified in the conditional analysis (blue: rs10175462; rs27069; rs272050; rs6938453; rs55986091; rs9266183) and derived from FinnGen r5 replication (red: rs10175462 or 113992800_TCC_T; rs27069; rs9272050) are represented.

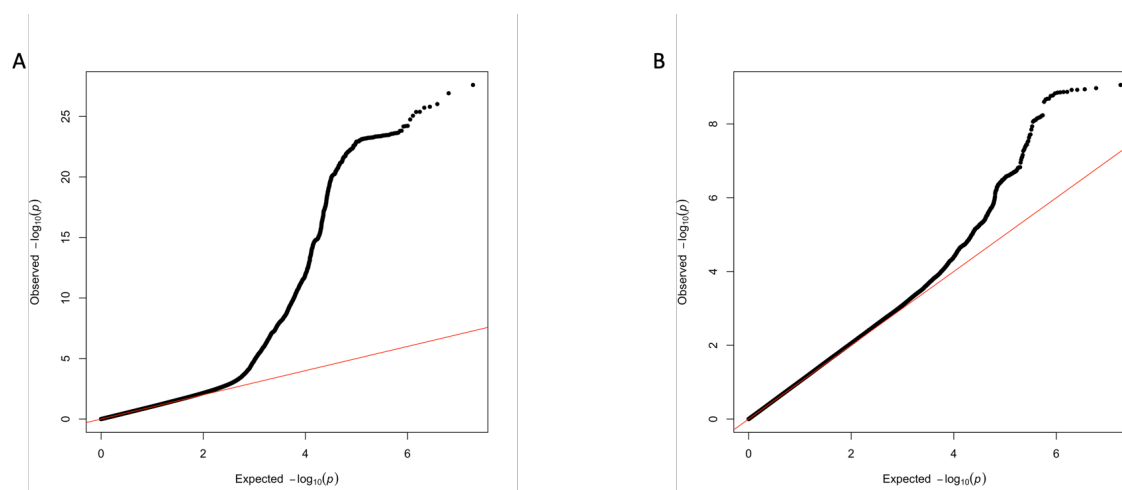


Figure 12. Quantile-quantile (QQ) plot of P-values from the genome-wide association study of cervical cancer in unrelated participants of European ancestry. The observed P-value of association between each variant and the cervical cancer outcome (Y-axis) is represented as a function of the expected P-value under the assumption of their uniform distribution (X-axis). The red line represents perfect agreement between the observed and expected values. A) Full genome including HCA region B) Excluding HCA region.

When restricting the phenotype to (N=764) ICC cases only, signals in the *PAX8* and *HLA* regions were attenuated and did not reach the genome-wide significance level, and a novel locus was detected at 12q24.11 (rs117960705; OR=2.41 (95%CI=1.81-3.23); $P=2.76 \times 10^{-9}$) located in the *ACACB* gene (appendix P 44). In a comparative random subset of 764 CIN3 cases, previously detected *PAX8* signals were no longer apparent, while *HLA* loci remained associated with CIN3 at a genome-wide significance level (Figure 13).

Analysis restricted to genotyped variants revealed the same pattern of association as the imputed and genotyped variants in *HLA* and *PAX8* regions, but the signal in *CLPTMIL* no longer reached the genome-wide significance level (Figure 14).

Regional plots were used to explore the LD of SNPs on the same chromosome (Figure 15). Regional plots of sentinel SNPs further suggested single signals at *PAX8* and *CLPTMIL* genes, whilst associations in the *HLA* were tightly clustered. Exploration of the LD structures of conditional signals showed single blocks in chromosomes 2 and 5, and a complex LD structure in the *HLA* region. Comparison of the three remaining conditionally associated SNPs in the *HLA-DQA1*, *HLA-DQB1* and *MICA* with GWAS Catalog, suggests they had not been previously identified, but were in high LD with reported variants ($r^2 > 0.8$), while the *HLA-B* low-frequency missense variant rs9266183 was not tagged by any previous study.

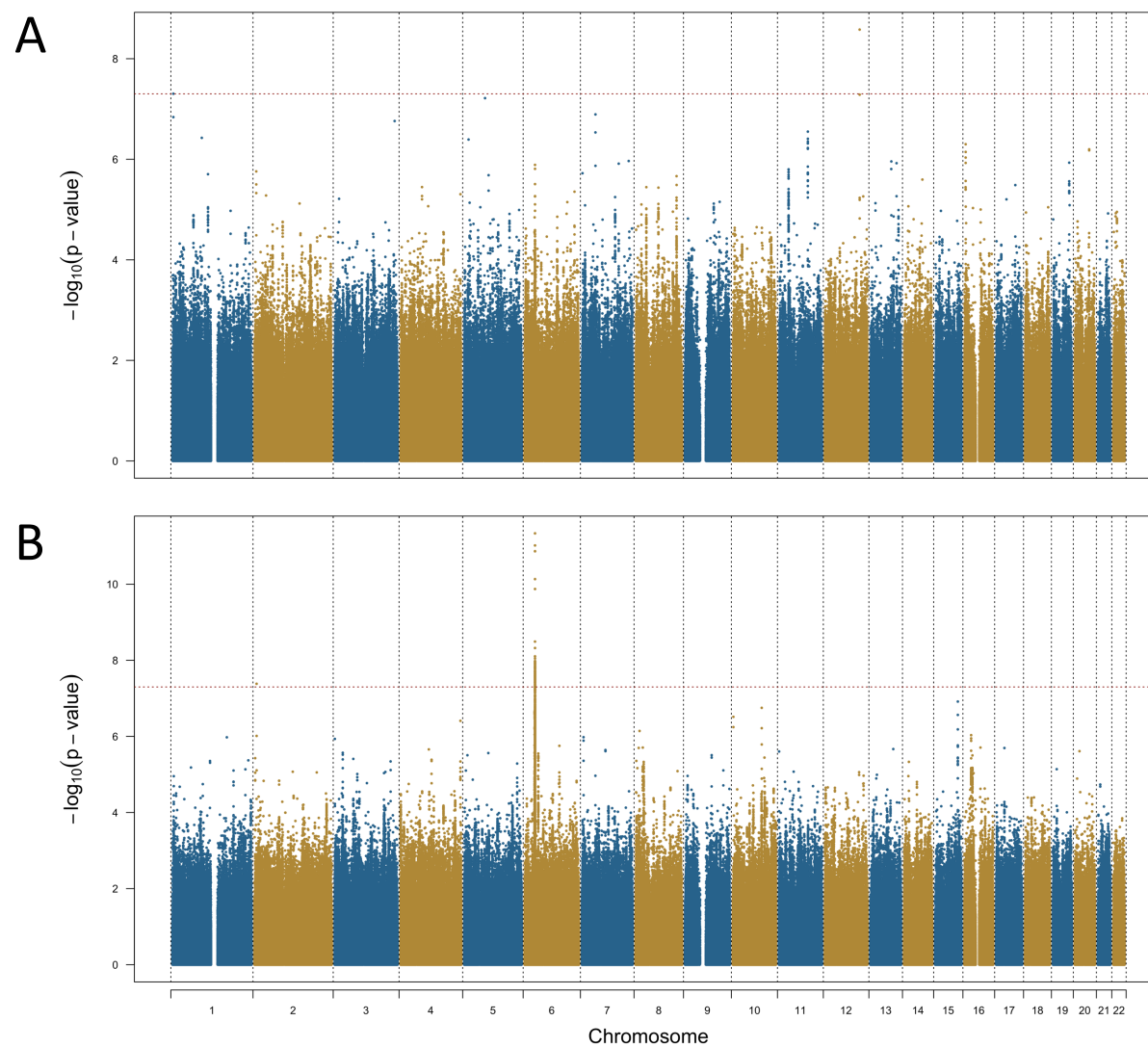


Figure 13. Manhattan plots showing the associations between genetic variants and ICC (A, N=764 cases and N=146,178 controls) or CIN3 status (B, subset of N=764 cases and N=146,178 controls) in the European unrelated population. Genetic variants (N=9,600,464) are ordered by their position on the genome (X-axis). The P-values, measuring the strength of association, are derived from additive logistic models, and are represented on the $-\log_{10}$ scale (Y-axis). The genome-wide significance level is set to 5×10^{-8} (horizontal red line)

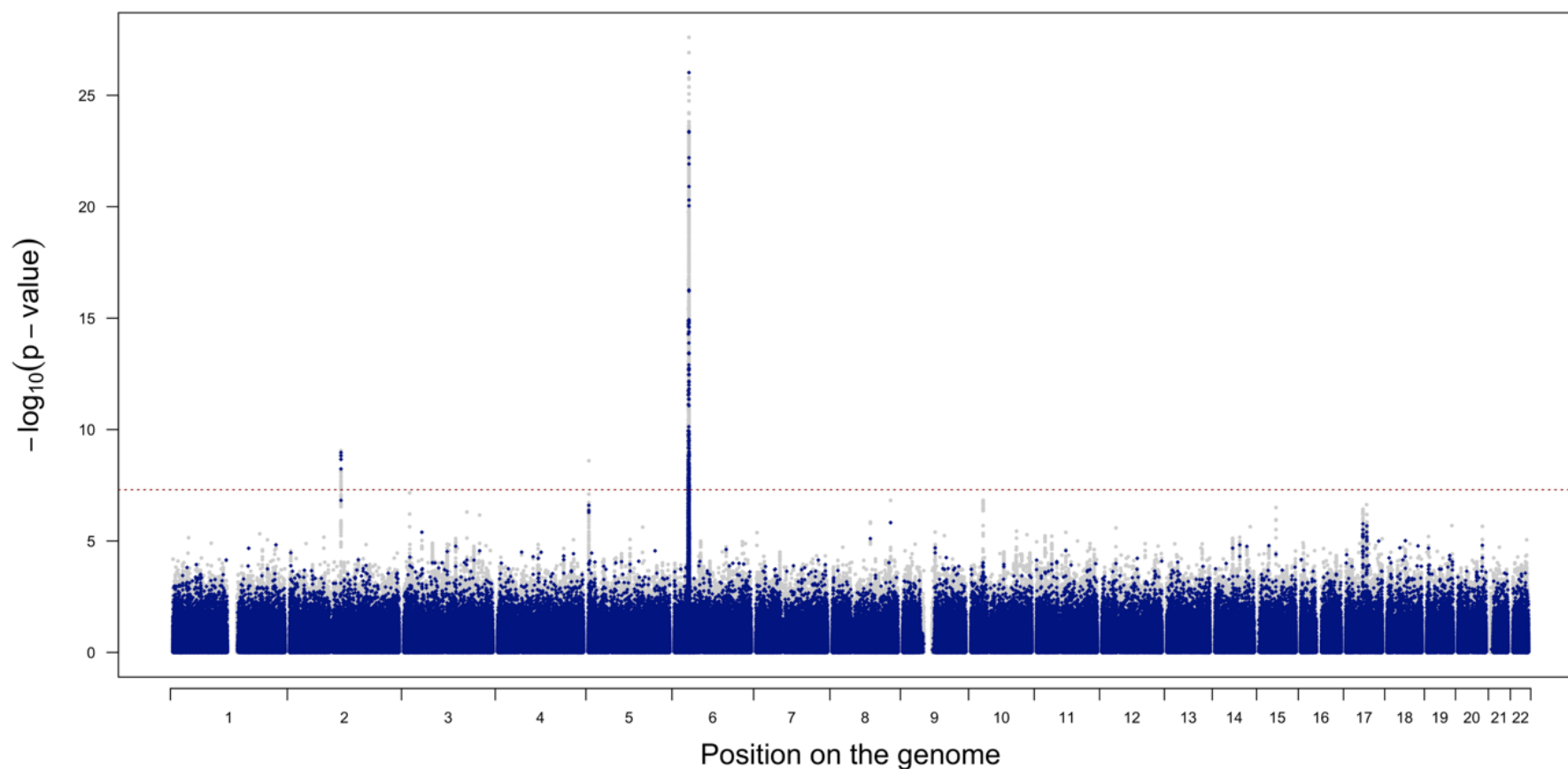


Figure 14: Manhattan plots showing the associations between genetic variants and cervical cancer status in the European unrelated population (N=4,797 cases, of which 764 ICC, vs N=146,178 controls). Genetic variants are ordered by their position on the genome (X-axis). The P-values, measuring the strength of association, are derived from additive logistic models and are represented on the $-\log_{10}$ scale (Y-axis). The genome-wide significance level is set to 5×10^{-8} (horizontal red line). Results are presented for all the (N=9,600,464) genotyped and imputed variants (grey), and the (N=656,284) genotyped only variants (blue).

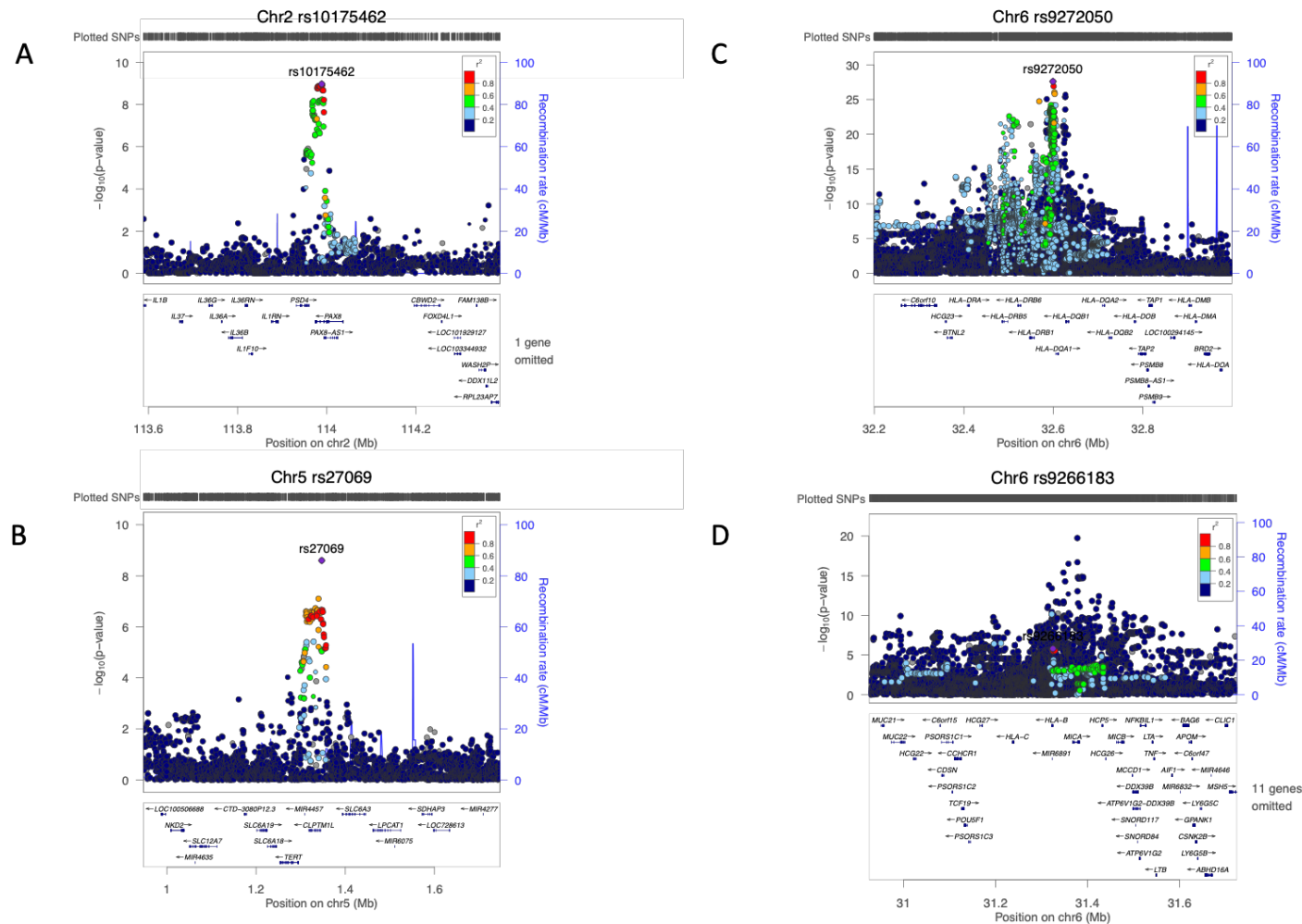


Figure 15. Regional Plots of sentinel SNPs from conditional analysis associated with CIN3/ICC in *PAX8* (Chr2, A), *CLPTMTL* (Chr5, B) and *HLA* (Chr6, C-D). The P-value of the association between variant and the cervical cancer status is represented by the log10 scale (Y-axis) in function of the genetic location. SNPs are colour coded in relation to their correlation (as measured by their recombination rate (r^2)) with the sentinel SNP (represented in purple) in the region. In the lower panel of each plot, gene annotations are provided according to the 1K genomes project 2014.

In the FinnGen release 5 (r5), in the absence of detailed information on disease subtypes, we performed three separate GWAS of cervical precancer and cancer phenotypes including a total of 128,123 participants. We investigated separately, (i) the ‘cervical dysplasia’ outcome (N=4,246, including mainly CIN3 but also CIN1-2 cases, Figure 3), (ii) carcinoma in situ (CIN3) outcome (N=298), and (iii) ICC outcome (N=1,648). Of our 6 conditional loci, three were assayed in FinnGen r5 (*PAX8* rs10175462 (and INDEL rs35724515); *CLPTMIL* rs27069; *HLA-DQA1* rs9272050). Replication was achieved for the *PAX8* SNP rs10175462 (and INDEL rs35724515) for ICC ($P=0.015$) and cervical dysplasia ($P=0.0002$) phenotypes with concordant direction of effect. The effect of rs10175462 on CIN3 phenotype, potentially due to the smaller sample size, failed to reach nominal statistical significance ($P=0.069$; the OR=0.86 (95%CI 0.73-1.01)), hence providing weak evidence for this association (appendix P 36). The *HLA-DQA1* lead SNP (rs9272050) replicated in ICC ($P=7.90 \times 10^{-8}$), CIN3 ($P=0.0005$) and cervical dysplasia ($P=5.33 \times 10^{-10}$) phenotypes. Lead SNP rs27069 in the *CLPTMIL* gene also replicated in ICC ($P=2.54 \times 10^{-7}$), CIN3 ($P=0.04$) and cervical dysplasia ($P=0.0004$) phenotypes (appendix 6).

Functional analysis

The strongest association on chromosome 2 was an INDEL (rs35724515), which was tagged to rs10175462 (LD $r^2=1$, distance 4.3 kb). When investigating the eQTL we detected that the whole region showed allele-specific long-range epigenetic silencing in cervical cancer tissues (Figure 16). Meanwhile examination of gene expression in healthy tissues suggested *PAX8* has a limited expression across tissue types and is mostly limited to cervix, thyroid and kidney tissues. For this locus, histone marks were also noted in HeLa (cervical cancer) cell lines, suggesting the SNP may have regulatory effects. Interrogation of the GWAS catalog revealed this SNP has been previously associated with a phenotype of reduced lymphocyte counts³³⁰. This SNP has not previously been associated with cervical or any other cancers, however increased *PAX8* gene expression has been associated with the mucinous ovarian cancer trait³³¹ and in epithelial ovarian cancer tumour tissue³³². Overall, there appears to be evidence of a regulatory function of this locus in predisposition to altered immune or carcinogenic pathways.

Annotation of rs27069 revealed an intergenic variant located 2029bp downstream from the *CLPTMIL* (also known as cisplatin-resistance gene), commonly co-expressed with the *TERT* gene. This SNP had a reported co-localised eQTL locus in oesophageal mucosal tissue and skin in GTEx however, no data were available for cervical tissue. HaploReg reported this SNP as motif-altering for YY1, a transcription factor which has been reported to be correlated with expression of the HPV16 oncogene E6 and

progression from CIN to cancer³³³. Previous SNP associations have been reported for this gene and HPV-driven plantar warts³³⁴, as well as oral cavity³³⁵ and nasopharyngeal³³⁶ cancers – a significant proportion of which are hrHPV-driven. Sequence variation at the *TERT-CLPTMIL* locus has been previously associated with cervical cancer as well as other squamous and adeno-carcinoma types including skin and lung cancers³³⁷.

We detected *HLA* associations including protective and risk variants in proximity of *HLA-DRB1*, *HLA-DQA1* and *MICA* genes. The protective association observed at rs9266183 in the *HLA-B* region (6p21.3) has not previously been associated to cervical cancer, and according to the dbSNP reference report, is a predicted missense variant located in the *MHC Class I* gene; this causes an amino acid change from Aspartate to Glycine. Upstream (3'UTR) from the variant is a co-localised microRNA (MIR6891), which has been reported to affect local RNA stability including transcripts for the IgA heavy chain³³⁸. Cervical mucosal IgA responses to HPV are recognised to be important in preventing infection, re-infection, spread to adjacent cells and HPV clearance³³⁹.

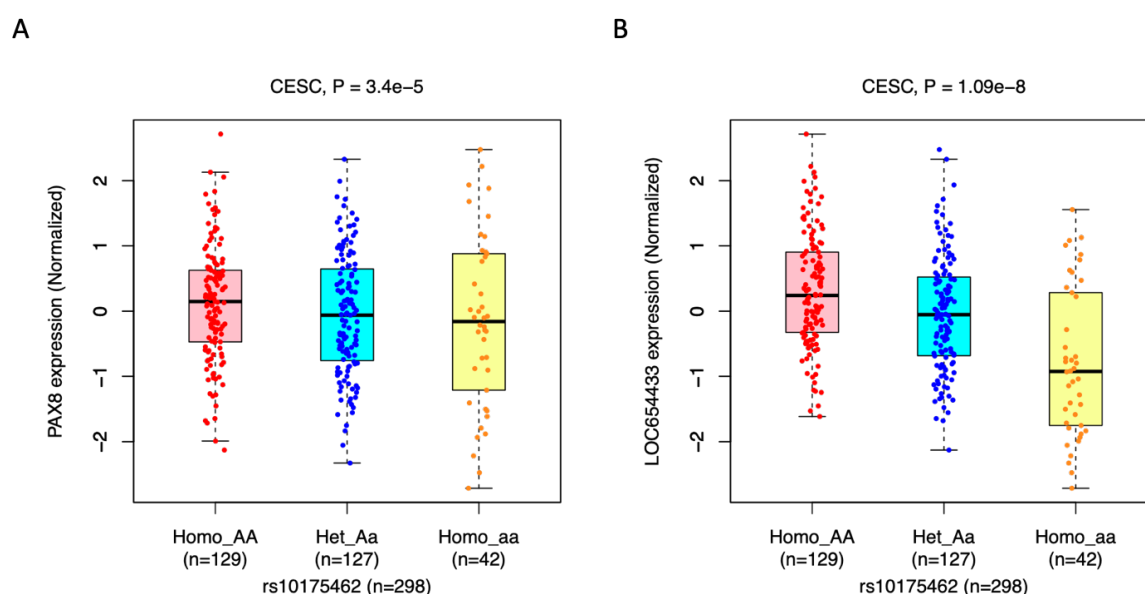


Figure 16. Significant SNPs were annotated for their effect on eQTL ratios in ICC from the PanCanQTL database. Box plots show the gene expression levels in *PAX8* (A) and *LOC654433* (B) genes, for 298 cervical cancer tissue samples for the three genotypes at rs10175462. Overall P-value for differential expression, as computed from an additive linear model (performed using Matrix eQTL), is given above the plot. Homozygosity for the T allele was associated with decreased gene expression at *PAX8* and *LOC654433*. The T allele in the discovery set conferred a decreased risk of cervical cancer trait for *PAX8* (OR=0.873; SE = -6.05; P= 1.41e-9) and *LOC654433* (OR=0.670; SE =-5.90; P=1.09e-8). Abbreviations: CESC: cervical squamous cell cancer tissue; Homo: homozygous; Het: heterozygous.

Mendelian randomisation

In the mendelian randomisation, 12 exposures had genetic instruments available to perform Mendelian randomisation, namely smoking³⁴⁰, reproductive behaviour in women as measured by age at first pregnancy³⁴¹, number of sexual partners³⁴², lupus³⁴³, rheumatoid arthritis, inflammatory bowel disease, alcohol consumption³⁴⁴, body mass index³⁴⁵, gestational diabetes mellitus, parity (number of living births) and height³⁴⁶. No HPV infection phenotypes were identified, however genetic instruments for both CIN3 and cervical cancer phenotypes were available.

The strongest associations with risk of cervical cancer were observed for smoking (OR 2.46, 95% CI=1.64–3.69) and number of sexual partners (OR=1.95, 95% CI=1.44–2.63). We additionally identified a protective effect for older age at first pregnancy (OR=0.80, 95%CI=0.68–0.95), while rheumatoid arthritis increased the risk of cervical cancer (OR=1.10 95%CI=1.05-1.15). The exposures of systemic lupus erythematosus, inflammatory bowel disease (on immunosuppression), alcohol consumption, body mass index (BMI), gestational diabetes mellitus (GDM), nulliparity and height all showed no significant association with CIN3 or cervical cancer (Figure 17).

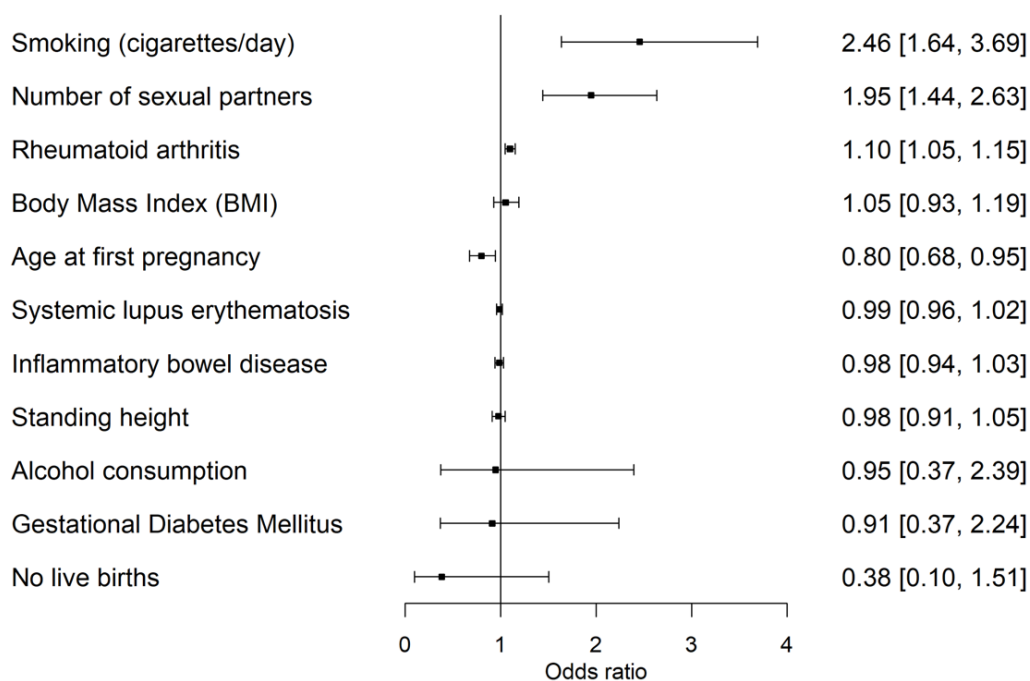


Figure 17. Forest plot shows inverse variance weighted mendelian randomisation for all identified known environmental risk or protective factors for cervical cancer with available GWAS, to determine effect sizes by OR and 95% CI (x-axis; n=11).

Sensitivity analyses using weighted-median and MR-Presso confirmed consistent effect sizes with the main analysis based IVW. To account for potential pleiotropic effects, we performed two attenuation analyses. We found that the effect of age at first pregnancy was independent of the number of sexual partners as there was no attenuation for age at first pregnancy when accounting for number of sexual partners. This suggests an independent protective exposure for increased age of first pregnancy on cervical cancer. In contrast, in the multivariable MR model including both smoking and number of sexual partners, the effect of smoking did attenuate when accounting for the number of sexual partners but remained significant (OR=1.74, 95%CI=1.08–2.81) (appendix 7).

2.4 DISCUSSION

In this study, we present results from the largest GWAS to date, analysing cervical cancer phenotypes in over 150,314 women of European ancestry and over 9 million imputed and genotyped genetic variants. We provide strong evidence for an association at 6 independent loci in the *PAX8*, *CLPTMIL* and *HLA* regions in the UKB. We were able to replicate three SNPs in *PAX8* (rs10175462), *CLPTMIL* (rs27069) and *HLA-DQAI* (rs9272050), in an independent Finnish dataset of 2,635 cases (FinnGen).

Previous GWAS studies have been limited by the modest sample sizes and rarity of ICC; cervical disease phenotypes have been largely dominated by precancerous disease with no separate analyses of cancers. Imputations to permit expansion of studied loci have rarely been performed, with the exception of the *HLA* region^{59,310,347}. Furthermore, the gene-environment interaction has to date not been adequately explored in cervical cancer, and our Mendelian randomisation supported a risk-increasing effect of smoking, number of sexual partners, and a protective effect of older age at first pregnancy on cervical cancer.

Our results in the *HLA* region are consistent with previous GWAS in cervical cancer, where allelic variation has been reported for *HLA-DRBI*^{59,70,73,347}, *-DQAI*⁷⁰, *-DQBI*^{59,347}, *-DPBI*³¹⁰, *-DPB2*³¹⁰, and *HLA-B*³⁴⁷, and both protective and risk haplotypes have been described. Associations at the nearby *major histocompatibility complex (MHC) class I polypeptide-related sequence A* gene (*MICA*) gene have also been replicated⁷⁰. *HLA-DRB*, *-DQAI* and *MICA* genes play important roles in the adaptive immune response to infection – coding for *MHC class I and II* alleles, which are responsible for antigen processing and exogenous peptide presentation. Such responses are particularly vital in the mediation of T-cell mediated activity and cytotoxicity, which are known to play key roles in immune responses to both HPV infection and tumour cells³⁴⁸. The *HLA* region is notorious for its complex LD structure. While we present four independent SNPS that have not been previously reported, LD patterns from our

analysis suggest high linkage with known variants. Our results therefore likely provide a replication of allelic variation for *HLA-DQA1*, *-DQB1*, *-B* and *MICA*. Notably gene annotations for non-coding variants are not certain. Of particular interest is our novel report of the association at a rarer SNP rs9266183 (MAF 0.037; *HLA-B*), which represents a missense variant resulting in a codon shift and amino acid change. Alteration of this allele may result in a shift in peptide presentation properties, distorting the T-cell response to HPV infection³⁴⁹. Owing to the lower minor allele frequency, this association has probably gone undetected in previous smaller studies. Despite the growing number of *HLA* associations reported for cervical cancer, minimal laboratory validation has been performed; given an anticipated coding effect, rs926618 would be one of the most interesting targets for further functional analyses.

In European populations, no SNPs outside of the *HLA* region have been validated prior to this study to our knowledge. While previous candidate gene studies of cervical cancer have explored regions related to immune function, cell cycle control, DNA repair and other carcinogenic processes, and identified genetic associations including *CTLA4*, *FANCA*, *OAS3*, *SULF1*, *IFNG*, *DUT*, *DMC1*, *GTF2H4* and *EVER1/2*⁶⁶, *ERAP1*, *LMP7* and *TAP2*, *TP53*, *TERT*, *IL10*⁶³, and *IL17* and *TNF*⁶², these findings have not been replicated in larger cohorts. In Chinese populations, GWAS detected loci in *EXOC1* and *GSDMB* genes, are suspected to influence immune response to viral infection³¹⁰. While a Japanese genome-wide study reports an association with *ARRDC3* and further evidence from knockdown in HeLa cells, that genetic variation may influence HPV entry to cells⁷³.

We identified novel variants in *PAX8* and *CLPTMIL* intronic regions in UKB, which replicated in FinnGen cervical cancer phenotypes. Our functional analysis demonstrated how these associations may represent carcinogenic or immune function pathways. The strongest association at the *PAX8* locus was an INDEL (del:rs35724515), which was in perfect LD with the lead SNP rs10175462. Enrichment for rs10175462 in cervical cancer tissue was co-localised with an eQTL, strongest effects were observed at LOC654433, suggesting long-range epigenetic regulation silencing may be controlling expression³⁵⁰. Specifically, decreased expression of the whole region was associated with a protective effect in the presence of the alternative T allele. Variant rs10175462 may be tagging the deletion variant rs35724515, resulting in decreased *PAX8* expression leading to a protective effect due to reduced uncontrolled cell growth and anti-apoptosis associated with *PAX8* overexpression^{351,352}.

Genetic variation detected at the *CLPTMIL* locus offers additional insight into potential carcinogenic pathways in hrHPV-driven cancers. *CLPTMIL* codes for a transmembrane protein and has been linked to cell growth promotion in non-cervical tumours³⁵³, while altering cisplatin-mediated apoptosis when

overexpressed³⁵⁴. Its previous association with multiple mucosal and hrHPV-driven cancers suggests a role in regulation of viral transmission across epithelial barriers. Additional investigations of anti-*CLPTMIL* monoclonal antibodies in chemo-resistant lung and pancreatic cancers, demonstrates a potential novel target for multi-cancer therapeutics³⁵⁵.

Our main GWAS was based on a broad definition of cervical cancer including both ICC and CIN3 cases. Conditional analyses of the CIN3/ICC phenotype identified four associated SNPs which could be viewed as independent genetic marks of cervical cancer that complementarily contribute to the explanation of the disease outcome. Although CIN3 is considered the immediate cancer precursor, it is estimated that 30% of women with CIN3 develop invasive cervical cancer within 10-15 years³⁵⁶. In the subgroup of ICC cases alone, there was a strong attenuation of the associations in *HLA* and *PAX8* regions, which were not found to be genome-wide associated with the outcome. Similar attenuation was observed for the associations linking genetic variants in the *PAX8* gene with the CIN3 outcome, both in our UKB participants, and in our replication study. Conversely, 132 associations in the chromosome 6 *HLA* region were identified in relation to the CIN3 outcome. Of these, only one SNP (rs9272245, *HLA*) survived in our conditional analyses, hence supporting the existence of a single strong genetic signal associated with CIN3. This is the first study with adequate power to separately explore variation in the invasive cancer phenotype alone. These results possibly uncover histological heterogeneity, with the *HLA* region being more strongly associated with non-progressive preinvasive lesions, rather than invasive cervical cancer. While *PAX8* signals may only be detectable in larger sample sizes as demonstrated in the ICC phenotype of the replication cohort. In our replication cohort, due to the original phenotype coding, the analyses pooling all cases included less severe phenotypes (CIN1 and CIN2). This potentially increased the heterogeneity of the cases and diluted the estimated effects. Longitudinal study of CIN lesions, could reveal valuable information on genetic variance associated with more aggressive phenotypes.

In keeping with the results from our MR models, smoking and an increased number of sexual partners (especially at a young age), are known risk factors for CIN and cervical cancer. We did find however that when applying a multivariable MR analysis, the effect of smoking attenuated when accounting for the number of sexual partners, suggesting that the strong associations seen with smoking, may in part be related to increased lifetime hrHPV exposure from other risky behaviours. Previous studies demonstrating an association between smoking cessation and cervical neoplasia regression³⁵⁷ and between HPV infection and current smoking but not former smoking³⁵⁸, do suggest that smoking is an independent causal factor for smoking that may be reversible and so smoking cessation advice should continue to be offered at colposcopy clinics.

We further confirmed that the effect of young age of pregnancy was not attenuated when controlling for number of sexual partners, suggesting a possible independent effect of young pregnancy on cancer risk, potentially through mechanisms of early cervical trauma or early hormonal changes. We were unable however, to confirm that risk associated to younger age of first pregnancy was not a surrogate for young age of hrHPV exposure and sexual debut, or related to higher parity¹⁹⁴. Additionally, although the effect size for nulliparity suggested a strongly protective effect, no statistical significance was observed in the MR model. Previous studies have shown a disappearance in effect after controlling for parity³⁵⁹, although in a large pooled analysis the effect of early pregnancy on cervical cancer remained after adjusting for sexual behaviours (lifetime sexual partners and age at first sexual intercourse)¹⁹⁵. Rheumatoid arthritis showed an increased risk for cervical cancer in our MR model which is not widely reported, the included study was small however with an effect size close to the null. Although not strong evidence, this does highlight a potentially causal association between autoimmune disorders, immunosuppressive therapy and cervical cancer. The effect of IBD on cervical cancer was not significant when explored by MR however, neither was SLE, height, alcohol consumption or gestational diabetes.

hrHPV status was not available in the UKB due to the invasive nature of sampling healthy volunteers and the absence of hrHPV-based screening data, which has only recently been introduced in most countries. Furthermore, hrHPV status at a given timepoint would be an unreliable surrogate of exposure or clearance, and serum antibodies tests have variable accuracy and are not commercially available. To reduce potential for this complex confounding, we included as controls, only women with no previously abnormal screening history - as this population was likely to have been exposed to hrHPV without developing any cervical lesion. Future adequately powered studies should investigate differential genetic variation in the presence of a known screening history of hrHPV clearance or persistence. The UKB only had sufficient cases to study European ancestry. Given the high burden of cervical malignancy in non-European populations, future studies should further explore genetic variation in other ethnicities, particularly Black and South Asian populations. Although this was the first MR, the analysis was based on publicly available summary-level data – we used female sex-specific data on genetic associations where possible, but this was not available for all exposures considered (smoking and number of sexual partners). For some of the exposures there is overlap in samples for the two-sample MR design. Yet since we base our analysis on genetic variants which are strongly associated with the exposure, representing strong instrumental variables, the induced bias can be considered negligible.

2.5 CONCLUSIONS

Our findings offer exciting insights into the genetic predisposition of the cervical cancer trait. This study supports a complex neoplastic model related to both altered immune response and carcinogenic processes, in the presence of hrHPV. Further study of the functional role of *HLA* and *PAX8* variants are warranted. Investigations which integrate the study of host susceptibility with viral genetic variation³⁶⁰, along with epigenetic behaviour, could further elucidate the differential risk related to complex host-viral interactions. In future, genetic and epigenetic markers could inform cervical screening strategies.

Chapter 3: A Meta-analysis of hrHPV DNA methylation markers and their diagnostic accuracy in detecting high-grade cervical disease.

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3.1 BACKGROUND

HPV infects over 70% of sexually active women during their lifetime³⁶¹. In most women, these infections are cleared whilst in a minority these persist and have the potential to lead to invasive cervical cancer²². HrHPV testing for primary cervical screening may offer 60-70% greater protection against invasive cancer than cytology-based screening¹²². hrHPV testing has modest specificity and positive predictive value (PPV) for the detection of high-grade cervical intra-epithelial neoplasia (CIN).¹⁰⁸ The best strategy to triage hrHPV positive women at screening and detect those truly at risk remains unclear. Novel molecular tests based on the hrHPV epigenome have the potential to permit a more comprehensive understanding of the disease process and as a result improve diagnostic accuracy within cervical screening programmes.

DNA hyper-methylation is an early and frequent event in many cancers and methylation of viral DNA has been recently proposed as a novel biomarker of cervical disease. Rapid developments in sequencing technologies have enhanced scientific ability to explore the hrHPV epigenome, together with its host interactions. Consequently, the number of studies investigating the role of hrHPV DNA methylation in disease progression has increased in recent years, the most comprehensive of which suggest an increase in methylation by disease grade in common high-risk HPV types^{139,147,148,150}.

Although results are encouraging,¹⁴⁶ variation in measurement and reporting of methylation has made comparisons difficult. Studies examining genome-wide HPV methylation have thus far been limited and methylation levels appear to vary by HPV genotype, gene (L1, L2, E2-4, URR) and even amongst adjacent CpG sites^{140,362}. Differences in case definitions and endpoints (e.g. cytology vs. histopathology, CIN2 vs. CIN3) further complicate comparisons and interpretation of the literature. To date, current evidence has not permitted analysis of the comparative performance of different hrHPV methylation sites. We conducted a systematic review and meta-analysis of the existing literature to explore how methylation levels of different HPV genotypes and genes correlate with disease severity (CIN histological grade) and determine the diagnostic accuracy of hrHPV methylation in detecting high-grade pre-invasive disease in hrHPV positive women.

3.2 METHODS

Selection criteria and Search strategy

The meta-analyses followed Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and Cochrane Collaboration recommendations for diagnostic test accuracy reviews^{363,364}. Ethical approval was not required. A systematic literature search was performed in the electronic bibliographic databases MEDLINE, EMBASE and CENTRAL from inception to 1st October

2019. Studies were identified by two independent reviewers and discrepancies resolved by a third author. We further hand-searched the references of the articles in the full-text stage and the proceedings of relevant conferences respectively.

Inclusion and exclusion criteria

We included all studies examining the degree of hrHPV DNA methylation across the viral epigenome with hrHPV infection and different grades of cervical disease as defined by cytology or histopathology. Any population of women representing a cervical screening or referral population were included without demographic restrictions, including retrospective biobanks of samples for women recruited from such populations and diagnostic accuracy studies examining the ability of hrHPV methylation to diagnose cytological or histological outcomes, regardless of HPV genotype, sample material and study methodology. We excluded studies where a reference standard (cytological and/or histological grade) was not reported and methylation experiment studies in cell lines only. Studies with less than five participants with CIN2 or worse were excluded due to high risk of bias. There was no language restriction.

Outcome measures

We explored two primary outcomes. Firstly, we established how gene-specific hrHPV methylation changes with CIN severity. The reported outcome measures presented across studies varied and included a) proportion of samples methylated at a CpG and/or gene (samples methylated/samples methylated plus samples unmethylated) b) percentage mean or median methylation at a CpG and/or gene (0-100% methylation) c) mean difference (MD) in methylation (mean methylation in high-grade disease minus mean methylation in low-grade disease) d) odds-ratio (OR) of high-grade vs. low-grade disease for high methylation. Secondly, we determined the diagnostic accuracy of a positive hrHPV methylation test (sensitivity, specificity or area under the curve (AUC)) in detecting $\geq$ CIN2 and $\geq$ CIN3.

Data Extraction

From each included study, two reviewers (SB, AM) extracted data on study design and setting, index test, sample material, HPV type, CpG sites analysed, reference standard, outcome measures and risk of bias using a predefined spreadsheet. Data were extracted for different HPV types separately and for individual CpG sites where available. Where data were missing (i.e. individual CpG sites data), we contacted the authors and collated additional data for a number of studies^{139,148,150,151,365}. Where only medians were available we estimated means and the relevant standard deviation (SD) using the Wan et al. method³⁶⁶. Where an SD was not provided or could not be calculated, we used the Furukawa et al imputation method³⁶⁷. Where additional data were not provided by authors and graphs were of high

quality, we used the *WebPlotDigitizer*³⁶⁸ to estimate data points from graphs. National centre for biotechnology information (NCBI) reference genomes were used to locate CpG sites for all HPV types.³⁶⁹ The full study protocol was registered prior to data extraction (appendix 9).

Quality Assessment of studies

We assessed the risk of bias for selected studies using a modified quality assessment tool for diagnostic test accuracy studies (QUADAS-2)^{113,370} which covers four main domains: patient selection, index test, reference standard, flow and timing. We assigned a judgement of low, moderate or high risk of bias to each of the domains according to the criteria outlined in QUADAS-2³⁶³. In detail, we used a modified QUADAS-2 tool in line with the Cochrane recommendation for quality assessment and risk of bias in diagnostic test accuracy studies. We modified this allow evaluation of all studies including experimental and proof of principle studies, as previously described by Arbyn et al (Lancet Oncology, 2014)³⁷¹. In the tailored QUADAS-2 criteria^{363,372}, we assessed four domains: A) Patient selection - P1: acceptable enrolment method, P2: acceptable inclusion criteria and inappropriate exclusions avoided. B) Index test - I1: acceptable sample material (LBC, cervical swab, cervical tissue), I2: acceptable methylation test giving quantitative results (Pyrosequencing, EpiTYPER, Next generation sequencing, Luminex) C) Reference test - R1: acceptable reference test (histology confirmation of grade for CIN and cancer, at least cytology confirmation of grade for normal and ascus), R2: masking of methylation analysis to reference test; D) Flow and timing - F1: acceptable interval between index test and reference standard; F2: differential verification avoided; F3: withdrawals and uninterpretable results explained. We excluded 'concerns regarding applicability' from the assessment as the studies were observational, non-randomised and none assessed diagnostic test accuracy of hrHPV methylation as a diagnostic test in a cervical screening population.

Subdomains were assessed according to QUADAS-2 guidance by answering yes, no or unclear (where unclear relates to insufficient data for assessment). A summary risk of bias score was calculated and was used for sensitivity analysis. The four domains were classified as high, moderate or low risk of bias after summarising the results of the subdomains.

Statistical Analyses

We analysed methylation changes with disease severity for the different outcome measures reported across studies; pooled estimates of study-specific means, proportions, MD and OR were calculated using an inverse variance random-effects meta-analysis model in the metafor R package v3.6.1. Our main analysis compared two groups based on standardised classification systems. CIN and squamous intraepithelial lesion (SIL) classifications have been commonly used to describe cervical disease (cytology and histology) and the choice has interchanged between different time periods and continents.

Similar grades of disease from either terminology were therefore grouped together: women with $\geq$ CIN2/HSIL (histologically confirmed CIN2 or worse/cytologically defined high-grade squamous intra-epithelial lesion (HSIL) or worse) vs. $\leq$ CIN1/LSIL (histologically confirmed CIN1 or less/cytologically defined low-grade squamous intra-epithelial lesion (LSIL) or less). We used histopathology to determine CIN grade if available; if histopathology (CIN) was not available or clinically indicated, the cytology result (SIL) was used.

We compared the proportions of methylated samples for $\geq$ CIN2/HSIL and $\leq$ CIN1/LSIL in a meta-analysis using the variance-stabilising Freeman-Tukey double arcsine transformation approach. Proportion of samples methylated at a CpG and/or gene was defined as: N samples methylated/ N samples methylated plus N samples unmethylated presented by studies. We used the individual author definition of methylated samples. Where proportions were provided per CpG site, we totalled proportion methylation per gene. Where a study only presented methylation data for a group of samples, this was divided by the total number of samples to estimate the proportion of samples methylated. Where a study provided percentages of samples methylated only the number of samples methylated was calculated by multiplying the percentage methylation by total number of samples, therefore in some cases this was not an integer. Where studies presented results for multiple CpG sites per gene, the mean result for the gene was meta-analysed.

We performed secondary analyses in more defined clinical groups to include normal, LSIL, HSIL and invasive cervical cancer (ICC). When several studies presented duplicate data from the same cohort,^{150,151,373,374} the study with the largest sample size and lowest risk of bias that presented data on the primary and secondary outcomes, was selected for inclusion. We assessed between-study heterogeneity across all meta-analyses using the Cochran's Q test, a visual inspection of forest plots, and the I^2 statistic along a 95% CI³⁷⁵⁻³⁷⁷. We estimated the between-study variance using the restricted maximum likelihood approach in R and the DerSimonian and Laird method in Stata.

Mean/Median methylation levels were determined by percentage mean or median methylation at a CpG and/or gene, from 0-100% methylation presented by studies. In the meta-analysis of means, where methylation levels were averaged by gene, standard deviations (SD) were averaged according to standard recommendations³⁷⁸. Mean difference values were calculated by subtracting Mean difference values in studies were calculated by subtracting mean methylation values from low-grade disease samples from high-grade disease samples, then individual CpG level data was averaged to give mean methylation levels by gene. Meta-analysis of MD was conducted on data averaged by gene and disease grade groups, assuming a normal distribution.

Odds ratios were determined as presented by individual studies - where the proportion of methylated samples in a high-grade disease group was compared to proportion of methylated samples in a low-grade disease group. When calculated from mean/median methylation, this was according to a chosen cut-off as determined by the study. When a study did not present an odds ratio, but a proportion of samples methylated were presented for high-grade vs. low grade disease, we calculated the underlying odds ratio with a corresponding 95% CI. For studies not reporting odds ratios but reporting means and SDs we calculated the standardised mean differences, which then were transformed to odds ratio that could be combined with existing study-specific odds ratio pooled analyses³⁷⁵.

We performed assessment for publication bias in the main analysis by visual assessment of the funnel plot and Egger's *P*-value for small study effects²⁰⁸.

For diagnostic accuracy meta-analysis, we used the true positive (TP), true negative (TN), false negative (FN) and false positive (FP), where available. When not available, these data were calculated from the total number of samples, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV). We used author defined cut-off thresholds throughout, where cut-off thresholds varied, the most overlapping thresholds were chosen, and we used a single threshold from each study in the meta-analysis. Where multiple CpGs were tested, we aimed to combine the most commonly analysed CpGs. Where possible, average data for the whole gene were chosen, otherwise the best performing CpG site was selected. Data were combined to calculate pooled estimates using two models. Where possible a bivariate model was applied in STATA using the *midas* routine³⁷⁹, taking the correlation between true-positive and false-positive rates and between-study variability into account. Where data were insufficient to perform a bivariate analysis, we analysed sensitivity and specificity separately using the logit transformation in a univariate analysis using the *metafor* R package³⁸⁰. In the bivariate mixed-effects binary regression model that was used in the diagnostic test accuracy studies, the DerSimonian and Laird estimator was used to estimate the between-study variance³⁸¹. Whereas in each univariate meta-analysis, the restricted maximum likelihood method was used^{382,383}. A 95% CI for the between-study variance was calculated using the Q-profile approach³⁸⁴.

Where insufficient data were available, we contacted authors for data by individual CpG site and varying cut-offs. Pre- and post-test probability analyses were calculated using the *ppp* function³⁸⁵. We used the diagnostic accuracy of the best performing HPV type, gene and CpG sites. Pre-test probability of CIN2/HSIL or worse in women with HPV16 was estimated from recently presented data³⁸⁶. Pre-test post-test probability plots decision thresholds were based on best performing CpG combinations and

benchmark risk levels of HPV16 positivity as reported across studies in Europe (2% and 20% for CIN2 or worse, 1% and 10% for CIN3 or worse, respectively)³⁸⁵.

We assessed between-study heterogeneity using the Cochran's Q test, a visual inspection of forest plots, and the I^2 statistic along a 95% confidence interval (CI)³⁸⁷. We estimated the between-study variance using the restricted maximum likelihood approach in R and the DerSimonian and Laird method in Stata.

We performed predefined sensitivity analyses. For the comparison of proportions and mean methylation by disease grade, we restricted to those that used histopathology alone as a reference (cytology was accepted to define normal alone). For the MD, OR and diagnostic accuracy analyses, we restricted to a) studies at low risk of bias; b) histopathology as reference test c) pyrosequencing as index test d) cervical exfoliated cells (CEC) as sample material e) cut-off limited to 10% mean methylation. In the MD meta-analysis, we additionally excluded imputed SD values for sensitivity analysis, whereas in the OR meta-analysis, we also excluded the transformed OR values from continuous data and performed a sub-group analysis by geographical region.

All analyses were performed in R v3.6.1 and R studio Version 1.2.5001³⁸⁸ and STATA v14³⁸⁹.

3.3 RESULTS

The literature search yielded 572 potentially eligible studies; 44 studies^{139,147,148,150-152,365,374,390-425} and 8819 women were included in the analysis (Figure 18). The detailed characteristics of the studies are summarised in appendix 10.

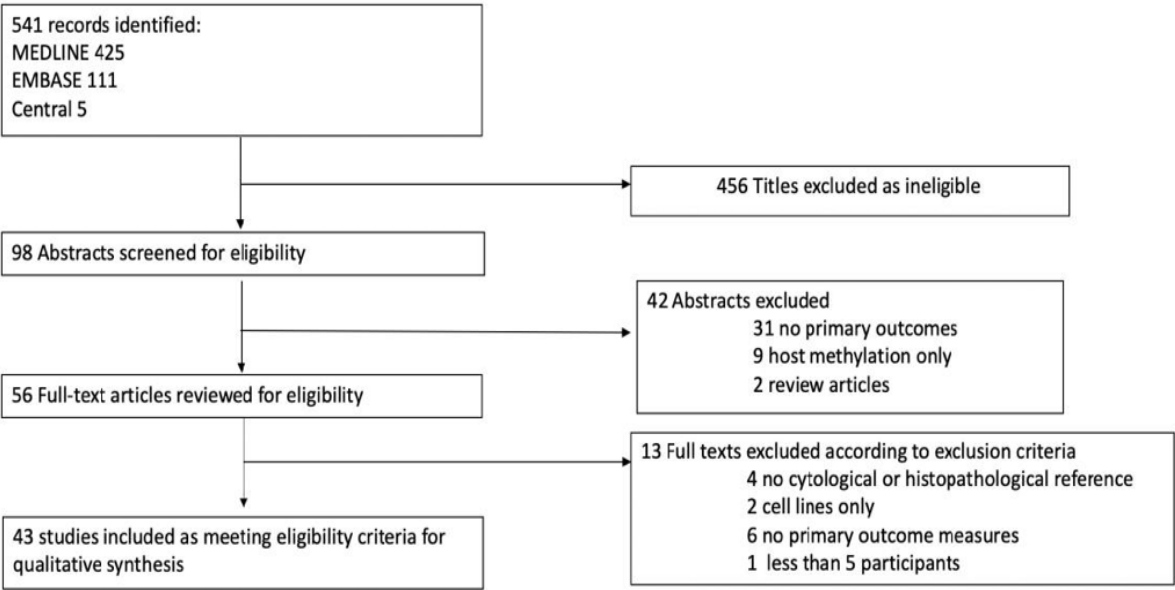


Figure 18. PRISMA flowchart of studies for hrHPV DNA methylation meta-analysis.

Quality assessment

The methodological quality of the studies was overall moderate (Table 11). Half were at low risk of bias, (50.0%, 22/44), whilst the rest had either moderate (45.5%, 20/44) or high risk (4.7%, 2/44). For patient selection, 43.2% (19/44) studies scored moderate to high risk of bias as samples were often selected from biobanks, case-controls without consecutive recruitment, with some having inappropriate exclusions. The index test scored moderate or high risk of bias in 34.1% (15/44); reasons included use of older bisulphite sequencing methods, no quantitative measure, and use of vaginal samples. Eighty-six percent (38/44) of studies scored high or moderate risk of bias on the reference test as there was no blinding of the person performing methylation, while in 13.6% (6/44) there was no histological confirmation for high-grade disease. For flow and timing, there was a delay between methylation and reference confirmation in 9.0% (4/44) of studies, whilst differential verification bias was present in 50.0% (22/44), as studies switched between cytology and histopathology to confirm disease grade. 38.6% (17/44) of studies explained withdrawals or uninterpretable results adequately, while 61.3% (27/44) were scored unclear for insufficient explanation.

Table 11. Quality assessment for risk of bias for all included studies with tailored QUADAS-2.

Author Year	Quality Assessment									Risk of Bias (ROB)				
	Patient selection		Index Test		Reference Standard		Flow and Timing			Patient selection	Index test	Reference Standard	Flow and Timing	Summary ROB score
	P1	P2	I1	I2	R1	R2	F1	F2	F3					
Badal 2003	✗	✗	✗	✗	✓	✗	✓	✗	✗	High	High	Moderate	Moderate	High
Bhattacharjee 2006	✗	✗	✗	✗	✓	✗	✓	✗	✗	High	High	Moderate	Moderate	High
Brandsma 2014	✗	✗	✓	✗	✓	✗	✗	✓	✗	High	Moderate	Low	Moderate	Moderate
Brentnall 2014	✓	✗	✓	✓	✗	✗	✓	✗	✓	Moderate	Low	High	Low	Moderate
Brentnall 2015	✓	✗	✓	✓	✗	✗	✓	✗	✓	Moderate	Low	High	Low	Moderate
Bryant 2014	✗	✗	✓	✓	✗	✗	✓	✗	✓	Unclear	Low	High	Low	Moderate
Bryant 2015	✓	✗	✓	✓	✗	✗	✓	✗	✓	Low	Low	High	Low	Low
Chaiwongkot 2013	✗	✗	✗	✓	✓	✗	✓	✗	✗	Unclear	Low	Moderate	Low	Low
Chaiwongkot 2017	✗	✗	✓	✓	✓	✗	✓	✓	✗	High	Low	Moderate	Low	Moderate
Clarke 2018	✗	✓	✓	✓	✓	✗	✓	✓	✗	Moderate	Low	Moderate	Low	Low
Ding 2009	✗	✓	✓	✓	✗	✗	✓	✓	✗	Moderate	Moderate	High	Low	Moderate
Dutta 2015	✓	✓	✓	✗	Low	Moderate	✓	✗	✗	Low	Moderate	High	Moderate	Moderate
Frimer 2015	✗	✓	✓	✓	✗	✗	✓	✗	✓	Moderate	Low	Low	Low	Low
Gasperov 2015	✗	✓	✓	✗	✗	✗	✓	✗	✗	Moderate	Moderate	High	Moderate	Moderate
Hong 2008	✗	✗	✓	✓	Unclear	✗	✓	✓	✓	Unclear	Low	Moderate	Low	Low
Hsu 2017	✓	✓	✓	✓	✓	✗	✓	✓	✓	Low	Low	Moderate	Low	Low
Hublarova 2009	✗	✓	✓	✗	✓	✗	✓	✓	✗	Low	Moderate	Moderate	Low	Low
Jacquin 2013	✗	✗	✓	✓	Unclear	✗	✓	✗	✗	Unclear	Low	High	Low	Moderate
Kalantari 2014	✓	✓	✓	✗	✗	✓	✗	✗	✓	Low	Moderate	Moderate	Moderate	Moderate
Kottaridi 2017	✓	✓	✓	✓	✓	✗	✓	✓	✓	Low	Low	Moderate	Low	Low
Leung 2015	✗	✓	✗	✓	✗	✗	✓	✗	✗	Low	Moderate	Unclear	Low	Low
Liu 2017	✗	✓	✓	✓	Low	✗	✓	✓	✗	Low	Low	Moderate	Low	Low
Lorincz 2013	✓	✓	✓	✓	✗	✗	✗	✓	✓	Low	Low	High	Low	Low
Lorincz 2016	✓	✓	✓	✓	✓	✓	✓	✓	✓	Low	Low	Low	Low	Low
Louvato 2015	✗	✓	✓	✓	✓	✓	✓	✓	✗	Moderate	Low	Low	Low	Low
Marongiu 2014	✗	✓	✓	✓	✗	✓	✓	✗	✗	Moderate	Low	Moderate	Moderate	Moderate
Mirabello 2012	✗	✓	✓	✓	✗	✗	✓	✓	✗	Moderate	Low	Low	Low	Low
Mirabello 2013	✗	✓	✓	✓	✗	✗	✓	✗	✗	Moderate	Low	Low	Moderate	Low
Mirabello 2015	✗	✓	✓	✓	✗	✓	✓	✗	✗	Moderate	Low	Low	Low	Low
Murakami 2013	✗	✓	✓	✗	✗	✗	✓	✗	✗	Low	Moderate	High	Moderate	Moderate
Niyazi 2017	✓	✓	✓	✓	✓	✗	✓	✓	✓	Low	Low	Moderate	Low	Low
Patel 2012	✗	✓	✓	✓	✗	✗	✓	✓	✗	Moderate	Low	High	Low	Moderate
Piyathilake 2011	✗	✓	✓	✓	✓	✗	✓	✗	✗	Low	Low	Moderate	Moderate	Low
Piyathilake 2014	✓	✓	✓	✓	✗	✗	✓	✗	✓	Low	Low	High	Low	Low
Qiu 2015 (1)	✓	✗	✓	✗	✗	✗	✓	✗	✗	Low	Moderate	High	Moderate	Moderate
Qiu 2015 (2)	✗	✗	✓	✗	✓	✗	✓	✓	✗	Unclear	Moderate	Moderate	Low	Moderate
Simanaviciene 2015	✗	✗	✓	✗	✓	✗	✓	✓	✗	High	Moderate	Moderate	Low	Moderate
Snellenberg 2012	✗	✓	✓	✓	✓	✗	✓	✗	✗	Moderate	Low	Low	Moderate	Low
Sun 2011	✓	✓	✓	✓	✓	✗	✓	✓	✗	Low	Low	Moderate	Moderate	Low
Vasiljevic 2014	✓	✗	✓	✓	✗	✗	✓	✗	✓	Low	Low	High	Low	Moderate
Wang 2017	✗	✗	✓	✗	✓	✗	✓	✓	✓	High	Moderate	Low	Low	Moderate
Wentzensen 2012	✓	✓	✓	✓	✓	✗	✓	✓	✗	Low	Low	Moderate	Low	Low
Xi 2011	✓	✓	✓	✗	✓	✗	✗	✓	✗	Low	Moderate	Moderate	Moderate	Moderate
Zhang 2019	✗	✓	✓	✓	✓	✗	✓	✗	✗	Low	Low	Moderate	Moderate	Moderate

QUADAS-2 domains. P1= acceptable enrolment method; P2= acceptable inclusion criteria and inappropriate exclusions avoided; I1= acceptable sample material (LBC, cervical swab, cervical tissue); I2= Acceptable methylation test giving quantitative results (Pyrosequencing, EpiTYPER, Next generation sequencing, Luminex-C); R1= Acceptable reference test (histology confirmation of grade for CIN and cancer, at least cytology confirmation of grade for normal and ascus); R2= Masking of methylation analysis to reference test; F1= Acceptable interval between index test and reference standard?; F2= Differential verification avoided; F3= withdrawals and uninterpretable results explained. Subdomains were assessed according to QUADAS-2 guidance by answering yes (green tick), no (red cross) or unclear (yellow exclamation mark), where unclear relates to insufficient data for assessment). Domains were assessed by summarising results of subdomains, into high, moderate or low risk of bias.

Characteristics of the studies

All studies were observational. Forty reported on HPV16, twelve on HPV18, and twelve on other HPV types. The majority of studies explored the L1 gene (N=30), followed by LCR (N=29), L2 (N=15), E6 (N=10), E2 (N=4), E7 (N=3), E5 (N=2) and E1 (N=2). Recent studies used pyrosequencing (27/44, 61.3%) and next generation sequencing (NGS) techniques (4/44, 9.0%) for further quantification. Cut-offs of positivity varied ranging from 0 to 90%, whilst some studies chose dichotomisation after division by tertiles. Seventeen studies (39.5%) reported histopathological diagnosis for all samples, twenty-two (50.0%) histopathology for $\geq$ CIN2/HSIL and cytology alone for $\leq$ CIN1/LSIL, three studies (7.0%) used cytology for CIN but histopathology for cancer^{394,399,400}, one study used cytology alone⁴¹⁴, and two studies did not define the reference test^{401,408}. The majority used exfoliated cervical cells (40/44, 90.9%). Assessment for publication bias revealed some asymmetry with large studies on the left showing a more conservative estimate, while Egger's P of <0.01 indicated small study effects (appendix 11).

Methylation levels and lesion severity

Within studies reporting proportions of methylated samples according to disease grade, we found that rates of positive methylation increased with disease grade. When we compared women with $\leq$ CIN1/LSIL to $\geq$ CIN2/HSIL the most marked difference in the pooled estimates for positive methylation rate were found at the HPV16 L1 gene (8 studies; pooled positive methylation rate: $\leq$ CIN1/LSIL=44.4% (95%CI 16.0-74.1) and $\geq$ CIN2/HSIL=72.7% (95%CI 47.8-92.2) (appendix 12a).

The contrast was consistent for HPV16 L2 gene, although the number of studies was smaller and between study heterogeneity was high (3 studies; 40.1% (95%CI 7.0-79.3) vs. 61.8% (95%CI 43.9-78.2)), whereas the differences between $\leq$ CIN1/LSIL vs. $\geq$ CIN2/HSIL were much smaller at the HPV16 LCR (12 studies; 37.5% (95%CI 20.2-56.5); vs. 44.5% (95%CI 30.9-58.5)). The differences were also marked for HPV 18 L1/L2 genes (4 studies; 52.6% (95%CI 13.8-89.8) vs. 86.0% (95%CI 77.7-92.8)) and for HPV31 L1/L2 (2 studies, 33.6% (95%CI 0.0-87.1) vs. 73.7% (95%CI 34.4-99.3)). There were not sufficient studies for pooled estimates for other HPV types (45,52,58), although the direction of effect was similar in the individual studies. We further analysed the data in more defined groups of normal, CIN1/LSIL, CIN2-3/HSIL, and invasive cervical cancer (ICC) and similar associations were observed (appendix 12b). The sensitivity analysis using studies that had histopathology alone as a reference standard were analysed separately (appendix 12c). We found consistent results with reduced heterogeneity and a more marked difference in the methylated rates for HPV16 L1 in $\geq$ CIN2 vs. $\leq$ CIN1 (71.5% (95%CI 24.8-99.8) vs. 24.1% (95%CI 5.3-50.0), suggesting that cytology as a reference may have misclassified disease. The quantification of the difference in mean methylation levels showed that HPV16 methylation was significantly higher in $\geq$ CIN2/HSIL vs. $\leq$ CIN1/LSIL in individual studies in

all genes ($P<0.05$) except the LCR ($P=0.169$) (Figure 19a). There were insufficient data for pooled analysis of other HPV types but the mean methylation levels for HPV18 L1, HPV18 L2, HPV18 LCR, HPV35 L2, HPV45 E2, HPV45 L1, HPV45 L2 and HPV59 L1 were systematically significantly lower in women with $\leq$ CIN1/LSIL when compared to those with $\geq$ CIN2/HSIL ($P<0.05$) (Figure 19b).

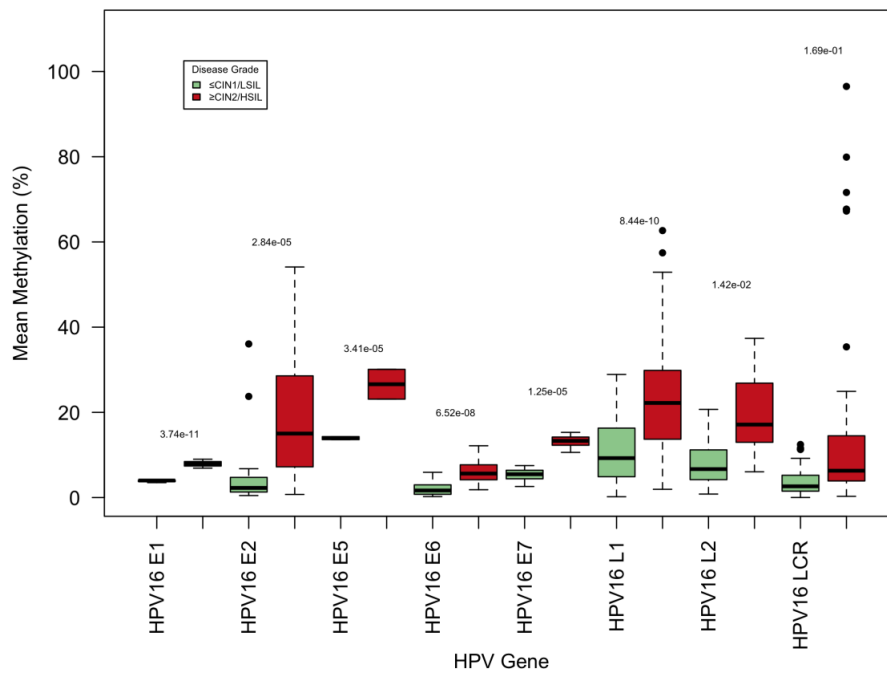
In HPV31 L2, HPV33 L1, HPV33 L2, HPV35 L1, HPV51 L1, HPV51 L2, HPV52 L1, HPV52 L2, HPV58 L1 and HPV58 L2, mean methylation levels in individual studies were higher in $\geq$ CIN2/HSIL but did not reach statistical significance. When restricting to studies using histopathology only as reference, pooled mean methylation levels also increased by disease grade (CIN1, CIN2, CIN3, ICC) in all HPV16 genomic regions (appendix 12d).

The pooled mean methylation levels for HPV16 were lower overall in women with CIN1/LSIL or less when compared to those with CIN2/HSIL or more (Table 12).

Mean Difference in methylation

The pooled difference in mean methylation level (MD) was significantly higher in $\geq$ CIN2/HSIL when compared to $\leq$ CIN1/LSIL for the HPV16 L1 gene (11.3% (95%CI 6.5-16.1)) followed by E2 (8.8% (95%CI 0.2-17.5)), E7 (7.7% (95%CI 3.7-11.7)), L2 (5.6% (95%CI 1.8-9.4)) and E6 (2.5% (95%CI 0.5-4.4)) (Table 12). In the LCR there was no significant difference observed (1.6% (95%CI -0.3-3.4)), while for HPV16 E1 and E5 there was only one study with the same direction of effect. High heterogeneity was observed in all MD meta-analyses and was explored with a series of sensitivity analyses that did not substantially improve heterogeneity (appendix 12d). There were insufficient data for meta-analysis in other HPV genotypes.

a)



b)

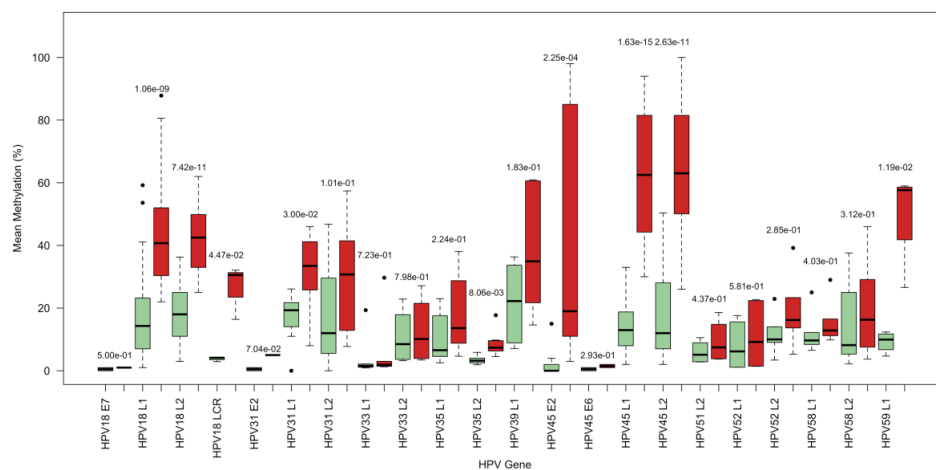


Figure 19. Mean methylation level in $\leq$ CIN1/LSIL vs. $\geq$ CIN2/HSIL for each individual study, by HPV gene. Each boxplot represents the median and interquartile range of the proportion of hyper-methylation per study, averaged by gene and grouped by disease grade. Whiskers represent 95% confidence interval bounds. Black dots represent outlier values. P-values obtained by student's t-test comparing mean methylation level in $\leq$ CIN1/LSIL (green) vs $\geq$ CIN2/HSIL (red). HPV genes ordered according to position in HPV genome but not distributed by actual gene size. $\leq$ CIN1/LSIL: normal, ASCUS, LSIL, CIN1; $\geq$ CIN2/HSIL: CIN2, CIN3, HSIL, ICC. a) HPV16 b) Other HPV types

Table 12. Pooled mean methylation levels for HPV16 in $\leq$ CIN1/LSIL vs $\geq$ CIN2/HSIL and pooled difference in mean methylation (MD). The grade was defined by histology; if this was not available, cytology was used.

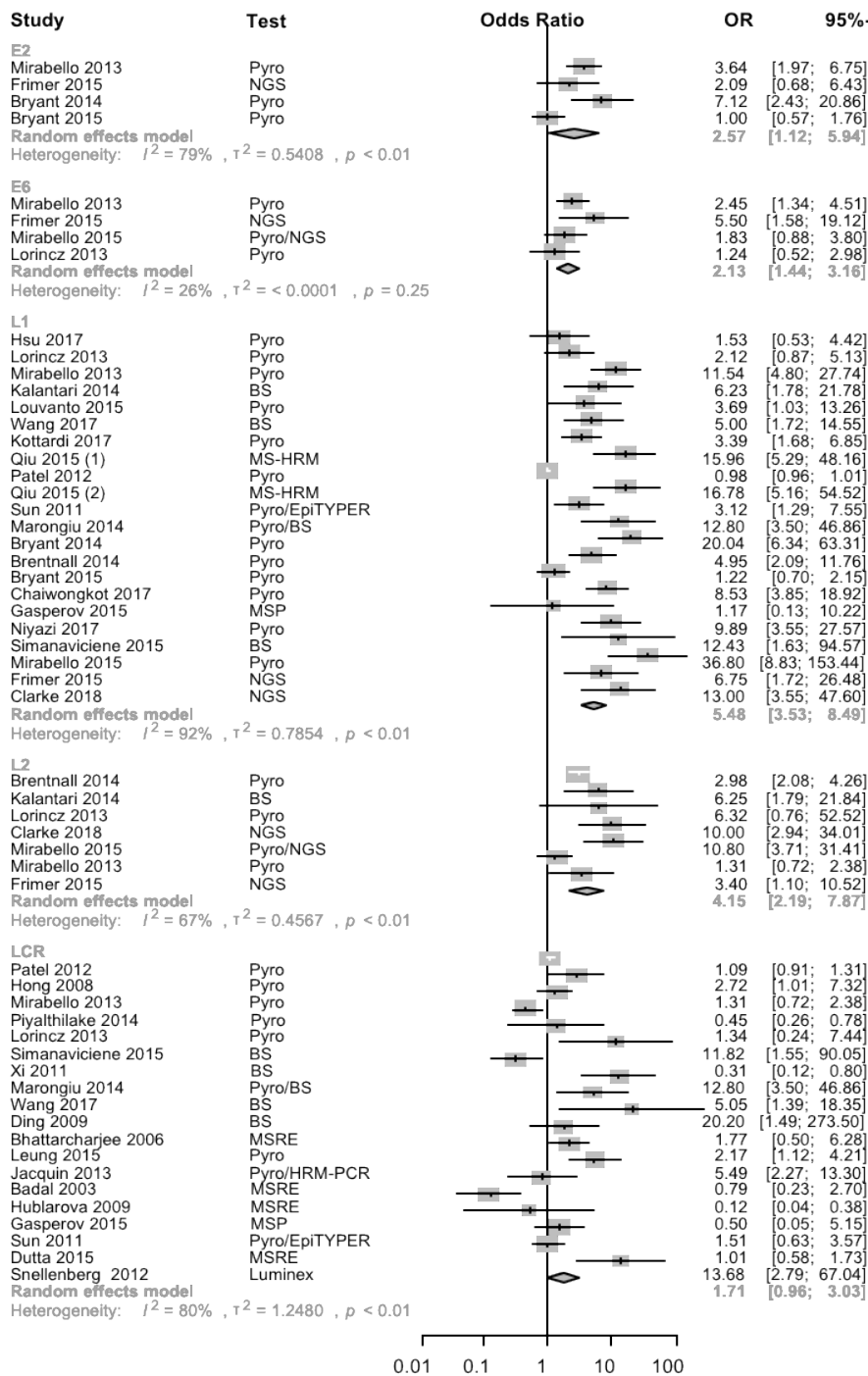
Gene (studies)	Pooled Mean $\leq$ CIN1/LSIL % (95%CI)	I ² (95%CI)	Pooled Mean $\geq$ CIN2/HSIL % (95%CI)	I ² (95%CI)	Pooled MD % (95%CI)	I ² (95%CI)
E1 (n=1)	3.9 (2.1-5.7)	N/A	14.8 (9.1-20.4)	N/A	4.0 (-1.6-9.6)	N/A
E2 (n=4)	5.6 (1.6-9.5)	89.0 (60.2-98.8)	14.1 (5.4-22.8)	87.9 (62.6-98.6)	8.8 (0.2-17.5)	88.0 (61.5-98.7)
E5 (n=1)	14.0 (9.8-18.1)	N/A	26.6 (17.7-35.5)	N/A	12.7 (2.9-22.4)	N/A
E6 (n=3)	2.1 (0.8-3.4)	72.0 (5.1-99.2)	4.8 (1.7-7.9)	57.0 (0.0-96.9)	2.5 (0.5-4.4)	13.3 (0.0-96.6)
E7 (n=1)	5.9 (4.0-7.9)	N/A	13.5 (9.1-17.8)	N/A	7.5 (2.4-12.7)	N/A
L1 (n=12)	10.8 (7.4-14.2)	96.3 (92.2-98.8)	23.3 (17.8-28.7)	96.7 (93.1-98.8)	11.3 (6.5-16.1)	93.0 (85.0-97.6)
L2 (n=5)	9.0 (4.8-13.2)	99.4 (98.2-99.9)	15.4 (9.3-21.4)	96.0 (87.8-99.6)	5.6 (1.8-9.4)	88.0 (54.3-98.7)
LCR (n=7)	4.0 (1.4-6.7)	96.7 (91.5-99.3)	6.6 (4.0-9.3)	94.1 (85.2-98.8)	2.5 (-0.1-4.8)	87.3 (64.4-97.9)

Abbreviations: CIN: cervical intraepithelial neoplasia; LCR: long control region; LSIL: low grade squamous intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion; Q-test: Cochran Q test. Mean methylation levels in studies were determined by percentage mean or transformed median methylation per group of samples, and averaged by gene (0-100% methylation). Mean difference values in studies were calculated by subtracting mean methylation values from low-grade disease samples from high-grade disease samples, then averaged by gene (0-100%), values were log-transformed before meta-analysis then back-transformed for presentation.

Odds ratios

Data to determine odds ratio (OR) comparing proportion of methylated samples or mean methylation for $\geq$ CIN2/HSIL vs. $\leq$ CIN1/LSIL in HPV16 was available in 21 studies; the study-specific ORs for HPV16 L1 ranged from 1.2 to 36.8. The cut-off used to define positive methylation varied between studies (range 0-16%). The pooled point estimate OR was greatest for the HPV16 L1 gene (22 studies, OR 5.5 (95%CI 3.5-8.5, $P < 0.0001$)) followed by L2 (7 studies, OR 4.2 (95%CI 2.2-7.8, $P = 0.0001$)), E2 (4 studies, OR 2.6 (95%CI 1.1-6.0, $P = 0.0266$)) and E6 (4 studies, OR 2.1 (95%CI 1.4-3.2, $P = 0.0002$)) (Figure 20).

The OR was not significantly different from unity for the HPV16 LCR region ($P = 0.0682$), with a few studies showing significant negative associations. When restricting the analysis to studies reporting only on proportions, the OR for HPV16 L1 gene was even higher (OR 6.5 (95%CI 3.8-10.9, $P < 0.0001$)). Restriction to studies with low risk of bias, histopathology only, or CEC only, all reduced observed heterogeneity. Restriction to pyrosequencing techniques only did not reduce heterogeneity. The pooled OR for HPV18 L1/L2 was similar (7 studies, OR 8.3 (95%CI 2.9-23.5, $P < 0.001$)). Fewer studies were available for other HPV types for the L1 gene alone. Subgroup analysis by geographical location was applied and revealed no significant difference in pooled estimates of OR according continent, including Americas, East Asia, Europe and South Asia.



BS: bisulphite sequencing; HRM-PCR: high-resolution melting polymerase chain reaction; MD: mean difference; MSP: methylation-specific polymerase chain reaction; MSRE: methylation-sensitive restriction enzyme; NGS: next-generation sequencing; OR: odd ratio; Pyro: pyrosequencing; τ^2 : between-study variance; 95%CI: 95% confidence intervals.

Figure 20. Meta-analysis of odds ratios of HPV16 methylation in genes L1, L2, LCR, E2, E6 for $\leq$ CIN1/LSIL vs. $\geq$ CIN2/HSIL.

Diagnostic accuracy in detecting high-grade CIN in women positive for individual high-risk HPV subtypes

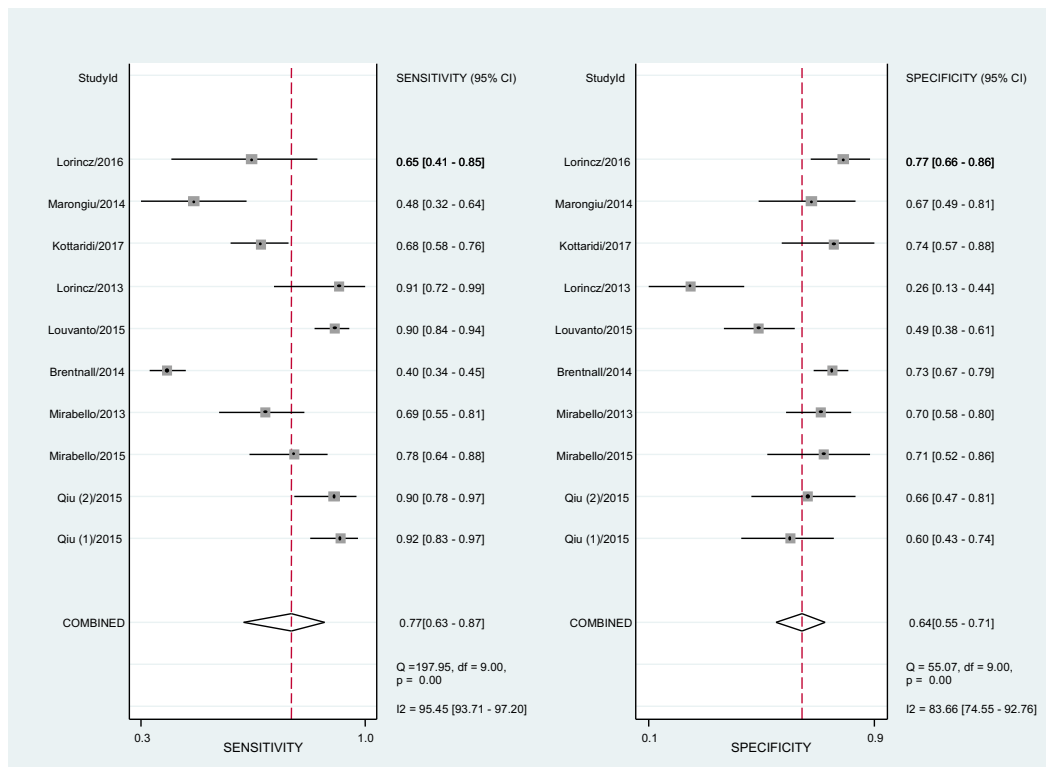
We calculated sensitivity and specificity of positive methylation (as defined by authors) in detecting CIN2 or worse and CIN3 or worse by pooling estimates by gene and by best performing CpG site. The cut-off for positive methylation varied across studies (0-15.9% or 3rd tertile). The highest sensitivity and specificity in CIN2+ detection in HPV16 positive women was observed by combination of positive methylation across CpG sites in the HPV16 L1/L2 genes (10 studies, sensitivity 77% (95%CI 63-87), specificity 64% (95%CI 55-71), AUC=0.73 (95%CI 69-76), $I^2=96\%$) (Figure 21).

When all HPV genes were included (L1/L2/E2/E5/E6/E7/LCR) the results were similar (sensitivity 75% (95%CI 59-86), specificity 65% (95%CI 57-73)). The pooled estimates for the L1 gene alone had improved sensitivity (82% (95%CI 73-88)) with a slight drop in specificity (57% (95%CI 49-65)). The analysis of L2 alone was similar to that of L1/L2 combined (5 studies). There were sufficient data to analyse pooled sensitivity and specificity of two single CpG site markers (L16367 and L16389) - performance was less than combined gene markers (Table 13). For prediction of CIN3 or worse, sensitivity was similar (76% (95%CI 66-84)), while specificity (75% (95%CI 57-87)) and AUC (0.90 (95%CI 0.80-0.96)) were greater.

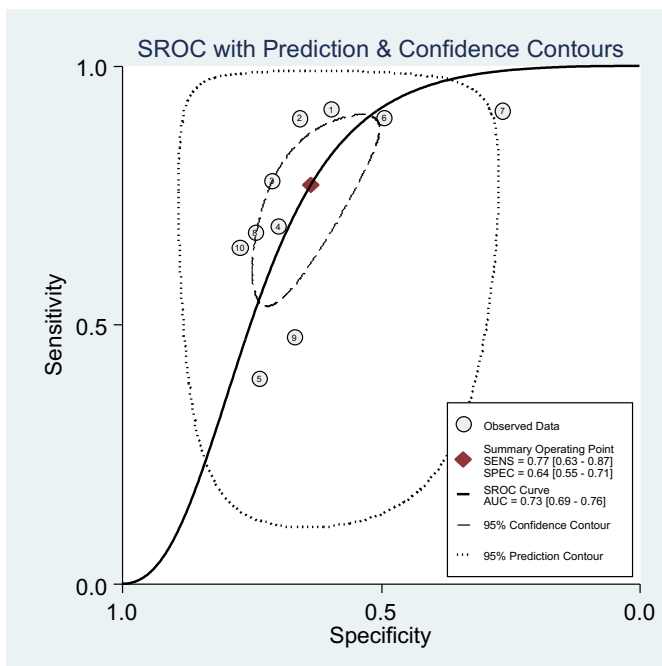
Sensitivity analyses

Restriction to studies at low risk of bias in the analyses of the HPV16 L1/L2 gene reduced heterogeneity ($I^2=81\%$); sensitivity was improved (80%) with no change in specificity (63%). Similarly, restriction to histopathology only studies increased sensitivity to 80.5%, while other analyses (including restriction to a 10% cut-off and pyrosequencing only techniques) did not change estimates (appendix 13).

a)



b)



1: Qiu (1) 2015; 2: Qiu (2) 2015; 3: Mirabello 2015; 4: Mirabello 2013; 5: Brentnall 2014; 6: Louvanto 2015; 7: Lorincz 2013; 8: Kottaridi 2017; 9: Marongiu 2014; 10: Lorincz 2016. 95%CI: 95% confidence intervals. df: degrees of freedom; I²: P statistic measure of heterogeneity; P: p-value; Q: Cochran Q-test.

Figure 21. Diagnostic Accuracy of HPV16 L1/L2 in predicting CIN2/HSIL or worse (bivariate model) a) Pooled sensitivity and specificity b) SROC curve.

Table 13. Meta-analysis of HPV16 methylation pooled sensitivity, specificity and area under the curve (by study defined cut-off) in detecting disease $\geq$ CIN2/HSIL and disease $\geq$ CIN3/HSIL compared to CIN1 or less (HPV+ and HPV-)

Gene	Studies (N)*	Threshold	Pooled Sensitivity % (95% CI)	Pooled Specificity % (95% CI)	Pooled estimated AUC (95% CI)	I^2 (95% CI)	τ^2
BIVARIATE							
$\geq$CIN2/HSIL							
All studies (L1/L2/E2//E5/E6/E7/LCR)	11	0.4-15.9/3 rd tertile	75 (59-86)	65 (57-73)	0.73 (0.69-0.76)	95.1 (93-97)	0.76 (0.00-1.89)
L1/L2/E5	11	0.4-15.9/3 rd tertile	76 (63-86)	64 (56-72)	0.73 (0.69-0.77)	95.0 (93-97)	0.43 (0.00-1.10)
L1/L2	10	0.4-15.9/3 rd tertile	77 (63-87)	64 (55-71)	0.73 (0.69-0.76)	95.5 (94-97)	0.00 (0.00-1.34)
L1	10	0.4-15.9/3 rd tertile	82 (73-88)	57 (49-65)	0.73 (0.69-0.77)	85.9 (78-93)	0.00 (0.00-2.99)
L2	5	0.4-15.9/3 rd tertile	75 (63-84)	66 (42-84)	0.77 (0.73-0.81)	32.9 (0-98)	0.00 (0.00-100)
L1 6367	5	3.1-10/3 rd tertile	74 (64-82)	53 (41-66)	0.70 (0.66-0.74)	73.0 (48-98)	0.35(0.00-0.98)
L1 6389	5	3.1-10/3 rd tertile	77 (67-84)	52 (38-65)	0.71 (0.67-0.75)	67.9 (38-98)	0.00 (0.00-1.64)
UNIVARIATE							
$\geq$CIN2/HSIL							
All studies (L1/L2/E2//E5/E6/E7/LCR)	14	0.4-15.9/3 rd tertile	74 (60-84)	65 (54-75)	0.782 (0.669-0.864)	39.5 (0-67)	0.50 (0.00-1.58)
L1/L2/E5	14	0.4-15.9/3 rd tertile	75 (62-84)	64 (54-74)	0.810 (0.712-0.880)	37.4 (0-61.7)	0.46(0.00-1.24)
L1/L2	13	0.4-15.9/3 rd tertile	75 (61-85)	64 (53-74)	0.808 (0.685-0.891)	39.7 (0.00-67.14)	0.49 (0.00-1.49)
L1	13	0.4-15.9/3 rd tertile	81 (73-87)	55 (44-66)	0.808 (0.685-0.891)	0.00 (0.00-70.7)	0.00 (0.00-2.62)
L2	5	0.4-15.9/3 rd tertile	81 (69-89)	69 (46-85)	-	0.00 (0.00-100)	0.00 (0.00-100)
LCR	3	0/3 rd tertile	45.3 (19-73.4)	75.2(47.6-91.0)	-	0.00 (0.00-100)	0.00 (0.00-100)
LCR/L1/L2	3	5.3/3 rd tertile	54.6 (26.1-80.4)	74.2 (45.8-90.7)	-	0.00 (0.00-100)	0.00 (0.00-100)
L1 6367	5	3.1-10/3 rd tertile	75 (62-85)	52 (37-67)	0.720 (0.665-0.769)	89.9 (68-99)	0.00 (0.00-1.45)

L1 6389	5	3.1-10/3 rd tertile	82 (68-91)	48 (33-63)	0.834 (0.691-0.918)	98.9 (97-100)	0.18 90.00-6.58)
L1 5611	3	10.8/3 rd tertile	-	-	0.830 (0.739-0.894)	96.4 (87-100)	0.22(0.05-9.24)
L1 6650	3	10.8/3 rd tertile	-	-	0.779 (0.653-0.868)	97.8 (92-100)	0.30 (0.08-12.06)
L1 7145	5	10.8/3 rd tertile	-	-	0.729 (0.649-0.796)	95.6 (88-100)	0.17 (0.06—1.55)
≥CIN3/HSIL							
L1	7	3.1-15.9	76 (66-84)	75 (57-87)	0.903 (0.804-0.955)	0.00 (0.00-58.6)	0.00 (0.00-0.77)

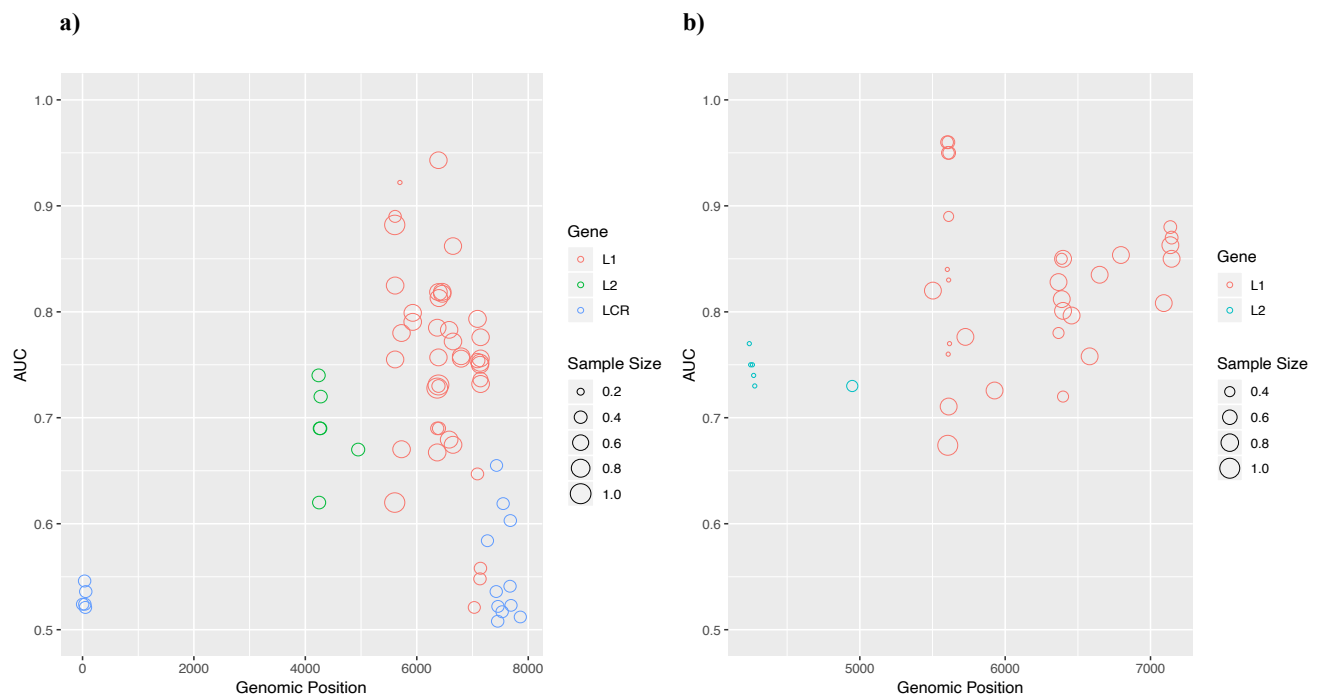
Abbreviations: B: bivariate model; CIN: cervical intraepithelial neoplasia; U: univariate model; WGA: Whole Genome Average.

Bivariate models applied wherever possible in preference. When not possible, univariate model were applied.

*Number of studies and heterogeneity refers to pooled sensitivity meta-analyses, on occasion the number of studies for AUC meta-analyses was less due to less data.

Area under the Curve (AUC)

We also performed meta-analysis of AUC for CpG sites L15611, L16367, L16389, L16650 and L17145, for detection of CIN2+ and of L1 5611 for CIN3+. The greatest pooled estimated AUC for CIN2+ was observed at L16389 (0.83 (95%CI 0.69-0.92)) and L15611 (0.83 (95%CI 0.74-0.89)) (Table 13). Where meta-analysis of AUC was not possible, we plotted AUC values for diagnostic accuracy of methylation in predicting CIN2+ and observed a clustering of higher values in L1/L2 regions when compared to LCR (Figure 22). This was similar for HPV18, 31, 33, 45 and 52, whilst for HPV31, the E1/E2 had greater AUC than the L1 gene (appendix 15).



AUC: area under the curve; LCR: long control region

Figure 22. Area under the curve values presented by individual studies to measure diagnostic accuracy of HPV16 methylation in predicting a) $\geq$ CIN2/HSIL and b) $\geq$ CIN3/HSIL by gene and according to genomic position, weighted by study sample size. The horizontal axis represents exact CpG site in the genome. Points near the top represents the studies with the best accuracy.

Pre- and post-test risk

We further compared the pre-test and post-test risk of CIN2 or worse and CIN3 or worse in a HPV16 positive screening population (Figure 23). Using the highest performing genes and sites (pooled sensitivity 77% - specificity 64%), a positive methylation test increased the risk of CIN2 or worse from 23.0% to 39.0%, whilst a negative test result decreased the risk to 9.7%. Risk of CIN3 or worse in a

HPV16+ woman with a positive methylation test rose from 16.0% to 35.8% whilst a negative test result decreased risk to 5.8%.

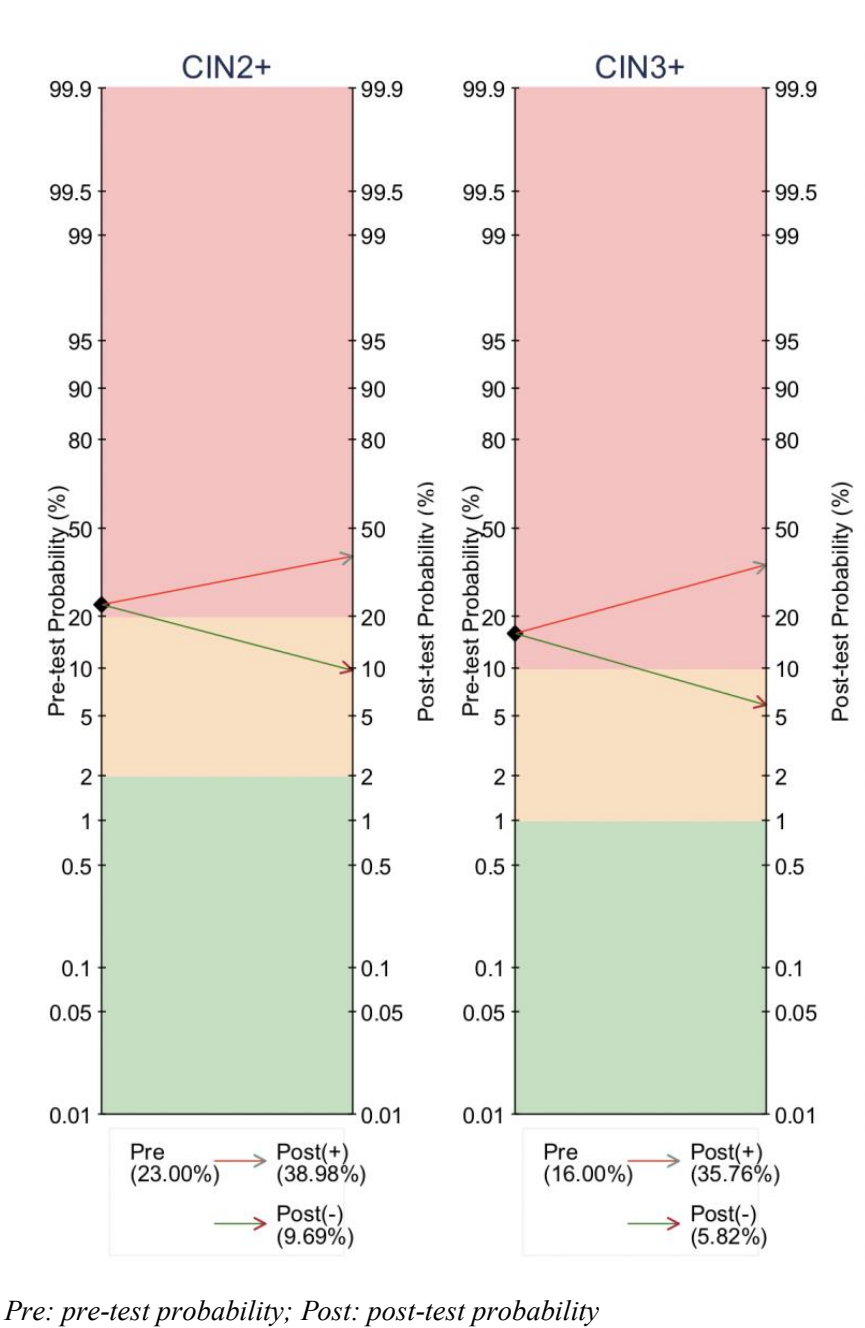


Figure 23. Pre- and post-test probability of HPV16 methylation testing for detection of a) $\geq$ CIN2 and b) $\geq$ CIN3 in HPV16+ women of a screening population. Colours represent clinical recommendations for degree of risk requiring referral to colposcopy where red= high risk, yellow=moderate risk and green=low risk. Pre-test probability of CIN2+ or CIN3+ estimated from a HPV16+ screening population PPV of CIN2+ or CIN3+.

3.4 DISCUSSION

Main results in context with published literature

We observed that the proportion of positive methylation and mean methylation levels increased with disease severity predominantly in L1 and L2 regions, but also in the E2 and E6 genes, while no correlation was present in the HPV16 LCR promoter/enhancer region. Pooled differences in the mean methylation levels and odds ratios for high methylation in $\geq$ CIN2/HSIL vs. $\leq$ CIN1/LSIL were greatest for HPV16 L1 gene followed by the L2. HPV16 L1/L2 genes had overall the best diagnostic accuracy in detecting CIN2 or worse. When applied in a pre- and post-test probability model with a 23% risk of CIN2 in HPV16 positive women, the risk of CIN2 or worse increased to 39% if methylation positive and decreased to 10%, if negative. Half of studies had moderate to high risk of bias according to QUADAS-2 criteria for diagnostic test accuracy. The heterogeneity was overall high for most analyses. We performed a series of sensitivity analyses to explore possible causes of heterogeneity however, residual heterogeneity remained high.

The published literature to date on hrHPV methylation in cervical disease has explored various HPV types, genes and adjacent CpG sites with conflicting results. The majority of studies explore HPV16 and consistently reports higher methylation levels with increasing disease severity for L1/L2, in line with our pooled results. Although human DNA methylation of promoter/enhancer regions have been previously associated with increased tumorigenesis in other cancers,⁴²⁶ the HPV LCR region revealed no correlation with disease severity in our analysis. The evidence in individual studies has been conflicting, with 15 studies reporting higher methylation in the LCR region in high-grade disease, while 6 studies observed lower methylation^{405,414-416}. The biological processes leading to variation in hrHPV CpG site performance are not well understood. The L1 and L2 genes code for late viral capsid proteins, particularly important for viral transmission during the early stages of infection. Possibly these genes are subsequently silenced, allowing prioritisation of E6/E7 oncogene expression for carcinogenesis.¹⁵ Studies examining the whole HPV genome have so far been limited but suggest a global increase in methylation by disease grade except for the LCR^{147,409}. As sequencing costs decrease, whole genome methylation analysis could prove of great benefit in determining CpG sites of best discrimination, avoiding bias and improving understanding of the variation in hrHPV methylation across the genome. Downstream functional studies would also be helpful for further mechanistic understanding of the role of hrHPV methylation in cervical precancer progression.

The detection of women at high risk of CIN2 or worse has been a great challenge for population-based screening. The introduction of the hrHPV DNA test in screening offers improved protection against

invasive cervical cancer when compared with cytology¹²² and is expected to replace cytology globally. hrHPV testing will inevitably increase the number of women that test positive at screening, while the optimal triage test to accurately select those warranting colposcopy remains unclear. Optimising detection, while minimising over-intervention in women at low risk, has major benefits. In many countries like the UK, reflex cytology will be used due to ease and existing infrastructure, although its sensitivity in the detection of CIN3 or worse, is at best 53-75% with a specificity of 78-86%⁴²⁷⁻⁴²⁹. Further molecular markers have been proposed to improve the specificity and NPV, although evidence on superiority of any one of these is lacking. P16/Ki-67 immunostaining appears to improve specificity but requires cytology reporting and highly-trained staff with additional costs.⁴³⁰ hrHPV genotyping has also been suggested to increase specificity (70-71%),⁴³¹ with the advantage of a streamlined, automated hrHPV workflow and potential for self-sampling, but suffers from the inability to distinguish between transient and persistent viral infection and therefore has a low PPV (13-29%), that is likely to fall further with the drop of hrHPV prevalence in vaccinated cohorts.⁴³² hrHPV mRNA tests compare well to hrHPV genotyping assays, with a high sensitivity but no apparent increase in specificity⁴³³ while E6/E7 protein assays have low sensitivity but may improve specificity over hrHPV genotyping and cytology.⁴³⁴

The diagnostic accuracy of HPV16 L1/L2 methylation in the detection of CIN2 or worse compares well to other proposed triage markers (sensitivity=77%, specificity=64%, AUC=0.73) with the highest pooled AUC for a single CpG site at HPV16 L16389 (0.83). hrHPV methylation has several practical advantages given that analysis can be streamlined in the same pipeline with that of hrHPV testing, allowing for future automation and potentially self-sampling. hrHPV methylation also performed well compared to host methylation for the detection of CIN2 or worse (sensitivity = 69-74%; specificity = 66-76%).³⁶²

There were sufficient data to meta-analyse sensitivity and specificity for two individual HPV16 CpG sites which performed well compared to combined markers. We were further able to perform pooled analysis for AUC values for 5 HPV16 CpG sites. We found the greatest AUC at L15611 and L16389 (0.83), outperforming AUC pooled estimates for all other combined markers including L1/L2 and individual genes L1 and L2. Pooled AUC estimates for L15611 and 6389 are also greater than the best performing estimates published for combined host and viral methylation markers for detection of CIN2 or worse in UK screening populations (AUC=0.78, sensitivity=74% (95%CI 59–85), specificity=65% (95%CI 60–70%)).⁴¹¹ Continued genome-wide exploration of hrHPV genotypes should determine the most accurate CpG sites alone or in combination that will yield greater discrimination. Next-generation sequencing methylation assays could allow for accurate whole genome coverage, while also improving

consistency of methylation testing. Eventual hrHPV methylation tests however should be targeted to sequencing the best performing CpG sites which will facilitate low-cost, high-throughput automation.

To date cervical screening targeted the detection of high-grade pre-invasive disease. Modern molecular markers have the potential to advance this beyond the current status quo and provide information on the true carcinogenic potential of hrHPV infections irrespective of histological disease grade, allowing prediction of women that are likely to have progressive or regressive infection and/or disease.²⁹⁵ As local cervical treatment increases reproductive morbidity, ability to avoid unnecessary treatments has major benefits.¹⁷⁰ Several studies explored the value of methylation in predicting hrHPV persistence or development of high-grade CIN. One of the largest longitudinal cohorts from Guanacaste, Costa Rica¹⁵⁰, demonstrated that high methylation in L1/L2 of 72 pre-diagnostic specimens was associated with increased risk of future CIN2+, with an OR of up to 9.3 (95%CI 2.3-45.1). Niyazi et al¹⁵² found similar results for L1 methylation in 145 women infected with HPV16, with an OR of up to 9.9 (95%CI 3.6–27.4). Longitudinal data could not be meta-analysed in our study.

Strengths and limitations

This the first systematic review and meta-analysis to appraise all published literature exploring the use of hrHPV methylation in cervical disease. We included 44 studies and over 8819 hrHPV positive women and explored data for all hrHPV subtypes, genes and CpG sites (including previously unpublished data) and provide a robust quality assessment of this literature. We performed meta-analytical pooling for a number of outcomes, including measures of positive methylation rate and mean methylation according to disease severity, along with odd ratios and diagnostic accuracy meta-analyses to determine test accuracy in detecting high-grade CIN in hrHPV positive women. To this end we have brought together a complex body of literature and present pooled estimates of methylation data by genotype, gene and CpG site, with corresponding risk of bias and heterogeneity assessments. We further calculated the pre- and post-test probability of high-grade CIN, providing a translational and understandable tool for policy makers, clinicians and patients to inform about risk.

There were however some limitations. Half of the studies were at moderate or high risk of bias and there was substantial heterogeneity for most analyses. The included studies used different eligibility criteria, sequencing tests, sampling techniques, and reference verification standards; many did not adhere to diagnostic test accuracy study principles including unbiased patient selection, investigator blinding to the reference test or avoidance of differential verification bias³⁶³. We performed a series of sensitivity analyses to explore heterogeneity. Restricting to studies at low risk of bias, or using histopathology for verification, or exfoliative cells only, reduced heterogeneity in analyses of mean

methylation and ORs ($\geq$ CIN2/HSIL vs. $\leq$ CIN1/LSIL). Restricting to pyrosequencing and NGS alone, did not. To further assess whether the transformation of median to means and imputation of SD values introduced bias, we performed sensitivity analysis to exclude imputed values, with a similar direction of effect observed. Most studies explored L1 and L2 genes that increased statistical power for these comparisons, and repeat assessment of selected CpG sites could have led to confirmation bias. Overall, there appears to be a clear association between increasing hrHPV methylation with cervical disease grade, however large variation in effect sizes exists and residual heterogeneity is yet to be fully explained. The heterogeneity was overall high for most analyses. Although heterogeneity is known to be higher in meta-analyses of continuous rather than those of binary data⁴³⁵ there are very likely other underlying reasons for the observed large heterogeneity. Methylation testing is still under development and not fully standardised. Furthermore, only a few studies assessed reproducibility by duplicate and triplicate analysis of experimental test results. Different studies have used different methylation techniques and reference standards. There was insufficient evidence to evaluate whether environmental factors, such as smoking⁴³⁶, modifies hrHPV methylation levels to cause heterogeneity; this would be an important research direction for the future. Additionally, small study effects were found to be present where investigated (Egger's $P < 0.01$) and although overall we could not formally test all analyses, similar bias of smaller studies showing more extreme estimates might still be present and explain some of the interstudy heterogeneity. We now need larger studies and a collaborative approach, which uses a standardised methodological approach and cut-off, in order to accurately determine the strength of this association.

The substantial heterogeneity noted in the diagnostic test accuracy (DTA) analyses did not improve when restricting to good quality studies. This is likely due to studies using different cut-off points for positivity and exploring various CpG sites, resultant pooled point estimates should be interpreted with caution. In our analysis, we aimed to explore this: where more than one cut-off was provided, we used the most overlapping cut-off thresholds between studies and performed a sensitivity analysis restricting to studies using 10% mean methylation as a cut-off. We used averages of CpG sites by gene but also analysed separately studies presenting single CpG sites where data were sufficient. The overall sensitivity and specificity did not differ greatly in either analysis, although AUC values for single CpG sites performed better than combined gene averages. Sensitivity analyses were small, and it is likely there was not sufficient power to adequately detect cut-off and CpG effects. Although not previously discussed, methylation 'classifiers' have attempted to bring the best performing CpG sites to a single marker and are currently under validation⁴¹¹. Future research should assess whether weighted combination of several best performing CpG sites has the potential to further improve the diagnostic accuracy. Following this it will be crucial to determine the best cut-off for an eventual diagnostic test,

which our review suggests may vary according to the included CpG sites. It should be noted that a cut-off of 0% should not be used, to avoid confusion with failed assays. Once cut-offs and CpG sites have been established, robust and blinded diagnostic test accuracy studies are needed to reduce risk of bias in methylation studies and determine the true chances of false positives and negatives, including in populations of different geographical regions and ethnicities. Additionally, just as genetic variation can alter measurement of methylation levels in the host, studies have demonstrated that methylation levels may be impacted by the number of CpG sites in the hrHPV genome, as sequence variations within hrHPV genotypes can create and destroy CpG sites⁴³⁷.

3.5 CONCLUSIONS

Despite these limitations, this meta-analysis provides the most comprehensive review and critical appraisal of the existing literature with stringent quality assessment of the methodological validity of existing studies; this information on the best performing techniques, cut-offs, genes and CPG sites should inform future design of diagnostic accuracy studies, including standardisation of assays. For translation to clinical practise, we urgently need larger and higher quality evidence. To provide this, a collaborative and standardised research approach, with consideration of data deposition on publicly available repositories, should be encouraged in the field.

We calculated pre- and post-test probabilities based on the rates of high-grade CIN in HPV16 positive women irrespective of cytology as an example of a screening population. We used the best performing sites and genes (HPV16 L1/L2) and saw that methylation added good additional discrimination between high and low-grade disease, although pre-test probabilities of HPV16 positive women were high. Multiplexed methylation assays combining CpGs across all hrHPV types are currently under development,⁴³⁸ while multiplexing for hrHPV genotyping is becoming well established⁴³⁹. To increase the clinical utility of viral methylation testing, multiplexed methylation assays are needed to cover all hrHPV types and could be applied to triage women screening as hrHPV positive, who have not been genotyped. In this scenario, pre-test probabilities would be lower³⁸⁵ and the value of methylation testing would increase. In addition to multiplexed hrHPV methylation assays, the addition of host methylation markers could further increase the accuracy for detection of CIN2 or more.

Chapter 4: Epigenetic variation in cervical preinvasive and invasive disease: an epigenome-wide association study (EWAS) on the EPIC 850k array.

Acknowledgments:

Assistance in statistical analyses were provided by Miss B Bodinier. Figure 25 (B and C) and Figure 26 were produced by Miss B Bodinier.

4.1 BACKGROUND

Cervical cancer remains the fourth most common cancer in women globally. Almost 99% of cervical cancer is caused by persistent infection with a high-risk genotype of human papillomavirus (hrHPV). The understanding that the majority of invasive cervical cancers are preceded by a detectable and treatable preinvasive lesion, cervical intraepithelial neoplasia (CIN), has allowed for cervical screening. Where implemented, cervical screening programmes have been highly successful in reducing the incidence and mortality from cervical cancer through monitoring and treatment of CIN. Traditionally cervical screening programmes have relied on cytology of exfoliated cervical cells. More recently, hrHPV DNA testing has replaced cytology in many national screening programmes, which is anticipated to increase sensitivity of screening and offer greater protection against invasive cancer¹¹⁰. However, the decreased specificity of hrHPV testing will increase the number of women screening positive. As such, further markers of high-grade CIN and cervical cancer are needed to increase the efficiency of screening and prevent overtreatment.

Methylation is a molecular process, which occurs at specific methylation sites in the DNA, called methylation 5-cytosine-phosphate-guanine-3 (CpG) sites, to regulate gene expression. Such sites occur where a cytosine is followed by a guanine nucleotide in the 5'-3' direction and are scattered throughout the human genome, although promoter and enhancer regions are particularly enriched in these patterns. Associations between DNA methylation change and cancer progression have been reported for multiple cancer types¹³²⁻¹³⁴, and is thought to reflect ongoing carcinogenesis through processes such as loss of tumour suppressor gene function and other aberrant cell cycle and cellular growth controls.

Several previous targeted epigenetic studies have identified CpG sites as candidate markers of cervical disease progression such as *CADM1*, *EPB41L3*, *FAM19A4*, *PAX1*, and *SOX1* genes^{138,140 135,139}. To date limited epigenome-wide exploration has been performed in cervical cancer, with previous studies focussing on CIN only, and it is likely that most methylation markers associated with high-grade cervical phenotypes remain unknown. Increased genome coverage could uncover a greater proportion of the epigenetic change ongoing in cervical carcinogenesis and identify novel markers for use in screening.

1.1 Methods

Sample size calculation

Sample size was calculated based on the intention to test for methylation on the Illumina 850K array including 850,000 tests, and previous estimates of differences in methylation values between cancer and normal cervical epithelial cells. Using an unmatched case-control study design with equal numbers of cases and controls and univariate logistic regression we had an 80% power to detect an effect size of $OR=2.2$ at an alpha of $6e-8$ (850,000 tests) with a study size of $n=240$. In the present study, we included 328 women to reach a sample size of $n=241$ for epigenome-wide sequencing.

Study design

Recruitment to the study was undertaken from 2017-2021, from two affiliated university teaching hospitals in London (UK) and from a third university teaching hospital in Thessaloniki (Greece) as part of the PRECAC study biobank held at Imperial College London, as previously described^{20,440}. Briefly, we included non-pregnant women, 18–70 years of age who attended the colposcopy, gynaecology clinics or accessed inpatient gynaecological services at Imperial College NHS Healthcare Trust, or colposcopy clinics at the Aristotle University of Thessaloniki. Normal controls were defined as women with a cytologically or histologically proven diagnosis of normal, whilst cases included women with a histologically-proven CIN3 or invasive carcinoma of the cervix graded by a senior cytopathologist or gynaecological pathologist according to the Bethesda (cytology) and NHSSCP systems (histopathology). All women were tested for hrHPV status using Abbott RealTime HR HPV assay (14 HR-HPV types) (Edinburgh, UK) and the Anyplex HPV 28 (28 hrHPV and hrHPV types) (Edinburgh, UK). Detailed medical and gynaecological history was collected. Women with other gynaecological cancers, who were HIV or hepatitis B/C positive or had another active genital tract bacterial infection were excluded. Ethnicity was self-reported as Caucasian, Asian, Black or other. Cervical samples were taken by a cytobrush used in the standard manner for cervical cytology i.e., using the ThinPrep Preservcyt system (Hologic, Crawley, UK) and stored at 4 °C until use. Samples were visually assessed for blood contamination and recorded as none, light, moderate or heavy in the study database by two investigators using a pre-designed colour chart. Study participants provided written informed consent. Recruitment continued until the study sample size was reached. Ethical approval was obtained from the National Research Ethics Service Committee London – Fulham (approval number 13/LO/0126) and the Aristotle University of Thessaloniki Bioethics Committee (approval number 123/08-12-2014).

DNA extraction and quantification

DNA extractions and QC were conducted in the same laboratory (Imperial College London, UK). Whole genomic DNA was extracted from 2-4mls of the Preservcyt solution using a QIAmp DNA Mini kit (Qiagen, Crawley, UK) according to manufacturer's instructions and eluted in DNA-free distilled

water. Samples were tested for genomic DNA quality and quantity using the Nanodrop spectrophotometer (ThermoFischer Scientific, MA) and PicoGreen protocol (Quant-iT PicoGreen dsDNA products Invitrogen). Where DNA quality was insufficient as measured by 260/280 and 260/230 ratios, samples were re-extracted. From a total of 328 samples collected, 84 samples were excluded due to inadequate DNA quality at second extraction. The remaining 244 samples were concentrated or diluted to a standard concentration of 50ng/ul prior to bisulphite conversion.

Bisulphite treatment and methylation array

Bisulphite conversion and methylation array processing were undertaken at the Oxford Genomics laboratory (Oxford, UK) where 500ng of DNA were bisulphite-converted with the EZ-96 DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Next, the Illumina Infinium Human Methylation 850k (EPIC) array (San Diego, CA) was hybridised as per the manufacturer's protocol. Methylation testing was performed blinded to hrHPV status, cytology and histology. This array measures DNA methylation at >850,000 cytosine positions across the human genome. Samples were randomised with respect to slide and position on methylation arrays to mitigate batch effects.

Derivation of normalised DNA methylation values and quality control

Raw data were analysed in the form of the Illumina Intensity Data (IDAT) files as input to an in-house analysis pipeline in R as previously described⁴⁴¹. Pre-processing included background subtraction and colour correction to account for the dye bias seen in Infinium II probes. This was done by equalising the intensities in the green and red channels to the average intensity across the two colours as measured by normalisation control probes present on the BeadChip. Samples failing quality control were identified by those lying 3 standard deviations outside of the sample distribution for bisulphite converted probes, and by measuring the average signals along the three types of beads (type 1 green, type 1 red and type II); all samples passed this initial quality control step (appendix 16). DNA methylation-inferred sex was evaluated via visualisation of the proportion of missing values on chromosome Y as a function of median methylation proportions on chromosome X; two samples were removed following identification of two outlying women with lower proportions of missing data on chromosome Y (appendix 17). We explored genetic data and measured SNPs, no individuals had more than 50% missing SNP data. 241 samples remained for analysis. Probes with missing values in more than 30% of the samples were excluded (N=22,248). The methylation level at each CpG was expressed as M-values, which were obtained from $\log_2$ -transformation of the fraction of methylated cytosines at that specific location (β value). To correct for technical variation, we used a series of linear mixed

models with methylation m-values for each of the CpG sites as the outcome, and chip and position as random intercepts. The residuals were extracted from these models and used as de-noised methylation levels, as previously described⁴⁴².

Epigenetic association analysis

To identify differentially methylated CpG sites, CIN3 or cervical cancer (ICC) status was regressed against de-noised methylation levels in a series of logistic models, with adjustment for DNA quality, hrHPV status and age. We corrected for multiple testing using the Bonferroni procedure.

We did a series of conditional analyses to identify CpGs that complementarily contributed to the disease risk prediction. For this, we used an iterative procedure further adjusting the models on the CpG site showing the strongest association with the CIN3 or ICC outcome. This procedure was repeated until no significant association could be detected after correction for multiple testing. The CpG sites showing the strongest association at each iteration are considered as independently associated with the cervical cancer outcomes. For each of these CpG sites, we report the conditional p value (p^{cond}), which is defined as the p value obtained from the model adjusted on CpG sites detected in previous iterations, i.e. the model in which the corresponding CpG site showed the strongest association.

We used a clustering approach to identify groups of CpG sites bringing redundant information to the discrimination between cases and controls. This was done by applying hierarchical clustering with complete linkage on the CpG sites significantly associated with the outcome. The absolute Pearson's correlation, conditioned on the disease status, was used as a similarity metric.

To account for disease heterogeneity and progression, we subsequently conducted stratified analyses comparing the methylation levels of participants with (i) CIN3 only, or (ii) ICC only, to controls.

As sensitivity analyses, we performed the logistic models without adjusting for hrHPV status. To further investigate the influence of hrHPV on genome-wide methylation in cases versus controls, we performed the same analysis in hrHPV positive women only. To identify CpG sites associated with hrHPV status, we performed a logistic regression of de-noised methylation levels against hrHPV status, adjusting for age, DNA quantity, restricting to controls only.

Quantifying the contribution to disease risk prediction

To quantify the ability of the identified CpG sites to discriminate cases from controls, we conducted a Receiver Operating Characteristic (ROC) analysis⁴⁴³. To limit over-fitting, the logistic models were

trained on 80% of the participants and performances were evaluated on the remaining 20% of the samples (test set). The proportions of cases and controls in the training and test sets were fixed to that observed in the full sample. The procedure was repeated over 1,000 iterations for a more reliable evaluation of the model performance. We reported the mean, 5th and 95th percentiles of the sensitivity, specificity and Area Under the Curve (AUC) to evaluate the clinical performance and extent of prediction of methylation markers for the detection of CIN3 or cervical cancer lesions. For independent validation, we conducted the same procedure using publicly available data from another study (accession number GSE143752¹⁴²) of 42 CIN3 cases and 54 control cervical LBC samples sequenced on the Illumina EPIC array.

2.0 Results

Participant characteristics

Characteristics of the included study population (N=241) are summarised in Table 14. Overall prevalence of hrHPV was 62.8%, with 92.1% of the cases and 24.6% of the controls testing hrHPV positive.

Table 14. Participant characteristics of the EWAS study population including assessed covariates

	Controls (N=114)	CIN3 cases (N=73)	Cancer cases (N=54)
Age (years) + SD	40.5 (12.3)	33.1 (7.3)	49.7 (15.3)
hrHPV infection			
<i>No</i>	86 (75.4%)	0 (0.0%)	10 (18.5%)
<i>Yes</i>	28 (24.6%)	73 (100.0%)	44 (81.5%)
Smoking status			
<i>Never smokers</i>	41 (36.0%)	33 (45.2%)	22 (41.0%)
<i>Ever smokers</i>	7 (6.14%)	7 (9.59%)	5 (9.3%)
<i>Missing</i>	66 (57.9%)	33 (45.21%)	27 (50.00%)
DNA quantity + SD	1.52 (0.5)	1.7 (0.4)	2.0 (0.4)
Blood contamination			
<i>Moderate</i>	89 (78.1%)	65 (89.0%)	28 (51.9%)
<i>Heavy</i>	25 (21.9%)	8 (11.0%)	26 (48.2%)

Abbreviations: CIN3: cervical intraepithelial neoplasia grade 3; hrHPV: high-risk genotype of human papillomavirus; ICC: invasive cervical cancer; SD: standard deviation

Epigenome-wide association analyses

Using univariate logistic models for the association between methylation level at each of the 843,611 CpG sites included for analysis, N=38,584 sites were differentially methylated in CIN3/ICC cases compared to controls in the models adjusted for age, DNA quantity and technical confounders, after correction for multiple testing using the Bonferroni procedure (Figure 24a). Of these, N=33,923 showed higher methylation levels in cases compared to controls (Figure 24b). There was some evidence of genomic inflation (Lambda 2.4) (appendix 18). When adjusting for hrHPV status, the strength of the associations was attenuated (Figure 24c). We identified N=409 CpG sites with differentially methylated levels at the Bonferroni corrected significance level (appendix 19), which were spread across the whole genome. 130 CpG sites demonstrated a differential methylation of greater or equal to 15%. These CpG sites were also strongly associated with the ICC/CIN3 outcome in the model not adjusted for hrHPV status (P-value $< 2.41 \times 10^{-7}$ (Lambda 1.6)).

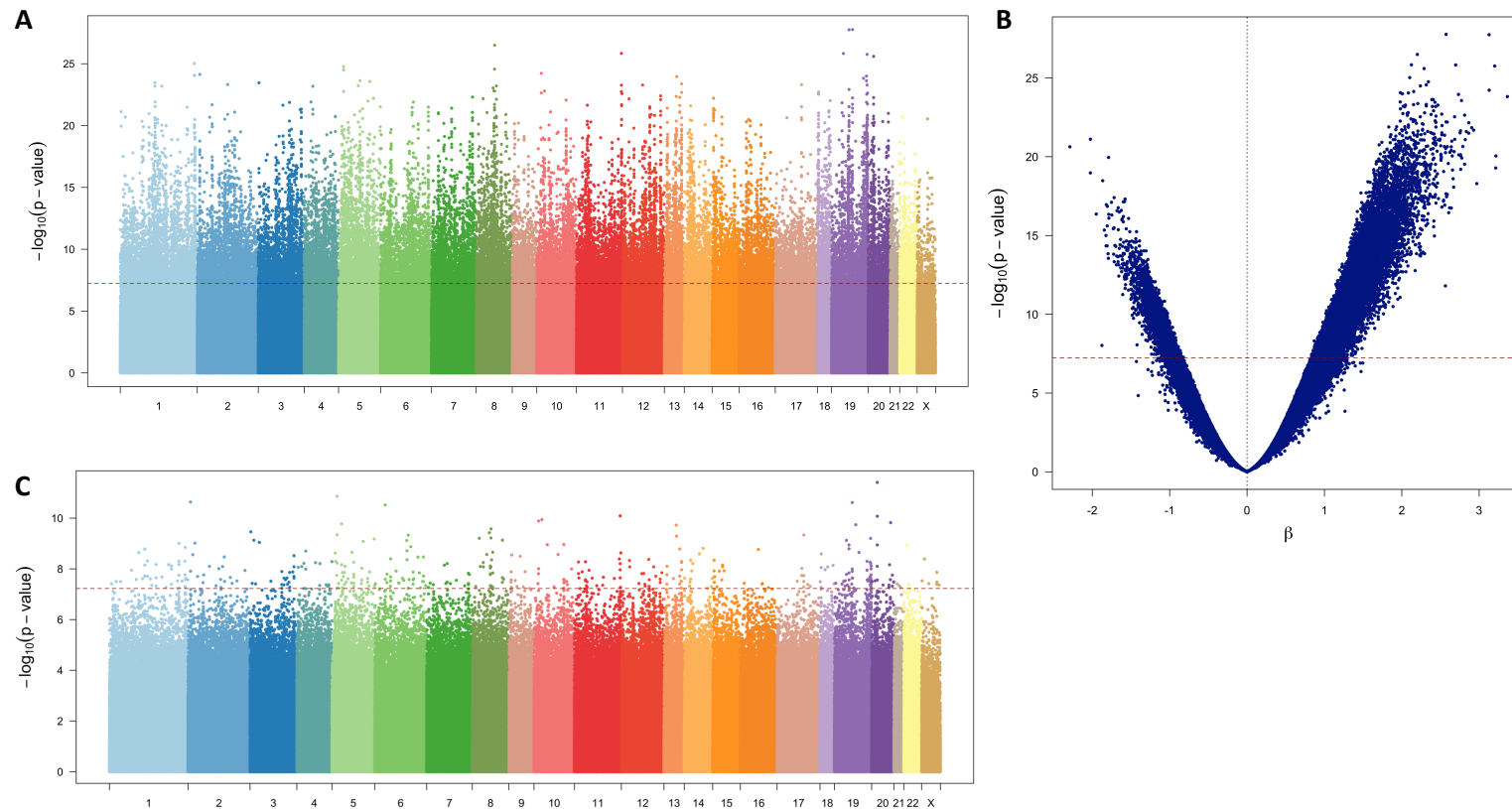


Figure 24: Association between CpG sites and CIN3 (N=73) or ICC (N=54) status (N=114 controls) unadjusted versus adjusted for hrHPV status in the study population. (A) Manhattan plot showing the P-values, measuring the strength of association, derived from logistic models **unadjusted for hrHPV status** and represented on the $-\log_{10}$ scale (Y-axis). CpG sites (N=843,611) are ordered by their position on the genome (X-axis). (B) Volcano plot demonstrating distribution of methylation beta-values by $-\log_{10}(P\text{-value})$ unadjusted for hrHPV status where 0 represents the null value and the red dashed line represents the Bonferroni-corrected EWAS significance level. Genome-wide a greater gain in methylation is observed in association with CIN3 or ICC case status across the assayed CpG sites on the EPIC array. (C) Manhattan plot showing the P-values, measuring the strength of association, derived from logistic models **adjusted for hrHPV status** and represented on the $-\log_{10}$ scale (Y-axis). CpG sites (N=843,611) are ordered by their position on the genome (X-axis). The epigenome-wide significance level is set to the Bonferroni-corrected threshold (horizontal red line).

Table 15: Top CpG sites in the main epigenome-wide association analysis, ordered by P-value in the logistic regression and restricted to a percentage methylation change of greater than 15% between cases (CIN3 and Cancer) versus normal controls (N=241).

CpG	Chr	Genomic Position	Gene	OR	OR lower 95% CI	OR upper 95% CI	Coefficient	SE	P-value	Δ beta
cg16767801	20	21686548	<i>PAX1</i>	6.32	3.20	12.50	1.84	0.35	3.9009E-12	16.39%
cg07195011	5	11904114	<i>CTNND2</i>	7.05	3.25	15.30	1.95	0.40	1.3655E-11	18.38%
cg17213402	2	5813650	–	5.80	3.04	11.08	1.76	0.33	2.3096E-11	19.48%
cg27058257	19	30019529	<i>VSTM2B</i>	13.84	4.08	46.93	2.63	0.62	2.425E-11	18.77%
cg11358689	11	131781260	<i>NTM</i>	5.40	2.93	9.97	1.69	0.31	8.1265E-11	24.88%
cg14587524	19	38183262	<i>ZNF781</i>	6.77	3.17	14.48	1.91	0.39	1.819E-10	19.01%
cg21183256	8	73163868	–	5.23	2.82	9.73	1.66	0.32	2.6508E-10	24.80%
cg25260137	8	65282185	–	4.66	2.62	8.31	1.54	0.29	3.7587E-10	19.47%
cg13368756	5	11904127	<i>CTNND2</i>	5.86	2.87	12.00	1.77	0.37	4.5191E-10	20.69%
cg02688304	17	50236104	<i>CA10</i>	5.47	2.84	10.56	1.70	0.34	4.6009E-10	18.20%
cg01758512	6	96463902	<i>FUT9</i>	4.27	2.54	7.20	1.45	0.27	4.676E-10	20.68%
cg25832771	8	72756058	<i>MSC</i>	6.37	2.94	13.79	1.85	0.39	6.0599E-10	16.78%
cg17403609	5	180075862	<i>FLT4</i>	7.66	3.31	17.74	2.04	0.43	6.6339E-10	15.26%
cg13912117	8	132054555	–	5.41	2.81	10.44	1.69	0.34	7.4124E-10	15.84%
cg17509967	19	13617094	<i>CACNA1A</i>	7.31	3.15	16.97	1.99	0.43	7.5355E-10	15.92%
cg07704959	3	40471662	–	4.36	2.54	7.50	1.47	0.28	9.0401E-10	16.39%
cg08448701	20	21686282	<i>PAX1</i>	5.01	2.66	9.44	1.61	0.32	1.138E-09	15.97%
cg01650149	1	240255141	<i>FMN2</i>	4.73	2.60	8.62	1.55	0.31	1.4109E-09	19.84%
cg15443663	16	48936072	–	4.19	2.46	7.14	1.43	0.27	1.716E-09	15.11%
cg10135483	6	87647527	<i>HTR1E</i>	5.14	2.66	9.93	1.64	0.34	1.9359E-09	16.28%
cg17233763	8	85095525	<i>RALYL</i>	4.71	2.56	8.66	1.55	0.31	2.2168E-09	18.21%

Abbreviations: Chr:Chromosome, CI: confidence interval, OR: odds ratio, SE: standard Error Δ: change in % methylation value between cases and controls. Beta represents the mean intensity of methylated beads/mean intensity of unmethylated + methylated beads. *Where intragenic, the gene is annotated from the UCSC reference gene browser. Intronic and intergenic CpGs sites annotated to genomic position only

Conditional analyses and hierarchical clustering

Conditional analyses adjusted on hrHPV status identified two CpG sites bringing complementary information discriminating CIN3/ICC cases from controls. These were (i) located in the first exon of *PAX1* on chromosome 20 (cg16767801; OR=6.32(95%CI=3.20-12.50); $P=3.90 \times 10^{-12}$; Δ beta =16.4%), and (ii) gene body of *NREP-AS1* (cg23642047; OR=0.33(95%CI=0.21-0.52); $P=3.91 \times 10^{-12}$ ($P_{cond}=2.44 \times 10^{-10}$; Δ beta=-15.9%) on Chromosome 5 (Table 16). A total of N=727 CpG sites were differentially methylated between CIN3/ICC cases and controls in the models adjusted for hrHPV status (Figure 25a) (N=409), and further conditionally adjusted for the strongest CpG site (cg161767801) (N=318).

Table 16: Differentially methylated CpG sites identified as independent from conditional and clustering analyses in CIN3 and ICC

CpG	Chr	Genomic Position	Gene	Anno	N	OR (95%CI)	P (P_{cond})	Δ beta
cg16767801	20	21686548	<i>PAX1</i>	First Exon	241	6.32 (3.20- 12.50)	3.90×10^{-12} (3.90×10^{-12})	16.4%
cg23642047	5	111265808	<i>NREP-AS1</i>	Gene Body	241	0.33 (0.21- 0.52)	3.34×10^{-7} (2.45×10^{-10})	- 15.9%

Abbreviations: Anno: Annotation; Chr: chromosome; N: number of women included; OR: odds ratio; P: P-value; P_{cond} : conditional P-value; Δ : change in % methylation value between cases and controls

Using hierarchical clustering, we could identify two clusters of strongly correlated CpG sites including cg16767801 (in blue) or cg23642047 (in red) (Figure 25B). Figure 25C shows that the P-values of CpG sites from the cluster with cg16767801 (or cg23642047) are strongly attenuated upon adjustment for the corresponding lead CpG site. None of the associations are significant after correction for multiple testing in models adjusted for both cg161767801 and cg23642047 (model 2).

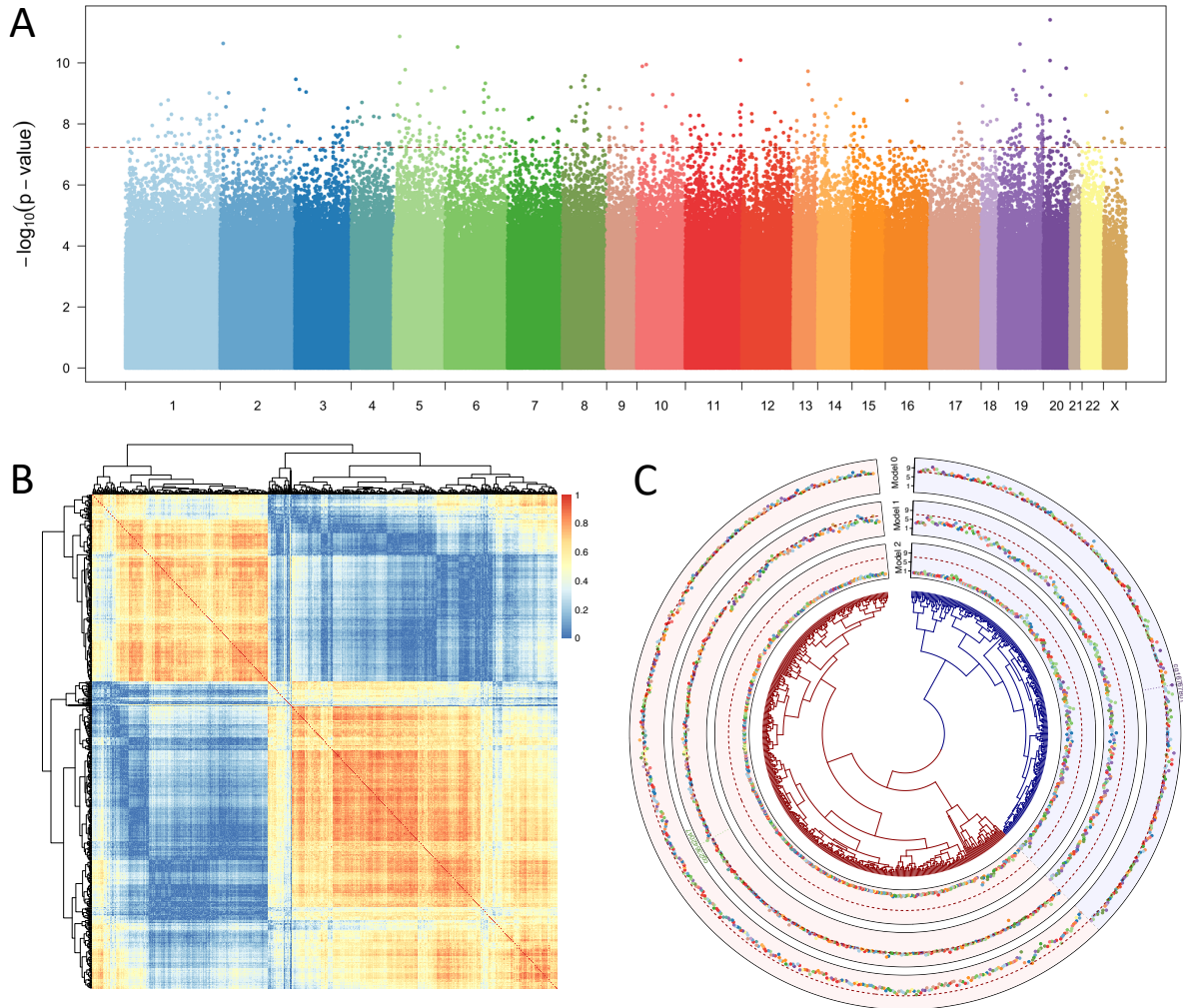


Figure 25: Associations between CpG sites and CIN3 (N=73) or ICC (N=54) status (N=114 controls) adjusted for hrHPV status in the study population. (A) Manhattan plot showing the P-values, measuring the strength of association, derived from logistic models adjusted for hrHPV status and represented on the $-\log_{10}$ scale (Y-axis). CpG sites (N=843,611) are ordered by their position on the genome (X-axis). The epigenome-wide significance level is set to the Bonferroni-corrected threshold (horizontal red line). (B) Heatmap of absolute correlations between the CpG sites (N=727) significantly associated with CIN3 or ICC status in the model adjusted on hrHPV status, or further adjusted on cg16767801. To avoid spurious correlations, these are adjusted on the case/control status. CpG sites are ordered by hierarchical clustering with complete linkage using the absolute correlation as a similarity metric. Horizontal and vertical axes both represent all possible CpG sites associated with CIN3/ICC in the main analysis. Red represents 1 where a perfect correlation is observed, blue represents 0 where no correlation is observed. (C) Circos plot showing the P-values from conditional analyses on $-\log_{10}$ scale. Models are adjusted on hrHPV status (Model 0), and further adjusted on cg16767801 (Model 1), and cg23642047 (Model 2). CpG sites (N=727) are ordered based on the same hierarchical clustering. Lead CpG sites from each of the models are indicated with vertical dotted lines. Points are coloured by chromosome as in panel (A). (Figure panels B and C were produced by B Bodinier).

Stratified analyses of preinvasive (CIN3) versus invasive phenotypes

When stratifying the analysis into CIN3 (N=73) or ICC (N=54) phenotypes, there was a reduction in the number of associations observed for the CIN3 than ICC (Figure 26A-B). A total of N=5,232 CpG sites were found differentially methylated between ICC cases and controls in logistic models adjusted for hrHPV status. The two CpG sites identified in conditional analyses were strongly associated with ICC status (P-value of 4.34×10^{-12} for cg16767801 and 9.74×10^{-7} for cg23642047). Analyses comparing CIN3 cases from controls revealed 62 differentially methylated CpG sites. While cg16767801 remained significant, the association was weaker for cg23642047 (P-value of 5.59×10^{-3}).

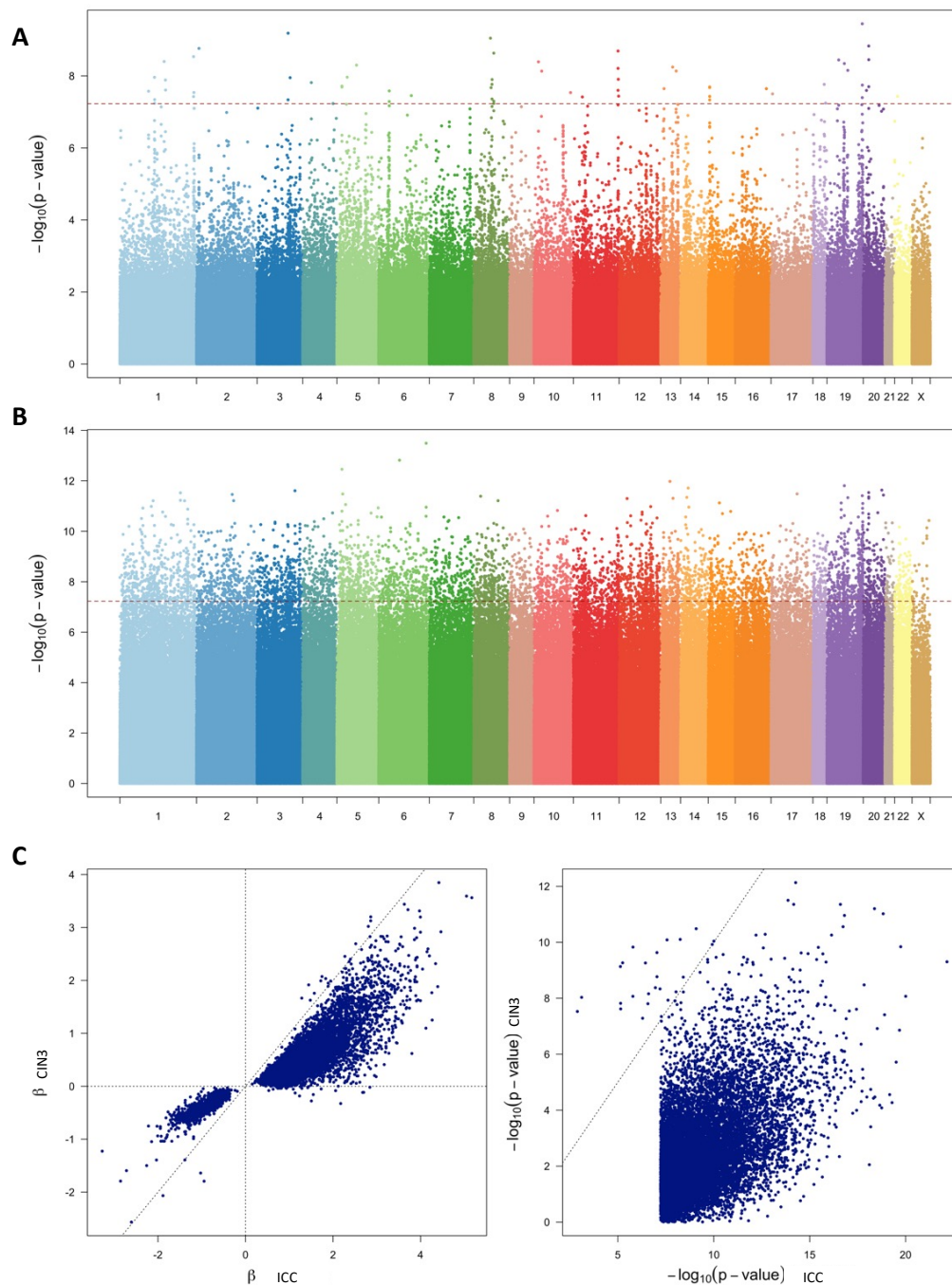


Figure 26: Manhattan plot showing the P-values, measuring the strength of association, derived from logistic models adjusted for HPV status and represented on the $-\log_{10}$ scale (Y-axis) for CIN3 only (A) and ICC only (B). CpG sites $N=843,611$ are ordered by their position on the genome (X-axis). The epigenome-wide significance level is set to the Bonferroni-corrected threshold (horizontal red line). Scatter plots of beta values (C, left) and $-\log_{10}(P\text{-value})$ (C, right) for all CpG sites identified as statistically significant ($N=5,263$) which contains the $N=5,232$ that are significant for ICC only (X-axis) and the $N=62$ for CIN3 only (y-axis).

Despite the smaller sample size (N=111 cases and N=25 controls), a total of N=719 CpG sites were found differentially methylated in hrHPV positive cases compared to hrHPV positive controls. The results were consistent with those from the models adjusted on hrHPV status on the full population (P-values $<1.68 \times 10^{-3}$ in the analyses restricted to hrHPV positives for the N=409 significant CpG sites identified in the full population) (Figure 27).

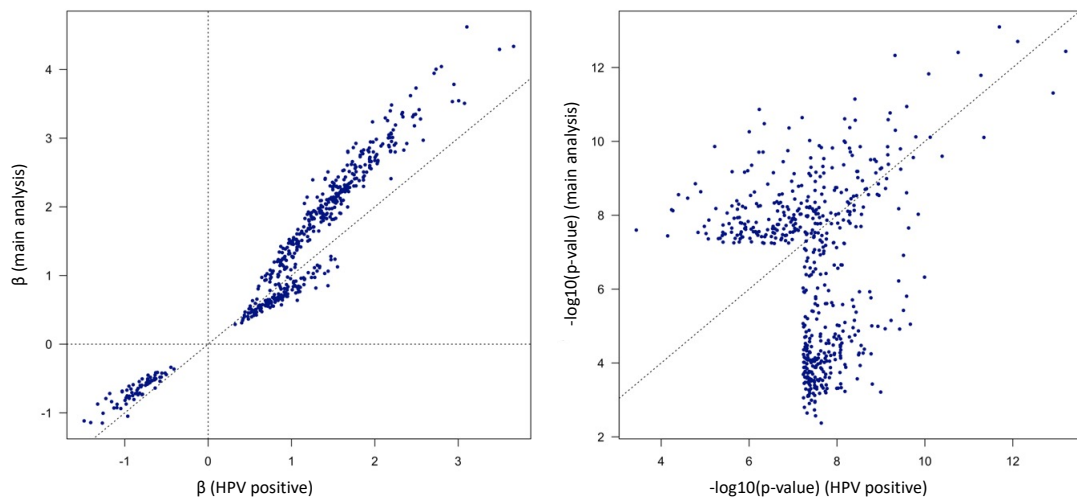


Figure 27: Scatter plots showing the correlation of beta methylation values (left) and $-\log_{10}(\text{P-value})$ (right) for Bonferroni significant CpG sites (N=409 CpG) from logistic models of the main analysis with the CIN3/ICC status as the outcome adjusted for hrHPV status (X-axis, N=114 controls and N=127 cases) or restricted to hrHPV positive participants (Y-axis, N=25 controls and N=111 cases).

Notably, DNA quantity was included in the final model as a higher DNA quantity was observed in cancer and CIN3 cases compared to controls. To test the hypothesis that cancer samples also contained more white blood cells than non-cancer samples as a result of tumour neoangiogenesis, we performed a stratified analysis on samples with the most blood contamination on visual inspection. Firstly, we analysed the level of contamination in a randomly selected subset of cases and controls, then we performed a logistic regression adjusted for visual contamination. An even distribution of contamination was observed across cases and controls, while no effect modification from visual contamination was observed. Standard packages to adjust for cell type in methylation analyses e/g/ the Houseman approach⁴⁴⁴, could not be applied as there were no reference data available for cell type distribution in cervical epithelial samples.

Due to the high degree of missing data on smoking we could not include smoking status in the final model, however, there was no significant difference in the proportion of known current or ever smokers between cases and controls (Table 13).

Diagnostic accuracy

As a demonstration of the predictive ability of the CpG sites cg16767801 and cg23642047 we used a ROC curve analysis. Prediction models based on the methylation levels of a single CpG site yielded very good discrimination performances between CIN3/ICC cases and controls (average AUC of 0.85 (95%CI 0.85-0.85) across 1,000 iterations for cg16767801, and 0.75 for cg23642047) (Figure 28A). The performances were further improved when using the two CpG sites as predictors in a logistic model (average AUC of 0.92 (0.92-0.92)). For both markers, we observed a better ability to discriminate controls from cervical cancer cases (AUC of 0.92 (0.91-0.92) (using both markers) than CIN3 cases (AUC of 0.88(0.87-0.88)) (Figure 28B-C). A sensitivity of 83%, resulted in a specificity of 87% (Table 14). For a set sensitivity, specificity was notably higher for cancer than CIN3 (appendix 20). Further assessment of the prediction performances of these two CpG sites in an independent validation set (N=54 controls; N=42 CIN3; N=0 ICC cases) (Figure 28D), revealed an average AUC of 0.77 (0.76-0.79) using the two CpG sites as predictors. Over 99% of the predictive ability of the bigenic marker for CIN3 was attributable to cg16767801 in this validation set (Table 17). Cg23642047 as a single marker did not validate. An independent validation set of ICC samples with available 850k array data for replication was not found at the stage of report writing.

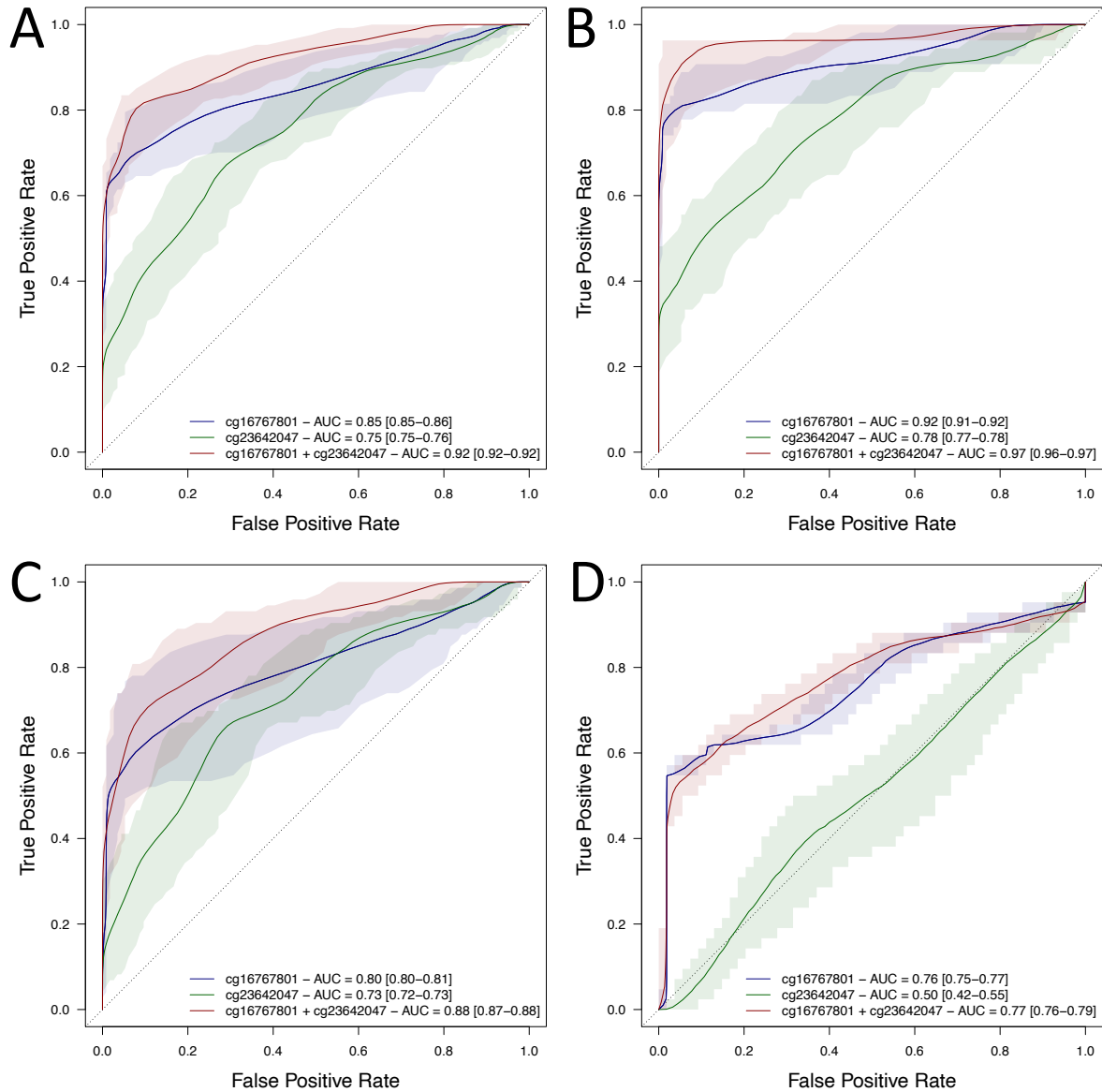


Figure 28: ROC curves for logistic models including cg16767801 (in blue), or cg23642047 (in green) or both (in red) as predictors and all cases (A), cancer (B) or CIN3 (C, D) as outcome. Models were fitted on 1,000 training sets with 80% of the cases and 80% of the controls and performances were evaluated on a test set with the remaining 20% of participants. The pointwise average, 5th and 95th percentiles of the True and False Positive Rates and Area Under the Curve (AUC) are reported. Results presented in panels A, B and C were obtained using our study population (N=114 controls, N=73 CIN3 cases and N=54 cancer cases). An independent validation set (accession number GSE14375s, with N=54 controls and N=42 CIN3 cases) was used in panel D.

Table 17. Prediction performance as measured by the sensitivity and specificity for logistic models including cg16767801 and/or cg23642047 as predictor(s) and comparing controls (N=114) to all cases (N=127). Performance metrics are reported as the mean, 5th and 95th quantiles computed over N=1,000 subsamples for different thresholds and proportions of the population predicted as cases.

cg16767801				cg23642047				cg16767801 + cg23642047			
Proportion	Threshold	Sensitivity	Specificity	Proportion	Threshold	Sensitivity	Specificity	Proportion	Threshold	Sensitivity	Specificity
90%	0.12 [0.11-0.12]	0.97 [0.96-0.97]	0.17 [0.17-0.18]	90%	0.21 [0.20-0.22]	0.95 [0.94-0.96]	0.15 [0.14-0.17]	90%	0.04 [0.03-0.04]	1.00 [0.99-1.00]	0.21 [0.20-0.21]
80%	0.22 [0.21-0.23]	0.91 [0.91-0.92]	0.32 [0.32-0.33]	80%	0.29 [0.28-0.30]	0.91 [0.90-0.91]	0.32 [0.31-0.32]	80%	0.09 [0.09-0.10]	0.96 [0.95-0.98]	0.38 [0.37-0.39]
70%	0.29 [0.28-0.30]	0.86 [0.85-0.87]	0.48 [0.46-0.49]	70%	0.38 [0.37-0.39]	0.85 [0.84-0.86]	0.47 [0.46-0.47]	70%	0.18 [0.17-0.19]	0.93 [0.93-0.94]	0.56 [0.55-0.57]
60%	0.38 [0.37-0.39]	0.83 [0.82-0.83]	0.65 [0.64-0.65]	60%	0.45 [0.44-0.45]	0.75 [0.74-0.76]	0.56 [0.55-0.57]	60%	0.32 [0.30-0.34]	0.88 [0.87-0.89]	0.71 [0.70-0.72]
50%	0.46 [0.45-0.47]	0.77 [0.76-0.78]	0.81 [0.80-0.82]	50%	0.54 [0.53-0.54]	0.68 [0.68-0.69]	0.71 [0.70-0.71]	50%	0.51 [0.49-0.53]	0.83 [0.83-0.83]	0.87 [0.87-0.88]
40%	0.57 [0.56-0.58]	0.70 [0.69-0.70]	0.94 [0.93-0.94]	40%	0.61 [0.60-0.62]	0.56 [0.55-0.57]	0.78 [0.77-0.79]	40%	0.73 [0.72-0.75]	0.72 [0.71-0.72]	0.95 [0.95-0.96]
30%	0.82 [0.81-0.83]	0.56 [0.56-0.56]	0.99 [0.99-0.99]	30%	0.68 [0.68-0.69]	0.46 [0.45-0.46]	0.88 [0.87-0.89]	30%	0.91 [0.90-0.92]	0.56 [0.56-0.57]	1.00 [0.99-1.00]
20%	0.93 [0.93-0.94]	0.38 [0.37-0.38]	1.00 [0.99-1.00]	20%	0.75 [0.75-0.76]	0.33 [0.32-0.33]	0.94 [0.94-0.95]	20%	0.97 [0.97-0.97]	0.38 [0.38-0.38]	1.00 [1.00-1.00]
10%	0.98 [0.97-0.98]	0.19 [0.19-0.19]	1.00 [1.00-1.00]	10%	0.84 [0.83-0.85]	0.19 [0.19-0.19]	1.00 [1.00-1.00]	10%	0.99 [0.99-0.99]	0.19 [0.19-0.19]	1.00 [1.00-1.00]

4.3 DISCUSSION

Using an epigenome-wide approach, we identified 409 CpG sites to be strongly associated with the cervical cancer or CIN3 phenotype through differential methylation; while both negative and positive differential methylation was observed, more CpGs demonstrated an associated gain in methylation. CpG sites were found in 216 unique genes. Conditional analyses allowed further exploration of the interdependence of methylated CpGs, with two clear independent CpGs identified at *PAXI* (cg16767801) and *NREP-ASI* (cg23642047). Clustering on P-values of CpG sites revealed that loci in clusters with cg16767801 (or cg23642047) were strongly attenuated upon adjustment for the corresponding lead CpG site, suggesting that CpG sites clustered together bring redundant information for disease prediction.

To our knowledge, this is the first study to adequately explore the differential methylation in cervical cancer samples versus normal cervical epithelium using a genome-wide approach. Few previous studies have undertaken epigenome-wide analysis of cervical cancer.

One small EWAS study of six cancer samples analysed on the Illumina 450k array demonstrated that most differentially methylated CpG sites were located in chromosomes 1, 6 and 19, with hypermethylated CpG sites more likely to be located in the CG islands, while hypomethylated CpGs were mostly located in the CG islands shore. Hypomethylation was particularly observed in chromosome 6 CpGs related to the *HLA* region, which has been previously identified as an important region of genetic variation in cervical cancer²⁵⁷. The small sample size, however, makes interpretation difficult. Our analysis expanded coverage through the Illumina 850k array, as well as expanding the number of cancer samples studied to fifty-four.

More EWAS studies of preinvasive samples have been conducted and suggest both hyper and hypomethylation associated with cervical lesions^{142,445-447} with differentially methylated regions scattered widely across the genome. This was consistent with our findings, where both hypo and hypermethylation was observed across the epigenome.

The strongest CpG in our analysis was identified at cg16767801 located in the *PAXI* gene (paired box gene 1). *PAXI* forms a member of nine well-characterised paired-box transcription factors important primarily for tissue development and cellular differentiation. As a group they are reported to be frequently expressed in cancer and play a necessary role in cancer cell survival⁴⁴⁸, with the silencing of *PAX* genes having been associated with apoptosis in cancer cell lines and endogenous *PAX* gene expression is thought to be required for the growth and survival of cancer cells. However, *PAXI*

specifically has been demonstrated to act as a tumour suppressor gene – one study has shown *PAX1* has the ability to inhibit the phosphorylation of kinase cascades, preventing the development of malignant phenotypes under oncogenic stress. *PAX1* promoter methylation was also associated with poor prognosis in cervical cancer. Loss of *PAX1* function, through DNA methylation may promote carcinogenesis through the disruption of kinase and phosphatase interaction in the cervical epithelium⁴⁴⁹.

PAX1 methylation has, however, been previously associated with cervical preinvasive and invasive disease in targeted studies of *PAX* genes, performed largely East Asian populations⁴⁵⁰⁻⁴⁵². This includes a systematic review and meta-analysis published in 2015⁴⁵³ which pooled the diagnostic test accuracy estimates for *PAX1* markers in the detection of CIN3 versus normal specimens in 1385 women across seven studies to report a sensitivity of 77% (95%CI 58-89%) and specificity of 92% (95%CI 88-94%). While Kelley *et al.*, report a sensitivity of 68.4% (95%CI 53.0-80.6) and specificity of 83.9% (74.2-90.4) for *PAX1* markers studied in 1217 women across 5 studies. The exact CpG sites were not defined and heterogeneity across studies was high. The definition used for determining methylation positivity was not provided and it is unclear whether different CpG sites and cut-off thresholds were combined in this meta-analysis (heterogeneity estimates were not reported). However, in summary, *PAX1* appears to have a promising performance as a methylation marker. Li *et al.* 2021 found that *PAX1* methylation combined with hrHPV E6/E7 mRNA, for the endpoint of HSIL+, had a sensitivity of 98.97%, specificity of 19.01%, and AUC=0.590. When comparing the AUC of each test alone, *PAX1* methylation alone had the best AUC of 0.919 vs 0.541 for hrHPV testing and 0.607 for hrHPV E6/E7 mRNA⁴⁵⁴. *PAX1* methylation has also been associated with a higher degree of hrHPV16 and 18 L1 methylation in CIN2 or worse. Possibly the higher oxidative stress has an impact of hrHPV epigenomics⁴⁰⁴. Consideration should be given to the combination of host and hrHPV16 and 18 assays to form a single methylation test.

When conditioning on *PAX1* cg16767801, one further distinct and independent association was observed at cg23642047 in the *NREP-ASI* gene. To our knowledge, this association to cervical cancer has not been previously reported. *NREP-ASI* is a long non-coding RNA related to the *NREP* gene and may have a role in anti-apoptosis. *NREP* itself may be involved in the activation of cancer-associated fibroblasts and the epithelial–mesenchymal transition (EMT), with transforming growth factor β 1 mediating both processes and variation in this gene has been associated with carcinogenesis of the breast⁴⁵⁵. In addition, *NREP* expression showed a positive correlation with the abundance of M2 macrophages, which are potent immunosuppressors. Interference in *NREP* function may therefore signify a relationship with hrHPV-associated carcinogenesis⁴⁵⁶.

As a bigenic methylation marker *PAX1-NREP* (cg16767801 + cg23642047) was able to detect CIN3 or cervical cancer with a sensitivity of 83%, specificity of 87% and an AUC of 0.92. This compares well to other methylation classifiers. In a study of 72 self-samples from hrHPV positive CIN and four cancers using the Illumina 450k array¹⁴¹, a 3-gene methylation classifier based on different genes (*ASCL1*, *LHX8* and *ST6GALNAC5*) showed a very good clinical performance for CIN3 detection in both lavage (AUC=0.88; sensitivity=74%; specificity=79%) and brush (AUC=0.90; sensitivity=88%; specificity=81%) self-samples. El Zein et al. (2020) performed genome-wide investigation of 98 cervical liquid-based cytology samples using the Illumina 850K array¹⁴², only preinvasive samples were sequenced genome-wide however. Two CpGs of interest were identified at *ATP10A* and *HAS1* genes and examined in a validation set including CIN3 and cancer: as a bigenic marker a perfect prediction of cancer (AUC 1.00) and a sensitivity and specificity of 68% and 60% for CIN3 were reported. Both markers appeared in the 409 associated CpGs identified in our main analysis.

To our knowledge, two methylation markers have reached the stage of commercial development for cervical cancer: (1) Gynect, an assay based on methylated DNA regions in the promoter 5' regions of the genes *ASTN1*, *DLX1*, *ITGA4*, *RFXP3*, *SOX17*, and *ZNF671* and (2) the QIASure Methylation Test with the associated methylation markers *FAM19A4* and *miR124-2*. In an independent comparative analysis⁴⁵⁷ a sensitivity of 60% for CIN3 and 100% for cancer has been reported for Gynect (N=123) while for QIASure a sensitivity of 74.3% for CIN3 and 98.3% (N=519) is presented⁴⁵⁷. The S5 classifier includes the *EPB41L3* gene as well as hrHPV genes and is reported to detect CIN3 or worse with a sensitivity of 77% in hrHPV positive women with abnormal cytology (specificity not provided)⁴⁵⁸. Of the previously reported genes, *ATP10A*, *LHX8*, *EPB41L3* appeared in the 409 Bonferonni-significant results identified in our analysis and the associations were in the same direction. Notably in previous studies Bonferonni-significance has not always been observed and the false positive rate may be raised and hrHPV-adjustment or restriction to hrHPV-positivity was not universally performed. Differences in sequencing arrays may also explain the lack of replication. Although *SOX17* and *ZNF671* associations were not found to be significant in the main analysis, genes from the same family containing statistically significant associations included *SOX1*, *SOX14*, *FAM155A*, *FAM135B*, *MIR129-2*, *ITGA1*, *ZNF773*, *ZNF808*, *ZNF583*, *ZNF536*, *ZNF831*, *ZNF135*, *ZNF702*, *ZNF582*, *ZNF606*, *ZNF491*, *ZNF492* and *ZNF814*. *ZNF* (zinc finger proteins) are abundant in the eukaryotic genome and represent a variety of functions including DNA binding and regulation of apoptosis, while *SOX* genes encode a variety of transcription factors important for DNA binding and cell regulatory processes.

CIN3 signals were found to be much attenuated when compared to cancer, despite the larger sample size. This validates previous studies such as Farkas *et al*¹³⁶ who report that CIN3 versus normal were all less than 20% methylation change between CIN3 and controls, with much greater deltas observed between cancer and normal tissue. It is likely that more global and uncontrolled methylation process occurring in cancer. Additionally high-grade CIN lesions represent a heterogeneous stage of disease, where only a subset will progress to cancer over a period of 15-30 years. It is, therefore, likely that the molecular profile of CIN3 differs between early and late CIN3 lesions disease. To further understand the heterogenous nature of CIN it is necessary to study methylation in longitudinal samples of disease that progressed and regressed. Methylation may, therefore, offer use as a prognostic marker in high-grade CIN.

Adjustment for hrHPV status made a large difference to our analysis, reducing the number of markers reaching statistical significance by a large number. This is in conflict with a previous report where methylation signals in hrHPV-positive women only, were similar to a mixed hrHPV positive and negative cohort – the distribution of hrHPV positivity between cases and controls was not clear in this analysis however and hrHPV prevalence in controls may have been unusually high¹⁴². Interrogation of cervical cancer specimens in The Cancer Genome Atlas (TCGA) has identified numerous HPV related methylation signatures. We further investigated the hrHPV effect by restricting to hrHPV positive only women, whereby comparing beta coefficients and P-values to the hrHPV adjusted analysis we confirmed the importance of hrHPV status in the association between methylation and cervical disease.

A weakness of this study is that incomplete data on smoking in clinical databases meant this could not be accounted for in the model. As smoking is known to affect methylation this may have introduced bias, however, the number of smokers was well balanced across cases and controls which should minimise any confounding. Additionally, contamination of the sample with other cell types could not be accounted for. It is common to adjust for blood cell distribution within epigenetic studies based on serum samples, however, as there is no reference for cervical epithelial cells, this could not be accounted for. The *NREP-ASI* CpG showed minimal validation in an external cohort of CIN3 samples; this may be explained by a reduced prediction for CIN3 (versus cancer) or by the small sample size and would warrant further investigation. Control samples were not tested for hrHPV positivity until later in the analysis, future studies would benefit from restricting to hrHPV positive women only, the population in which methylation markers are likely to be applied. An additional potential benefit of methylation markers from cervical specimens is that they may feasibly detect endometrial and ovarian cancers through the tracking of epithelial and endothelial cells down the reproductive tract⁴⁵⁹. We did not study for endometrial and ovarian outcomes however this would be an interesting investigation in future.

4.4 CONCLUSIONS

In addition to hrHPV methylation markers, human methylation signals are associated with CIN3 and cervical cancer. We detected one human methylation marker with independent validation with predictive potential for both CIN3 and cancer, which may be a useful diagnostic tool for the detection of high-grade disease in hrHPV positive women identified at screening. In combination with hrHPV methylation markers, host methylation has the potential to improve the accuracy of cervical screening and reduce unnecessary anxiety, follow-up and treatments, through identifying truly progressive lesions from transient hrHPV infection.

OVERALL CONCLUSIONS

1. Summary of hypotheses

The environmental factors influencing the relationship between hrHPV infection and development and progression of CIN to cancer are incompletely understood. We hypothesised that due to the inherent biases of observational studies, some widely accepted risk factors for cervical cancer may in fact be biased and based upon weak evidence, while lesser-known risk factors may have strong evidence. Uncovering the strength of evidence and degree of bias in the associations elicited by meta-analyses investigating environmental and behavioural risk factors and cervical cancer, may influence public health information and policy decisions.

We hypothesised that through the study of genetic variation in women with and without CIN and cervical cancer, we may uncover genetic signals associated with the risk of developing cervical cancer in the presence of hrHPV infection. Understanding the genetic component of cervical cancer may help guide risk-based screening intervals, or influence novel therapeutics.

We further hypothesised that through the study of epigenetic associations to cervical preinvasive and invasive disease, novel molecular markers could be identified for potential use in improving the accuracy of detection of high-grade CIN and early invasive cervical cancer. In future such markers could be used as a triage test in hrHPV positive women, to identify pre-invasive lesions with true progressive potential.

2. Summary of findings, translational implications, and final conclusions

2.1 *Environmental risk of disease*

There is consistently strong and highly suggestive evidence that HIV positivity reduces hrHPV clearance rates and increases the risk of hrHPV infection and cervical cancer development. Vaginal dysbiosis and immunosuppressive medications for women with inflammatory bowel disease are also strongly associated with increased risk of progression from normal to LSIL or HSIL and cervical cancer. While for smoking we found highly suggestive evidence in the sensitivity analyses only, which is likely due to a lack of high-quality research in smokers and difficulty in accurate reporting of smoking in observational studies. Our research highlights that providing treatment for HIV, as well as other

sexually transmitted infections, including bacterial vaginosis and an abnormal vaginal microbiome may reduce the risk of cervical cancer.

2.2 Genetic risk of disease

Our findings of strong associations in *PAX8*, *CLPTMIL* and *HLA* regions offer exciting insights into the genetic predisposition of the cervical cancer trait. This study supports a complex neoplastic model related to both altered immune response and carcinogenic processes, in the presence of hrHPV. Heterogeneity between CIN3 and invasive cervical cancer phenotypes warrants further investigation in larger cohorts; meta-analyses of existing biobanks with linkage to cervical cancer registries may be a cost-efficient alternative to sequencing until costs reduce further. Genetic risk screening panels for multiple cancers are already offered directly to consumers from the private medical market, in future it is likely we will see widespread uptake of genetic screening as costs decrease and patients seek more personalised medical approaches. Our work can contribute to informing individuals seeking genetic screening of their potential underlying risk of cervical cancer, however careful counselling regarding limitations of such genetic markers will be paramount. Meanwhile, further investigation of functional effects of loci, within and outside of the *HLA*, is needed to better understand the pathogenesis of cervical neoplasia, and implications for potential therapeutic targets in women with cervical preinvasive and cancerous lesions.

2.3 Epigenetic markers for triage of high-grade disease

Our meta-analysis of hrHPV methylation provides a comprehensive summary of how different hrHPV methylation CpGs, genes and combinations perform. We demonstrated that hrHPV methylation correlates with disease severity and calculated the diagnostic accuracy of methylation for individual hrHPV genotypes in detecting high-grade CIN in hrHPV positive women. The best performing markers were identified in L1 and L2 genes, where cg6367 had the strongest area under the curve. One of the major challenges is that viral methylation is type-specific and future tests should allow for a multitude of markers, targeting all hrHPV types, in one assay. Next-generation multiplex sequencing assays containing all hrHPV genotypes are under development and have the potential to allow rapid, automated and low-cost methylation testing.

In our epigenome-wide association study we detected 409 sites from ~850,000 assayed, that strongly associate with CIN3 or ICC phenotypes. Our data suggests that *PAX1* (cg16767801) and *NREP-ASI* (cg23642047) methylation is strongly associated to cervical cancer, and to a lesser extent CIN3, which validated in an independent cohort. This suggests that a *PAX1* assay may perform as well as current

methylation markers currently under trial including Qiasure and GynTest tests (promoter/5' regions of the genes *ASTN1*, *DLX1*, *ITGA4*, *RXFP3*, *SOX17*, and *ZNF671*).

Viral and host methylation may be useful epigenetic markers for cervical cancer prevention as triage markers in cervical screening. While HPV vaccination is being shown to be highly effective in reducing cervical cancer where implemented⁴⁶⁰, accurate screening is still needed whilst we await higher levels of hrHPV immunity across all vulnerable age cohorts, and in geographical settings where the scale up of vaccination remains slow. Host and hrHPV markers could be combined into a single combined test, based on a weighting score for each individual CpG site. With the assumption that these will perform at least as well as the pooled diagnostic accuracy for HPV16 L1/L2, combined methylation testing has the potential to evolve to a useful candidate triage marker in women that test positive of hrHPV at primary screening, determining who will warrant further colposcopy.

It is anticipated that technical improvements and standardisation of protocols will further increase clinical accuracy of epigenetic markers. When such tests are validated in laboratory conditions, large high-quality clinical trials in screening populations comparing various triage tools head-to-head will be needed. Exploration of methylation in longitudinal cohorts has the potential to also enlighten its possible predictive value in disease progression.

3. Future work

3.1 Targeted pyrosequencing of *PAX1*, *NREP-AS1* and HPV16 L1 methylation in a longitudinal series of HPV16 positive women, to determine prognostic potential

Introduction

Molecular markers may improve the triage of HPV16 positive women, to determine those truly at risk of cervical cancer. Our work demonstrates that the strongest associations between CpG methylation and CIN3 or cervical cancer were observed at host genes *PAX1* and *NREP-AS1*, and HPV16 L1 (CpG). Area under the curve ranged from 0.81-0.92.

Methods

Population: A) We will use remaining HPV16 positive samples from the PRECAC cohort to validate the findings of previous studies for host genome CpG sites cg16767801, cg23642047 and HPV16 cg6367, in a small subset of 50 women with CIN3/Cancer or normal samples.

B) We will investigate the prognostic potential of methylation in a unique cohort of 94 women with CIN2 (high-grade CIN) who were not treated for 24-months and either progressed to CIN3 (15%),

regressed to low-grade CIN or back to normal (68%), or had persistent CIN2 (17%)²³. There are 4 to 6 serial samples available for each woman (n=608 samples). The samples are currently held at Imperial College London in collaboration with Prof Moscicki at University of California (UCLA) (president of the American Society of Cervical Pathology and Colposcopy (ASCCP)). We hold well-characterised clinical metadata (including age, ethnicity, smoking status, sexual health history and practices, contraception use, illicit drug use) hrHPV genotyping and other soluble biomarker information (hrHPV genotyping, vaginal microbiome, metabolome) for this cohort.

hrHPV DNA typing: The LMNX Genotyping kit HPV GP HR kit was be used for hrHPV detection and genotyping. This is a reverse hybridization assay using GP5+/6+ primers for the quantitative identification of the 14 high-risk types HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. Our collaborators in the HPV reference laboratory, Edinburgh University, who have validated this assay in their laboratory, perform this test.

Methylation analysis: We will bisulphite convert DNA using Zymo EZ-96 DNA Methylation kit. Specific primer pairs will be designed using Pyromark Assay design. We will optimise PCR and thermal cycling conditions in our laboratory for each assay. Pyrosequencing will be performed using Pyromark assays and established techniques in our laboratory^{461,462}.

Statistical analysis: We will calculate the mean methylation levels and SE at individual CpG sites at time of diagnosis in women with CIN2 that progressed or regressed in the UCSF/UCLA cohort. We will use linear mixed models accommodating repeated measurements to investigate markers driving the dynamics of disease progression.

Potential confounders of this association will be explored using multivariable logistic regression of grade with methylation as the predictor, adjusting for potential technical and biological confounders. Analyses will be generalised to multivariate models by adopting penalised regression, aimed at determining an optimal prognostic score using target methylation sites. To protect against overfitting and ensure reproducibility, we will assess sensitivity and stability using sub-sampling techniques.

Pilot work: Analysis of 500mg DNA from a CIN1 sample at CpG 7034bp revealed a methylation of 17%, which is expected (Figure 18 and 19). Further development of assays will be undertaken with biobanked samples before analysis of cross-sectional (PRECAC) and then the longitudinal (UCSF/UCLA) series.

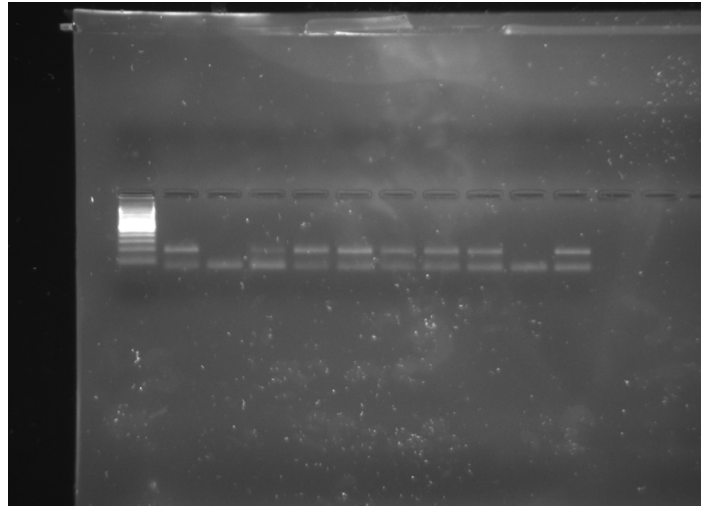


Figure 18. Agarose gel of Agarose gel of 7 LBC samples from women with CIN with negative and positive control against DNA ladder.

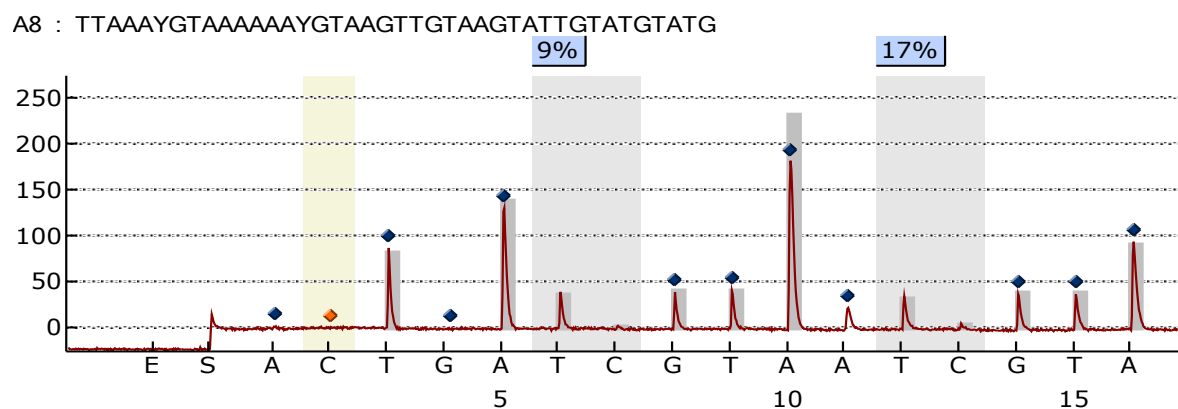


Figure 19. Pyrogram produced using pyromark software from our analysis of a HPV16 positive CIN1 sample examining CpG site 6367 in the L1 gene, demonstrating methylation level of 17% at this site.

3.2 A diagnostic test accuracy review of hrHPV and Host Methylation markers

There is a rapidly increasing number of methylation markers undergoing investigation for triage of HPV positive women. However, the quality of these studies is highly variable. We will assess the quality of evidence, and where possible pool accuracy estimates, for all methylation markers with diagnostic accuracy data in a diagnostic test accuracy review of existing hrHPV and Host methylation tests for the detection of high-grade CIN and cervical cancer as outlined in the attached protocol (appendix 21).

3.3 Risk prediction model for cervical screening

Based on any validated epigenetic markers, and genetic markers from GWAS studies, we would like to assess complementarity and create a polygenic and epigenetic risk model for the prediction of CIN progression in cervical screening. We will evaluate predictive performances and absolute and relative abilities of pooled markers. Series of sensitivity analyses excluding (groups of) markers will help identify relating contribution to prediction. In addition, we will include variable grouping in our model (e.g. genetic or epigenetic markers, or functionally informed grouping) and perform group selection⁴⁶³ to identify the most influential set(s) of markers.

3.4 Rapid diagnostic tests for point-of-care (POC) testing

Continued advances in genomic technologies are anticipated and could offer rapid point-of-care testing, that can be applied for out of hospital or low-resource settings. For example, the Oxford Nanopore MinION, a third-generation sequencing technology, is a portable device that can perform real-time whole genome sequencing. This has already been trialled for rapid analysis of Ebola virus and Salmonella outbreaks^{464,465}. Although reported accuracy for sequencing of methylation levels at CpG sites is lower, this is a potentially exciting technology for rapid methylation testing. Loop mediated isothermal amplification and lateral flow test technology which was underway prior to the COVID-19 pandemic, has now rapidly expanded and can be applied to multiple types of viruses including HIV, malaria and yellow fever⁴⁶⁶. Such technologies are already being investigated for HPV⁴⁶⁷. A point-of-care HPV test would majorly expand access to cervical screening in low resource settings. Potential assays could be based on DNA or viral proteins⁴⁶⁸. We are working on development of HPV point-of-care tests for use in sub-Saharan Africa.

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APPENDICES

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Appendix 3b. Evaluation of heterogeneity, small study effects and excess significance bias in the 87 meta-analyses investigating the risk factors associated with HPV, cervical pre cancer and cancer outcomes - only cohort studies included.

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Appendix 3d. Details of evidence grading for meta-analysis of risk factors for HPV, cervical precancer and cancer outcomes – only cohort studies included*

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Appendix 14. Diagnostic accuracy of HPV16 L1/L2_in prediction of CIN2+: Sensitivity analyses restricted to Histology only samples (univariate model applied as Hessian matrix was not positive therefore between-study correlation of estimate -1 causing the determinant to be zero)

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Appendix 21. Protocol for DTA meta-analysis of human and HPV DNA methylation in the triage of HPV positive women

Appendix 1: Search strategy for Umbrella review

We searched the electronic databases PubMed, Ovid MEDLINE and Embase Classic, and the Cochrane database of systematic reviews for systematic reviews and meta-analyses of that investigated the association between non-genetic risk factors and incidence or prevalence of HPV, CIN or cervical cancer from inception to 1st May 2021. The search strategy was developed with the Imperial College London (Hammersmith Hospital campus) librarian. The Pubmed search terms were as follows, the same search was adapted for the other databases:

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(((((Uterine Cervical Neoplasms [MeSH Terms] OR Uterine Cervical Dysplasia [MeSH Terms] OR female genital tract cancer OR uterine cervix cancer OR Cervical Intraepithelial Neoplasia [MeSH Terms] OR CIN [tw] OR CINII* [tw] OR CIN2* [tw] OR CINIII* [tw] OR CIN3* [tw] OR SIL [tw] OR HSIL [tw] OR H-SIL [tw] OR LSIL [tw] OR L-SIL [tw] OR ASCUS [tw] OR AS-CUS [tw]) OR (papillomaviridae [MeSH:NoExp] OR alphapapillomavirus [MeSH Terms] OR "DNA, viral" [MeSH Terms] OR Papillomavirus Infections [MeSH Terms] OR Tumor Virus Infections [MeSH Terms] OR "Cervix Uteri/virology" [MeSH Terms] OR HPV [tw] OR "human papillomavirus" [tw] OR papillomaviridae [tw]))) OR ((cervix[Tw] OR cervical[Tw] OR cervico*[Tw] OR Cerv*[tw]) AND (cancer*[Tw] OR carcinoma[Tw] OR adenocarcinoma[Tw] OR neoplas*[Tw] OR dysplas*[tw] OR dyskaryos*[Tw] OR squamous[Tw]))) AND (Meta-analysis[pt] OR systematic[sb] OR (Meta-analysis[tw] OR systematic review[tw] OR systematic[sb] OR meta-analysis[pt] OR meta-analysis as topic[mh] OR meta-analysis[mh] OR meta analy*[tw] OR metanaly*[tw] OR metaanaly*[tw] OR met analy*[tw] OR systematic review*[tiab]))) NOT (animal[mh] NOT human[mh]) Sort by: PublicationDate
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Appendix 2: Summary quality assessment for all included systematic reviews using the ASMTAR 2 tool (A Measurement Tool to Assess Systematic Reviews).

AMSTAR 2 Questions	PICO	'A priori' design and deviations justified	Study design	Literature search	Duplicate study selection	Duplicate data extraction	Excluded studies	Description of included studies	Risk of bias assessment	Funding	Statistical methods for meta-analysis	Impact of RoB from meta-analysis	Risk of bias of individual studies	Heterogeneity	Small study bias	Conflict of interest	Overall rating
Study Author, year																	
Wang 2020	●	⊙	●	⊙	○	●	○	○	⊙	○	●	○	●	●	○	●	critically low
Xie 2018	●	●	●	⊙	○	●	●	⊙	⊙	○	○	●	●	○	●	●	low
Yang 2018	○	○	●	⊙	○	○	●	●	○	○	NA	NA	○	●	○	●	critically low
Ye 2018	●	○	●	⊙	●	●	●	●	○	○	●	●	○	●	●	●	critically low
Zard 2014	●	⊙	●	⊙	●	●	●	⊙	○	○	○	○	○	○	●	●	critically low
Zeng 2012	●	○	●	⊙	●	○	○	⊙	⊙	○	○	●	●	●	●	●	critically low
Zhang 1997	●	○	○	○	○	○	○	○	⊙	○	○	○	●	○	○	○	critically low
Zhang 2012	●	⊙	●	⊙	○	●	○	⊙	⊙	○	●	●	○	●	●	●	low
Zhang 2018	●	⊙	●	⊙	●	●	○	⊙	○	○	○	●	○	●	●	●	critically low
Allegreti 2015	●	●	●	⊙	●	●	○	⊙	⊙	○	●	●	●	●	●	○	moderate
Appleby 2005	●	⊙	○	⊙	○	○	○	⊙	○	○	●	●	●	●	○	○	critically low
Zhu 2016	●	●	●	⊙	○	●	○	⊙	⊙	○	●	●	●	●	●	●	moderate
Appleby 2006	○	○	○	○	○	○	○	⊙	⊙	○	●	○	○	●	○	○	critically low
Hu 2017	●	●	●	⊙	●	○	○	⊙	○	○	○	●	●	●	●	○	critically low

Cao 2014	●	○	○	◎	○	●	○	◎	◎	○	●	●	○	●	○	○	critically low
Cao 2016	●	◎	●	◎	●	○	○	◎	○	○	●	●	●	●	●	○	critically low
Castellsague 2011	●	○	○	○	○	○	○	●	○	●	●	●	●	●	○	●	critically low
Cortessis 2017	●	◎	●	◎	●	○	○	●	◎	○	●	●	●	●	●	○	critically low
Debeaudrap 2019	●	◎	○	●	●	●	○	●	◎	○	●	●	●	●	●	●	critically low
De Lima 2018	●	○	●	◎	●	○	○	●	◎	○	●	●	●	●	○	○	critically low
Gillet 2011	●	○	●	◎	●	○	○	◎	◎	○	●	●	●	●	●	●	critically low
Gillet 2012	●	○	○	◎	○	○	○	◎	●	○	●	●	●	●	●	●	critically low
Grulich 2007	●	○	○	◎	●	○	○	◎	◎	○	●	○	○	●	●	●	critically low
Haverkos 2003	●	◎	○	◎	○	○	●	◎	◎	○	○	●	●	○	○	○	critically low
He 2017	●	◎	●	◎	○	●	○	◎	◎	○	○	●	○	●	●	●	critically low
Helm 2013	●	◎	●	◎	●	●	●	●	●	○	●	●	●	●	●	●	high
Josyula 2015	●	○	●	◎	○	○	○	◎	◎	○	●	●	○	●	●	●	critically low
Kaderli 2014	●	○	○	○	○	○	○	◎	◎	○	○	○	●	○	○	○	critically low
Kelly 2018	●	◎	○	●	○	○	○	●	◎	○	●	●	○	●	●	●	low
Lee 2016	○	◎	○	○	○	○	○	●	●	○	●	●	●	●	●	○	critically low
Li 2013	●	◎	●	◎	●	●	○	●	●	○	●	●	●	●	●	●	moderate
Liu 2014	●	◎	●	◎	○	●	●	●	●	○	●	●	●	●	●	●	moderate
Liu 2015	●	◎	●	◎	○	●	○	◎	◎	○	●	●	●	●	●	○	low
Liu 2018	●	◎	●	◎	○	○	○	◎	●	○	●	●	●	●	●	●	low
Looker 2018	○	◎	○	◎	○	●	○	◎	●	○	○	●	●	●	●	●	critically low

Manhart 2002	●	◎	●	○	○	○	○	◎	●	○	●	●	●	●	○	○	critically low
Myung 2011	●	◎	○	◎	●	○	○	○	○	○	●	○	●	●	○	●	critically low
Naldini 2019	●	◎	●	◎	●	●	○	●	◎	○	●	●	●	●	●	●	low
Peng 2017	●	◎	●	◎	●	●	○	●	●	○	●	●	●	●	●	●	critically low
Plummer 2003	○	○	○	○	○	○	○	◎	◎	○	○	○	●	●	●	○	critically low
Simon 2015	●	◎	●	◎	○	○	○	○	○	○	●	●	●	●	○	●	critically low
Smith 2003	○	○	○	○	○	○	○	●	◎	○	●	●	●	●	●	●	critically low
Tamarelle 2018	●	○	●	◎	○	○	○	○	●	○	●	●	●	●	●	●	critically low
WCRF Cup Cervical Cancer	○	◎	○	○	○	○	○	●	◎	○	●	●	●	●	●	○	critically low
Brusselaers 2019	●	●	●	●	●	●	●	◎	◎	○	●	●	●	●	○	●	moderate
Appleby 2007	●	○	●	○	○	○	○	◎	◎	○	●	○	○	●	○	●	critically low
Chen 2020	●	○	●	●	●	●	○	●	◎	○	●	○	○	●	○	●	critically low
Tomita 2020	●	●	●	●	●	●	●	●	◎	○	●	○	○	●	●	●	low
Liang 2019	●	○	●	◎	○	○	○	○	◎	○	●	○	○	●	●	●	critically low

Key: ● yes
 ◎ partial yes
 ○ no

Appendix 3a: Description of 87 meta-analyses investigating risk factors associated with HPV, cervical pre cancer and cancer outcomes - only cohort studies included.

Author, year	Exposure	Exposure contrast	Outcome	N ^a		N cases/cohort	Summary relative risk (95% CI)			Fixed P- value ^b	Random P-value ^c	95% Prediction interval ^d
				N	cohorts		Fixed Effects	Random Effects	Largest Study			
Appleby 2006	Age during FTP	Per 1-year decrease	CIN3 incidence	15	5	620/1782	1.03(0.98- 1.07)	1.03(0.98- 1.07)	1.04(0.99- 1.1)	2.38E- 01	2.38E- 01	0.96-1.10
WCRF CUP	Alcohol intake	Highest vs lowest	Cervical cancer incidence	4	4	1549/2686978	1.00(0.87- 1.16)	1.00(0.87- 1.17)	1.00(0.84- 1.19)	9.56E- 01	9.56E- 01	0.73-1.39
Gillet 2012	Bacterial vaginosis	Yes vs no	CIN prevalence	20	12	109572/1652847	1.28(1.23- 1.33)	1.62(1.30- 2.04)	1.19(1.13- 1.26)	6.26E- 31	2.43E- 05	0.78-3.39
Gillet 2011	Bacterial vaginosis	Yes vs no	HPV prevalence	12	2	199/667	2.12(1.50- 3.00)	1.94(1.08- 3.47)	2.42(1.64- 3.57)	1.95E- 05	2.67E- 02	NA
WCRF CUP 2018	BMI	Highest vs lowest level	Cervical cancer mortality	4	4	420/3144461	1.64(1.25- 2.15)	1.82(1.07- 3.1)	1.15(0.79- 1.7)	3.89E- 04	2.75E- 02	0.22-15.20
WCRF CUP 2018	BMI	Highest (>30) vs lowest (<25)	Cervical cancer incidence	7	7	4837/7875703	1.14(0.99- 1.32)	1.13(0.9- 1.380)	1.02(0.8- 1.31)	7.40E- 02	2.31E- 01	0.71-1.79
WCRF CUP 2018	BMI	per 5kg/m2 increase	Cervical cancer incidence	9	9	5035/9680798	1.02(1.00- 1.04)	1.02(0.97- 1.07)	0.98(0.95- 1.02)	4.22E- 02	4.45E- 01	0.09-1.17
WCRF CUP 2018	BMI	per 5kg/m2 increase	Cervical cancer incidence	7	7	4837/7875703	1.01(0.99- 1.03)	1.01(0.96- 1.06)	0.98(0.95- 1.02)	3.61E- 01	6.72E- 01	0.87-1.17
WCRF CUP 2018	BMI	per 5kg/m2 increase	Cervical cancer mortality	3	3	307/3032760	1.02(0.90- 1.14)	1.02(0.83- 1.25)	1.04(0.88- 1.24)	7.87E- 01	8.35E- 01	0.11-9.52
Wang 2019	Cervicovaginal lactobacilli	LP CSTs vs non LP CSTs	HR HPV incidence	9	2	51/104	0.68(0.29- 1.59)	0.58(0.16- 2.14)	0.98(0.37- 2.64)	3.76E- 01	4.16E- 01	NA
Wang 2019	Cervicovaginal lactobacilli	LIP CST III vs non	HR HPV incidence	8	2	16/83	0.57(0.18- 1.82)	0.57(0.18- 1.82)	0.76(0.16- 3.58)	3.40E- 01	3.40E- 01	NA

Wang 2019	Cervicovaginal lactobacilli	LIP CST III LCP CST I vs non LCP CST I	HR HPV incidence	8	3	59/155	0.57(0.24-1.35)	0.57(0.24-1.35)	0.5(0.18-1.39)	2.03E-01	2.03E-01	0-156.11
Zhu 2016	Chlamydia tr and HPV, coinfection	Yes vs no	Cervical cancer incidence (CIN1+)	6	3	492/2528	3.29(2.47-4.4)	3.3(2.47-4.4)	3.23(2.39-4.35)	6.42E-26	6.42E-16	NA
Naldin 2019	Chlamydia tr infection	Yes vs no	HPV incidence	40	6	2753/7426	1.51(1.07-1.24)	1.74(1.28-2.38)	1.2(1.09-1.32)	6.81E-05	4.94E-04	0.64-4.73
Naldin 2019	Chlamydia tr infection	Yes vs no	HR HPV incidence	15	2	912/2878	2.47(1.23-4.99)	2.07(1.57-2.72)	1.84(1.36-2.47)	2.00E-07	1.13E-02	NA
Zhu 2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence (CIN3+)	3	3	832/4305	2.22(1.88-2.62)	2.21(1.62-3.03)	2.21(1.84-2.65)	2.63E-21	6.43E-07	0.09-53.4
Zhu 2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence (SCC)	11	2	500/3077	2.34(1.92-2.85)	2.33(1.90-2.87)	2.4(1.95-2.94)	5.52E-17	6.59E-16	NA
Zhu 2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence (CIN1+)	11	4	875/4718	1.86(0.91-3.79)	1.86(0.91-3.79)	1.9(0.82-4.29)	8.70E-01	8.70E-01	0.39-8.85
Appleby 2007	COCP (current users)	Per year of use	Invasive cervical cancer incidence	3	3	45/311	1.03(0.97-1.09)	1.03(0.97-1.09)	1.01(0.94-1.09)	3.86E-01	3.86E-01	0.69-1.53
Appleby 2007	COCP (1-9ys since last use)	Per year of use	Invasive cervical cancer incidence	3	3	50/252	1.03(0.98-1.08)	1.03(0.98-1.09)	1.04(0.97-1.11)	2.30E-01	2.74E-01	0.65-1.64
Appleby 2007	COCP (>10ys since last use)	Per year of use	Invasive cervical cancer incidence	3	3	120/1591	1.01(0.97-1.04)	1.01(0.98-1.04)	1.00(0.96-1.04)	7.59E-01	7.59E-01	0.81-1.25
Smith 2003	COCP	<5 years users vs Never	Cervical cancer incidence (CIN3+)	5	2	94/193	1.24(0.64-2.4)	1.24(0.64-2.4)	1.2(0.6-2.4)	5.26E-01	5.26E-01	NA
Smith 2003	COCP	5-9 years users vs Never	Cervical cancer incidence (CIN2+)	5	2	101/215	0.8(0.43-1.51)	0.8(0.43-1.51)	0.8(0.4-1.5)	4.89E-01	4.89E-01	NA
Smith 2003	COCP	>10 years users vs Never	Cervical cancer incidence (CIN2+)	3	2	129/228	1.59(0.85-3.97)	1.59(0.85-2.97)	1.5(0.8-2.9)	1.44E-01	1.44E-01	NA

Smith 2003	COCP	5-9 years users vs Never	Invasive cervical cancer incidence	21	4	448/17910	2.22(1.72-2.87)	1.95(1.138-3.36)	3.1(2.1-4.5)	1.00E-09	1.51E-02	0.18-20.80
Smith 2003	COCP	<5 years users vs Never	Invasive cervical cancer incidence	20	4	470/17983	1.82(1.40-2.35)	1.81(1.40-2.35)	2.1(1.4-3.0)	6.12E-06	6.12E-06	1.03-3.20
Smith 2003	COCP	>10 years users vs Never	Invasive cervical cancer incidence	13	3	322/16209	3.27(2.37-4.51)	3.02(1.56-5.83)	4.7(2.9-7.5)	6.91E-13	1.02E-03	0-6554
Lee 2016	Environmental tobacco smoke	Yes vs no	Cervical cancer incidence (CIN2+)	17	7	432/65031	1.26(1.03-1.55)	1.41(1.01-1.96)	0.9(0.65-1.24)	2.45E-02	4.32E-02	0.58-3.44
Wang 2020	GDM	Yes vs no	Cervical cancer incidence	3	3	63694/1163875	1.02(0.82-1.29)	1.02(0.82-1.29)	0.90(0.65-1.26)	8.43E-01	8.43E-01	0.23-4.49
WCRF CUP	Height	Per 5 cm increase	Cervical cancer incidence	4	4	1182/990226	1.01(0.93-1.10)	1.03(0.87-1.20)	1.01(0.92-1.11)	7.73E-01	7.65E-01	0.57-1.83
Looker 2018	HIV	HIV+ vs HIV-	HR HPV incidence	11	11	2323/8668	2.22(1.99-2.47)	2.20(1.89-2.54)	2.35(2.03-2.72)	0	3.01E-26	1.63-2.97
Looker 2018	HIV	HIV+ vs HIV-	Clearance of HPV	15	15	2977/7448	0.56(0.51-0.61)	0.53(0.43-0.64)	0.67(0.56-0.81)	2.43E-36	4.32E-10	0.26-1.06
Debeaudrap 2019	HIV	HIV+ vs HIV-	Treatment failure for any grade lesions	18	15	1339/4300	4.34(3.74-5.17)	4.90(3.89-6.18)	3.2(2.4-4.3)	0	0	2.64-9.10
Grulich 2007	HIV	HIV+ vs HIV-	Cervical cancer incidence	9	9	1184/142676	5.35(4.45-6.43)	3.98(2.24-7.05)	5.2(3.81-6.93)	0	2.32E-06	0.59-26.73
Liu 2018	HIV	HIV+ vs HIV-	CIN regression (LSIL)	2	2	1968/4554	0.68(0.56-0.81)	0.68(0.56-0.81)	0.69(0.57-0.84)	3.46E-05	3.46E-05	NA
Looker 2018	HIV	HIV+ vs HIV-	HPV incidence	15	15	1722/8342	1.71(1.48-1.98)	1.73(1.39-2.16)	1.35(0.99-1.84)	2.50E-13	1.01E-06	0.91-3.31
Looker 2018	HIV	HIV+ vs HIV-	Clearance of HR HPV	11	11	3581/9903	0.70(0.65-0.75)	0.66(0.55-0.80)	0.67(0.59-0.77)	1.58E-20	8.03E-06	0.37-1.20
Liu 2018	HIV	HIV+ vs HIV-	HPV 18 incidence	2	2	54/1050	2.56(1.17-5.60)	2.56(1.17-5.60)	3.19(1.17-8.7)	1.92E-02	1.92E-02	NA
Liu 2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (Any HPV)	2	2	424/696	0.43(0.32-0.57)	0.34(0.16-0.75)	0.46(0.34-0.62)	5.25E-09	7.18E-03	NA
Liu 2018	HIV	HIV+ vs HIV-	Clearance of prevalent and	3	3	675/1053	0.64(0.52-0.79)	0.58(0.39-0.87)	0.75(0.59-0.96)	1.89E-05	7.83E-03	0.01-44.81

Liu 2018	HIV	HIV+ vs HIV-	newly detected HPV (HR HPV) Clearance of prevalent and newly detected HPV (HPV 16)	3	3	485/994	0.61(0.47-0.80)	0.64(0.44-0.93)	0.57(0.41-0.79)	4.23E-04	1.99E-02	0.02-20.1
Looker 2018	HIV	HIV+ vs HIV-	Clearance of HPV 16	9	9	2283/4964	0.63(0.53-0.75)	0.67(0.50-0.90)	0.56(0.39-0.81)	2.95E-07	7.28E-03	0.28-1.61
Liu 2018	HIV	HIV+ vs HIV-	HPV incidence	3	3	528/1694	2.64(2.05-3.42)	2.64(2.05-3.42)	2.98(2.07-4.29)	1.13E-13	1.13E-13	0.5-13.94
Liu 2018	HIV	HIV+ vs HIV-	HR HPV incidence	2	2	263/1210	2.35(1.64-3.37)	2.35(1.64-3.37)	2.3(1.57-3.37)	3.62E-06	3.62E-06	NA
Liu 2018	HIV	HIV+ vs HIV-	HPV 16 incidence	2	2	91/1064	3.05(1.71-5.43)	3.047(1.711-5.426)	3.09(1.39-6.86)	1.55E-04	1.55E-04	NA
Liu 2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (HR HPV)	2	2	617/1992	0.56(0.45-0.69)	0.53(0.37-0.75)	0.6(0.47-0.76)	9.75E-08	3.86E-04	NA
Liu 2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (HPV 18)	2	2	348/624	0.48(0.32-0.72)	0.48(0.32-0.72)	0.5(0.31-0.81)	4.31E-04	4.31E-04	NA
Liu 2018	HIV	HIV+ vs HIV-	CIN incidence (LSIL)	2	2	362/2744	3.73(2.62-5.32)	3.73(2.62-5.32)	4(2.61-6.13)	3.46E-13	3.46E-13	NA
Debeaudrap 2019	HIV	HIV+ vs HIV-	Treatment failure for high grade lesions	10	9	304/2555	2.47(1.83-3.35)	2.57(1.83-3.35)	3.5(2-6.1)	4.32E-09	4.32E-09	1.72-3.56
Liu 2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (HPV 16)	2	2	105/121	0.88(0.49-1.59)	0.82(0.21-3.20)	1.61(0.74-3.51)	6.82E-01	7.72E-01	NA
Looker 2018	HIV	HIV+ vs HIV-	Clearance of HPV 18	7	7	2170/4523	0.78(0.62-0.97)	0.77(0.52-1.15)	0.91(0.62-1.33)	2.89E-02	2.06E-01	0.24-2.48
Looker 2018	HIV	HIV+ with CD4>200 vs HIV-	HPV incidence	5	5	1151/2335	2.89(2.55-3.27)	3.09(2.17-4.40)	3.61(3.01-4.32)	0	3.75E-10	0.91-10.55

Looker 2018	HIV	HIV+ with CD4≤200 vs HIV-	HPV incidence	3	3	703/1212	5.33(4.48-6.34)	5.76(3.65-9.08)	5.19(4.3-6.26)	0	4.73E-14	0.04-844.24
Looker 2018	HIV	HIV+ with CD4>200 vs HIV-	HR HPV incidence	4	4	327/758	1.94(1.45-2.61)	2.03(1.3-3.17)	1.41(0.89-2.24)	8.18E-06	1.78E-03	0.36-11.36
Looker 2018	HIV	HIV+ with CD4≤200 vs HIV-	HR HPV incidence	2	2	152/334	1.32(0.82-2.08)	1.31(0.82-2.08)	1.26(0.77-2.06)	2.62E-01	2.62E-01	NA
Looker 2018	HIV	HIV+ with CD4>200 vs HIV-	Clearance of HR HPV	4	4	299/468	1.23(0.97-1.56)	1.19(0.89-1.58)	1.49(1.09-2.04)	8.30E-01	2.40E-01	0.51-2.77
Debeaudrap 2019	HIV	HIV+ vs HIV-	Treatment failure for any grade lesions	18	15	1339/4300	4.34(3.74-5.17)	4.90(3.89-6.18)	3.2(2.4-4.3)	0	0	2.64-9.10
Kelly 2018	HIV+	On ART vs no ART	CIN regression (SIL)	10	10	3074/10241	1.57(1.38-1.78)	1.62(1.32-1.99)	1.3(1-1.7)	1.38E-11	4.41E-06	0.93-2.82
Kelly 2018	HIV+	On ART vs no ART	CIN incidence (SIL)	12	11	1826/6475	0.70(0.57-0.85)	0.72(0.54-0.95)	0.62(0.42-0.91)	4.27E-04	2.15E-02	0.35-1.49
Kelly 2018	HIV+	On ART vs no ART	CIN progression (SIL)	10	10	3493/8568	0.70(0.63-0.78)	0.74(0.61-0.90)	0.66(0.54-0.81)	3.94E-10	2.63E-03	0.42-1.31
Kelly 2018	HIV+	On ART vs no ART	Invasive cervical cancer incidence	3	2	2340/411779	0.44(0.27-0.74)	0.40(0.18-0.87)	0.5(0.29-0.87)	1.64E-03	2.03E-02	NA
Liu 2018	HIV+	CD4 <200 vs >500	Clearance of HPV	2	2	812/2218	0.46(0.37-0.57)	0.46(0.37-0.57)	0.47(0.38-0.59)	1.11E-12	1.11E-12	NA
Liu 2018	HIV+	CD4 200-500 vs >500	Clearance of HPV	2	2	812/2218	0.76(0.64-0.89)	0.76(0.64-0.89)	0.76(0.64-0.9)	1.01E-03	1.01E-03	NA
Liu 2018	HIV+	On ART vs no ART	CIN regression (LSIL)	3	3	522/1604	0.65(0.52-0.82)	0.65(0.52-0.82)	0.66(0.47-0.92)	2.78E-04	2.78E-04	0.16-2.91
Kelly 2018	HIV+	On ART vs no ART	HR -HPV prevalence	20	19	3901/7996	0.76(0.70-0.83)	0.83(0.68-1.01)	0.83(0.74-0.94)	6.77E-11	6.65E-02	0.4-1.71
Kelly 2018	HIV+	On ART vs no ART	Cervical cancer prevalence (HSIL, CIN2+)	14	7	886/3724	0.72(0.54-0.96)	0.76(0.49-1.18)	0.54(0.32-0.91)	2.52E-01	2.20E-01	0.22-2.62
Liu 2018	HIV+	On ART vs no ART	CIN incidence (LSIL)	2	2	358/1254	0.67(0.45-0.99)	0.70(0.40-1.25)	0.55(0.34-0.89)	4.54E-02	2.30E-01	NA

Liu 2018	HIV+	On ART vs no ART	CIN regression (LSIL)	2	2	325/1149	2.29(1.56-3.37)	1.11(0.16-7.61)	2.61(1.75-3.89)	2.57E-05	9.13E-01	NA
Allegreti 2015	IBD on immunosuppressive medications	Yes vs healthy controls	Cervical cancer incidence	8	5	10829/244724	1.33(1.27-1.39)	1.33(1.27-1.39)	1.35(1.28-1.43)	7.78E-37	7.78E-37	1.24-1.43
Li 2013	IVF	Yes vs no	Cervical cancer incidence	4	4	33735/1453927	0.63(0.55-0.74)	1.07(0.45-2.55)	0.61(0.52-0.71)	5.01E-09	8.71E-01	0.03-34.71
Appleby 2006	Parity	Per increase of 1 FTP	CIN3 incidence	15	5	957/3209	1.10(0.99-1.20)	1.05(0.92-1.21)	1.22(1.06-1.41)	5.42E-02	4.08E-01	0.72-1.56
Liu 2014	Pregnancy	Pregnant vs non pregnant	HPV incidence	14	14	1474/9518	1.43(1.26-1.62)	1.42(1.03-1.96)	0.87(0.66-1.14)	2.86E-08	3.33E-02	0.42-4.80
Helm 2013	Retinoid use	Yes vs no	Complete or partial regression of CIN2/3 at 3-12 months	3	3	186/314	0.98(0.56-1.72)	0.98(0.56-1.72)	0.92(0.39-2.16)	9.50E-01	9.50E-01	0.03-36.66
Helm 2013	Retinoid use	Yes vs no	Complete regression of CIN2/3 at 9-27 months	2	2	160/406	0.79(0.51-1.23)	0.79(0.51-1.23)	0.71(0.41-1.24)	2.94E-01	2.94E-01	NA
Helm 2013	Retinoid use	Yes vs no	Complete regression of CIN2 at 9-27 months	2	2	116/260	0.74(0.42-1.29)	0.81(0.29-2.28)	0.5(0.25-1.02)	2.86E-01	6.93E-01	NA
Simon 2015	Rheumatoid arthritis	Yes vs general population	Cervical cancer incidence	15	15	297/297887	0.86(0.84-0.89)	0.85(0.73-0.99)	0.86(0.84-0.89)	4.19E-26	4.73E-02	0.54-1.34
Liu 2015	Sexual partners	Multiple vs few partners	CIN incidence	26	2	345/1345	1.15(0.70-1.90)	1.15(0.69-1.90)	1.18(0.6-1.75)	5.90E-01	5.90E-01	NA
Kaderli 2014	Smoking	Yes vs no	HPV incidence	10	10	9442/28187	1.33(1.21-1.46)	1.37(1.21-1.56)	1.2(1-1.3)	2.51E-09	1.04E-06	1.06-1.77
Appleby 2005	Smoking	Past vs never smoker	Cervical cancer incidence (CIN3+)	19	4	798/2873	1.54(1.13-2.09)	1.53(1.12-2.10)	1.83(1.19-2.82)	6.14E-03	8.10E-03	0.73-3.2
Kaderli 2014	Smoking	Yes vs no	HPV prevalence	5	5	4451/10853	1.19(1.08-1.33)	1.24(1.03-1.50)	1.1(1-1.4)	4.07E-04	2.63E-02	0.66-2.31

Appleby 2005	Smoking	Current vs Never	Cervical cancer incidence (CIN3+)	19	4	798/2874	1.85(1.47- 2.34)	1.85(1.47- 2.34)	1.68(1.17- 2.41)	2.22E- 07	2.22E- 07	1.11-3.09
Grulich 2007	Transplant recipient	Yes vs no	Cervical cancer incidence	3	3	24/18800	2.13(1.38- 3.3)	2.13(1.38- 3.3)	2.5(1.33- 4.27)	7.09E- 04	7.09E- 04	0.12-36.38
Brusselsaers 2019	Vaginal dysbiosis	Yes vs no	Progression from normal to ASCUS+	9	9	27405/460746	1.58(1.42- 1.75)	1.59(1.40- 1.82)	1.44(1.22- 1.71)	2.55E- 18	5.34E- 12	1.2- 2.12
Brusselsaers 2019	Vaginal dysbiosis	Yes vs no	HPV incidence	4	4	1926/ 5280	1.33(1.18- 1.50)	1.33(1.18- 1.49)	1.24(1.04- 1.47)	2.24E- 06	2.24E- 06	1.03-1.73
Brusselsaers 2019	Vaginal dysbiosis	Yes vs no	HPV incidence	7	7	1719/4711	1.07(1.02- 1.12)	1.14(1.01- 1.28)	1.03(0.97- 1.09)	1.08E- 02	3.20E- 02	0.86-1.5
Tamarelle 2018	VMB	LL-VMB vs HL- VMB	HPV incidence	7	4	1661/6995	1.65(1.38- 1.96)	1.60(1.22- 2.10)	2.11(1.54- 2.91)	1.94E- 08	7.27E- 04	0.75-3.39
Tamarelle 2018	VMB	LL-VMB vs HL- VMB	HPV incidence	13	2	113/146	1.99(0.91- 4.36)	1.99(0.91- 4.36)	1.91(0.83- 4.39)	8.51E- 02	8.51E- 02	NA

^a Number of studies

^b P value of summary fixed effects estimate

^c P value of summary random effects estimate

^d Prediction intervals are reported only for meta-analyses including at least 3 studies

All statistical tests were two-sided

Abbreviations: FTP: full term pregnancy; NA: Not available; LP: lactobacillus predominant; CSTs: community state types; HPV: human papilloma virus; HR HPV: high risk HPV; LIP: Lactobacillus iners predominant; LCP: Lactobacillus crispatus predominant; Chlamydia tr: Chlamydia trachomatis; CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma; COCP: combined oral contraceptive pill; ys: years; GDM: gestational diabetes mellitus; HIV: human immunosufficiency virus; ART: antiretroviral treatment; SIL: squamous intraepithelial lesion; HSIL high grade SIL; LSIL: low grade SIL; IBD: inflammatory bowel disease; IVF: in vitro fertilization; VMB: vaginal microbiota; LL-VMB: Low lactobacillus vaginal microbiota; HL-VMB: high lactobacillus vaginal microbiota.

Appendix 3b: Evaluation of heterogeneity, small study effects and excess significance bias in the 87 meta-analyses investigating the risk factors associated with HPV, cervical pre cancer and cancer outcomes - only cohort studies included.

Author, year	Exposure	Exposure contrast	Outcome	Egger's P ^a	I ² (95% CI) P ^b	Observed	Expected ^c , P-value ^d					
							Fixed effects	Random effects	Largest study			
Appleby 2006	Age during FTP	Per 1-year decrease	CIN3	0.54	0(0-64)0.46	0	0.26	NP	0.26	NP	0.27	NP
WCRF CUP	Alcohol intake	Highest vs lowest	Cervical cancer incidence	0.14	0(0-68)0.99	0	0.20	NP	0.20	NP	0.20	NP
Gillet 2012	Bacterial vaginosis	Yes vs no	CIN prevalence	0.20	92(88-94)0	8	5.19	0.10	8.20	NP	4.33	0.03
Gillet 2011	Bacterial vaginosis	Yes vs no	HPV prevalence	NA	51(1.08-3.47)0.15	1	1.69	NP	1.53	NP	1.84	NP
WCRF CUP	BMI	Highest vs lowest level	Cervical cancer mortality	0.63	65(0-86)0.04	2	2.93	NP	3.35	NP	0.50	0.02
WCRF CUP	BMI	Highest (>30) vs lowest (<25)	Cervical cancer incidence	0.94	33(0-71)0.18	1	2.52	NP	2.34	NP	0.41	0.35
WCRF CUP	BMI	per 5kg/m2 increase	Cervical cancer incidence	0.79	72(32-84)<0.01	2	0.53	0.04	0.51	0.03	0.52	0.04
WCRF CUP	BMI	per 5kg/m2 increase	Cervical cancer incidence	0.96	68(3-84)<0.01	1	0.37	0.29	0.37	0.29	0.42	0.36
WCRF CUP	BMI	per 5kg/m2 increase	Cervical cancer mortality	0.86	65(0-88)<0.01	0	0.15	NP	0.16	NP	0.17	NP
Wang 2019	Cervicovaginal lactobacilli	LP CSTs vs non LP CST IV	HR HPV incidence	NA	49(0.16-2.14)0.16	0	0.21	NP	0.33	NP	0.10	NP
Wang 2019	Cervicovaginal lactobacilli	LIP CST III vs non LIP CST III	HR HPV incidence	NA	0(0.18-1.82)0.58	0	0.24	NP	0.24	NP	0.13	NP
Wang 2019	Cervicovaginal lactobacilli	LCP CST I vs non LCP CST I	HR HPV incidence	0.19	0(0-73)0.89	0	0.48	NP	0.48	NP	0.65	NP
Zhu 2016	Chlamydia tr and HPV, coinfection	Yes vs no	Cervical cancer incidence (CIN1+)	NA	0(2.47-4.4)0.63	2	1.98	0.89	1.98	0.89	1.98	0.89
Naldin 2019	Chlamydia tr infection	Yes vs no	HPV incidence	0.04	90(82-94)0	5	1.42	<0.01	5.73	0.16	2.07	<0.01
Naldin 2019	Chlamydia tr infection	Yes vs no	HR HPV incidence	NA	73(1.23-4.99)0.06	2	2	NP	2.0	NP	1.98	0.88
Zhu 2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence	0.96	46(1.62-	2	2.86	NP	2.86	NP	2.86	NP

			(CIN3+)		3.03)0.16							
Zhu 2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence (SCC)	NA	0.9(1.9-2.87)0.32	1	1.83	NP	1.83	NP	1.83	NP
Zhu 2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence (CIN1+)	0.59	0(0.91-3.79)0.99	0	3.19	NP	3.19	NP	3.26	NP
Appleby 2007	COCP (current users)	Per year of use	Invasive cervical cancer incidence	0.86	0(0.97-1.1)0.55	0	0.15	NP	0.15	NP	0.15	NP
Appleby 2007	COCP (1-9ys since last use)	Per year of use	Invasive cervical cancer incidence	0.56	19(0-78)0.29	0	0.15	NP	0.15	NP	0.15	NP
Appleby 2007	COCP (>10ys since last use)	Per year of use	Invasive cervical cancer incidence	NA	0(0-73)0.88	0	0.15	NP	0.15	NP	0.15	NP
Smith 2003	COCP	<5 years of use vs Never	Cervical cancer incidence (CIN3+)	NA	0(0.64-2.4)0.77	0	0.18	NP	0.18	NP	0.16	NP
Smith 2003	COCP	5-9 years of use vs Never	Cervical cancer incidence (CIN2+)	NA	0(0.43-1.51)1	0	0.19	NP	0.19	NP	0.19	NP
Smith 2003	COCP	>10 years of use vs Never	Cervical cancer incidence (CIN2+)	NA	0(0.85-2.97)0.47	0	0.52	NP	0.52	NP	0.43	NP
Smith 2003	COCP	5-9 years of use vs Never	Invasive cervical cancer incidence	0.18	75(0-89)<0.01	3	3.76	NP	3.49	NP	3.98	NP
Smith 2003	COCP	<5 years of use vs Never	Invasive cervical cancer incidence	0.23	0(1.4-2.35)0.57	2	3.04	0.22	3.04	0.22	3.47	0.03
Smith 2003	COCP	>10 years of use vs Never	Invasive cervical cancer incidence	0.36	75(1.56-5.83)0.02	2	2.99	NP	2.99	NP	3.0	NP
Lee 2016	Environmental tobacco smoke	Yes vs no	Cervical cancer incidence (CIN2+)	0.19	52(0-78)0.05	2	1.99	0.99	2.70	NP	1.44	0.60
Wang 2020	GDM	Yes vs no	Cervical cancer incidence	0.04	0(0-73)0.55	0	1.14	NP	1.14	NP	3	NP
WCRF CUP	Height	Per 5 cm increase	Cervical cancer incidence	0.76	44(0-80)0.15	0	0.21	NP	0.22	NP	0.20	NP
Looker 2018	HIV	HIV+ vs HIV-	HR HPV incidence	0.77	22(0-61)0.2316	10	9.43	0.62	9.38	0.60	9.68	0.77
Looker 2018	HIV	HIV+ vs HIV-	Clearance of HPV	0.37	73(52-83)0	8	11	0.13	1.15	<0.01	7.35	0.74
Debeaudrap_2019	HIV	HIV+ vs HIV-	Treatment failure for any grade lesions	<0.01	38(0-65)0.07	14	14.5	0.42	1.47	0.22	1.36	0.74
Grulich 2007	HIV	HIV+ vs HIV-	Cervical cancer incidence	0.30	86(75-91)0	5	6.19	NP	5.37	NP	6.12	NP
Liu 2018	HIV	HIV+ vs HIV-	CIN regression (LSIL)	NA	0(0.56-0.81)0.42	1	1.92	NP	1.92	NP	1.89	NP

Looker_2018	HIV	HIV+ vs HIV-	HPV incidence	0.57	47(0-70)0.02	6	8.24	NP	8.45	NP	3.64	NP
Looker_2018	HIV	HIV+ vs HIV-	Clearance of HR HPV	0.40	77(54-86)0	9	5.26	0.02	6.13	<0.01	5.96	<0.01
LIU_2018	HIV	HIV+ vs HIV-	HPV 18 incidence	NA	0(1.17-5.6)0.49	1	1.36	NP	1.36	NP	1.68	NP
Liu_2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (Any HPV)	NA	63(0.16-0.75)0.1	2	1.89	0.73	1.98	0.88	1.82	0.66
Liu_2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (HR HPV)	0.50	61(0-87)0.08	2	1.81	0.82	2.31	0.67	0.94	0.18
Liu_2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (HPV 16)	0.37	30(0-80)0.24	1	1.90	NP	1.68	NP	2.19	NP
Looker_2018	HIV	HIV+ vs HIV-	Clearance of HPV 16	0.25	60(0-79)<0.01	4	4.55	NP	3.97	0.98	5.49	NP
Liu_2018	HIV	HIV+ vs HIV-	HPV incidence	0.97	0(0-73)0.63	2	2.91	NP	2.91	NP	2.96	NP
Liu_2018	HIV	HIV+ vs HIV-	HR HPV incidence	NA	0(1.64-3.37)0.74	1	1.82	NP	1.82	NP	1.80	NP
Liu_2018	HIV	HIV+ vs HIV-	HPV 16 incidence	NA	0(1.71-5.43)0.96	2	1.88	0.73	1.88	0.73	1.89	0.74
Liu_2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (HR HPV)	NA	47(0.37-0.75)0.17	2	1.49	0.41	1.56	0.45	1.39	0.35
Liu_2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (HPV 18)	NA	0(0.32-0.72)0.71	2	1.81	0.65	1.81	0.65	1.75	0.59
Liu_2018	HIV	HIV+ vs HIV-	CIN incidence (LSIL)	NA	0(2.62-5.32)0.57	2	2	NP	2.00	NP	2.00	NP
Debeaudrap_2019	HIV	HIV+ vs HIV-	Treatment failure for high grade lesions	0.26	0(0-54)0.80	3	5.94	NP	5.94	NP	7.58	NP
Liu_2018	HIV	HIV+ vs HIV-	Clearance of prevalent and newly detected HPV (HPV 16)	NA	81(0.21-3.2)0.02	1	1.06	NP	1.06	NP	1.14	NP
Looker_2018	HIV	HIV+ vs HIV-	Clearance of HPV 18	1.0	61(0-81)0.02	3	1.86	0.33	1.93	0.36	0.56	<0.01
Looker_2018	HIV	HIV+ with CD4>200 vs HIV-	HPV incidence	0.71	82(2.17-4.4)0	5	4.97	0.87	4.97	0.9	5.0	0.96
Looker_2018	HIV	HIV+ with CD4≤200 vs HIV-	HPV incidence	0.75	62(3.65-9.08)0.07	3	3.00	1	3.00	1.0	3.0	1.0
Looker_2018	HIV	HIV+ with CD4>200 vs HIV-	HR HPV incidence	0.40	55(1.30-3.18)0.09	2	2.61	NP	2.74	NP	1.12	0.33
Looker_2018	HIV	HIV+ with CD4≤200 vs	HR HPV incidence	NA	0(0.82-	0	0.36	0.5	0.36	0.51	0.29	0.56

		HIV-			2.08)0.67								
Looker 2018	HIV	HIV+ with CD4>200 vs HIV-	Clearance of HR HPV	0.14	19(0.89-1.58)0.3	1	0.47	0.42	0.38	0.29	1.19	NP	
Debeaudrap_2019	HIV	HIV+ vs HIV-	Treatment failure for any grade lesions	<0.01	38(0-65)0.07	14	14.5	0.42	14.7	0.22	1.36	0.74	
Kelly_2018	HIV+	On ART vs no ART	CIN regression (SIL)	0.38	50(0-74)0.04	6	7.13	NP	7.3	NP	5.33	0.67	
Kelly_2018	HIV+	On ART vs no ART	CIN incidence (SIL)	0.48	40(0-69)0.08	3	5.06	NP	4.53	NP	6.65	NP	
Kelly_2018	HIV+	On ART vs no ART	CIN progression (SIL)	0.13	60(0-78)<0.01	5	5.97	NP	5.07	NP	6.87	NP	
Kelly_2018	HIV+	On ART vs no ART	Invasive cervical cancer incidence	NA	32(0.18-0.87)0.23	2	1.96	0.85	1.99	0.92	1.89	0.73	
Liu_2018	HIV+	CD4 <200 VS >500	Clearance of HPV	NA	0(0.37-0.57)0.34	2	1.99	0.92	1.99	0.92	1.99	0.91	
Liu_2018	HIV+	CD4 200-500 VS >500	Clearance of HPV	NA	0(0.64-0.89)0.79	1	1.20	NP	1.20	NP	1.18	NP	
Liu_2018	HIV+	On ART vs no ART	CIN regression (LSIL)	0.65	0(0-73)0.94	2	1.83	0.84	1.83	0.84	1.77	0.79	
Kelly_2018	HIV+	On ART vs no ART	hrHPV prevalence	0.35	72(53-81)0	4	5.35	0.49	3.15	0.60	3.18	0.61	
Kelly_2018	HIV+	On ART vs no ART	Cervical cancer prevalence (HSIL CIN2+)	0.53	53(0-78)0.05	2	2.13	NP	1.77	0.84	3.97	NP	
Liu_2018	HIV+	On ART vs no ART	CIN incidence (LSIL)	NA	49(0.4-1.25)0.17	1	1.25	NP	1.04	NP	1.78	NP	
Liu_2018	HIV+	On ART vs no ART	CIN regression (LSIL)	NA	84(0.16-7.61)0.01	1	1.52	NP	0.20	0.06	1.65	NP	
Allegreti_2015	IBD on immunosuppressive medication	Yes vs healthy controls	HSIL+	0.29	0(0-64)0.42	3	3.41	NP	3.41	NP	3.45	NP	
Li_2013	IVF	yes vs no	Cervical cancer incidence	0.29	69(0-87)0.022	2	1.40	0.53	1.16	0.35	1.44	0.56	
Appleby_2006	Parity	Per increase of 1FTP	CIN3 incidence	0.11	43(0-78)0.14	1	0.43	0.36	0.32	0.21	1.11	NP	
Liu_2014	Pregnancy	Pregnant vs non pregnant	HPV incidence	0.93	82(71-88)0	6	4.65	0.44	4.53	0.40	1.50	<0.01	
Helm_2013	Retinoid use	Yes vs no	Complete or partial regression of CIN2/3 at 3-12 months	0.16	0(0-73)0.41	0	0.15	NP	0.15	NP	0.16	NP	
Helm_2013	Retinoid use	Yes vs no	Complete regression of CIN2/3 at 9-27 months	NA	0(0.51-1.23)0.532	0	0.29	NP	0.29	NP	0.51	NP	
Helm_2013	Retinoid use	Yes vs no	Complete regression of CIN2 at 9-27 months	NA	69(0.19-2.28)0.07	0	0.30	NP	0.19	NP	1.11	NP	

Simon 2015	Rheumatoid arthritis	Yes vs general population	Cervical cancer incidence	0.97	63(26-78)<0.01	3	1	0.04	1.01	0.04	1.0	0.04
Liu 2015	Sexual partners	Multiple vs few partners	CIN incidence	NA	0(0.7-1.9)0.77	0	1.17	NP	1.17	NP	1.22	NP
Kaderli_2014	Smoking	Yes vs no	HPV incidence	0.48	22(0-62)0.24	4	5.91	NP	6.15	NP	4.49	NP
Appleby 2005	Smoking	Past vs never smoker	Cervical cancer incidence (CIN3+)	0.13	3(0-69)0.38	1	2.92	NP	2.89	NP	3.61	NP
Kaderli_2014	Smoking	Yes vs no	HPV prevalence	0.46	66(0-85)0.02	3	2.49	0.65	3.03	NP	2.52	0.67
Appleby 2005	Smoking	Current vs Never	Cervical cancer incidence (CIN3+)	0.84	0(0-68)0.87	2	3.64	NP	3.64	NP	3.64	NP
Grulich 2007	Transplant recipient	Yes vs no	Cervical cancer incidence	0.39	0(1.38-3.3)<0.01	1	0.66	0.63	0.66	0.63	0.89	0.89
Brusselaers 2019	Vaginal dysbiosis	Yes vs no	Progression from normal to ASCUS+	0.63	26(0-65)0.21	5	7.34	NP	7.40	NP	6.75	NP
Brusselaers_2019	Vaginal dysbiosis	Yes vs no	HPV incidence	0.31	0(0-68)0.63	3	2.76	0.79	2.76	0.79	1.97	0.30
Brusselaers_2019	Vaginal dysbiosis	Yes vs no	HPV incidence	0.23	44(0-75)0.1	3	0.51	<0.01	0.95	<0.01	0.38	<0.01
Tamarelle 2018	Vaginal microbiota	LL-VMB vs HL-VMB	HPV incidence	0.39	51(0-79)0.07	3	4.46	NP	4.28	NP	5.38	NP
Tamarelle_2018	Vaginal microbiota	LL-VMB vs HL-VMB	HPV incidence	NA	0(0.91-4.36)0.77	0	0.49	NP	0.49	NP	0.45	NP

^a P-value from the Egger's regression asymmetry test (P<0.10)

^b I² metric of inconsistency (95% confidence interval) and the P-value of the Q test

^c Expected number of statistically significant studies using the point estimate of each meta-analysis (from fixed effect, random effect of largest study accordingly) as the plausible effect size

^d P value of the excess statistical significance test

All statistical tests were two-sided

Abbreviations: NA: Not available, due to <3 included studies; NP: Not Pertinent, because the estimated is larger than the observed, and there is no evidence of excess statistical significance based on the assumption made for the plausible effect size; LP: lactobacillus predominant; CSTs: community state types; HPV: human papilloma virus; HR HPV: high risk HPV; LIP: Lactobacillus iners predominant; LCP: Lactobacillus crispatus predominant; CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma; COCP: combined oral contraceptive pill; GDM: gestational diabetes mellitus; HIV: human immunodeficiency virus; ART: antiretroviral treatment; SIL: squamous intraepithelial lesion; HSIL high grade SIL; LSIL: low grade SIL; IBD: inflammatory bowel disease; IVF: in vitro fertilization; FTP: full term pregnancy; VMB: vaginal microbiota; LL-VMB: Low lactobacillus vaginal microbiota; HL-VMB: high lactobacillus vaginal microbiota.

Appendix 3c: Sensitivity analysis using credibility ceilings when the association is non-significant of the 87 studies investigating the risk factors associated with HPV, cervical pre cancer and cancer outcomes - only cohort studies included.

Author, year	Exposure	Exposure contrast	Outcome	N ^a	Credibility ceiling when association non-significant
Appleby 2006	Age at FTP	Per 1-year decrease	CIN incidence	5	<1%
WCRF CUP 2018	Alcohol intake	Highest vs lowest	Cervical cancer incidence	4	<1%
Gillet 2012	Bacterial Vaginosis	Yes vs no	CIN prevalence	12	18%
Gillet 2011	Bacterial Vaginosis	Yes vs no	HPV prevalence	2	<1%
WCRF CUP 2018	BMI	Highest vs lowest level	Cervical cancer mortality	4	2%
WCRF CUP 2018	BMI	>30 vs <25	Cervical cancer incidence	7	<1%
WCRF CUP 2018	BMI	per 5kg/m ² increase of BMI	Cervical cancer incidence	9	<1%
WCRF CUP 2018	BMI	per 5kg/m ² increase of BMI	Cervical cancer incidence	7	<1%
WCRF CUP 2018	BMI	per 5kg/m ² increase of BMI	Cervical cancer mortality	3	<1%
Wang 2019	Cervicovaginal lactobacilli	LP CSTs vs non LP CST IV	HPV incidence	2	<1%
Wang_2019	Cervicovaginal lactobacilli	LIP CST III vs non LIP CST III	HPV incidence	2	<1%
Wang 2019	Cervicovaginal lactobacilli	LCP CST I vs non LCP CST I	HPV incidence	3	<1%
Zhu 2016	Chlamydia tr and HPV, coinfection	Yes vs no	Cervical cancer incidence	3	12%
Naldin 2019	Chlamydia tr infection	Yes vs no	HPV incidence	6	3%
Naldin 2019	Chlamydia tr infection	Yes vs no	HPV incidence	2	6%
Zhu 2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence	3	2%
Zhu 2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence	2	4%
Zhu_2016	Chlamydia tr infection	Yes vs no	Cervical cancer incidence	4	<1%
Smith 2003	COCP	<5 years users vs Never	Invasive cervical cancer incidence	4	10%
Smith 2003	COCP	5-9 years users vs Never	Invasive cervical cancer incidence	4	2%
Smith 2003	COCP	>10 years users vs Never	Invasive cervical cancer incidence	3	7%
Smith 2003	COCP	<5 years users vs Never	Cervical cancer incidence	2	<1%
Smith 2003	COCP	5-9 years users vs Never	Cervical cancer incidence	2	<1%
Smith_2003	COCP	>10 years users vs Never	Cervical cancer incidence	2	<1%
Appleby 2007	COCP (current user)	Per year of use	Invasive cervical cancer incidence	3	<1%
Appleby 2007	COCP (1-9ys since last use)	Per year of use	Invasive cervical cancer incidence	3	<1%
Appleby 2007	COCP (>10ys since last use)	Per year of use	Invasive cervical cancer incidence	3	<1%
Lee_2016	Environmental tobacco smoke	Yes vs no	Cervical cancer incidence	7	<1%
Wang 2020	GDM	Yes vs no	Cervical cancer incidence	3	<1%
WCRF CUP 2018	Height	Per 5 cm increase	Cervical cancer incidence	4	<1%
Looker 2018	HIV	HIV+, CD4>200 vs HIV-	HPV incidence	5	17%

Looker 2018	HIV	HIV+, CD4<=200 vs HIV-	HPV incidence	3	12%
Looker 2018	HIV	HIV+, CD4>200 vs HIV-	HPV incidence	4	14%
Looker 2018	HIV	HIV+, CD4<=200 vs HIV-	HPV incidence	2	<1%
Looker 2018	HIV	HIV+, CD4>200 vs HIV-	HPV clearance	4	<1%
Looker 2018	HIV	HIV+, CD4<=200 vs HIV-	HPV clearance	2	<1%
Looker 2018	HIV	HIV+ vs HIV-	HPV incidence	11	26%
Looker 2018	HIV	HIV+ vs HIV-	HPV clearance	15	19%
Grulich_2007	HIV	HIV+ vs HIV-	Cervical cancer incidence	6	7%
Liu 2018	HIV	HIV+ vs HIV-	CIN regression	2	7%
Looker 2018	HIV	HIV+ vs HIV-	HPV incidence	15	20%
Looker 2018	HIV	HIV+ vs HIV-	HPV clearance	11	17%
Debeaudrap 2019	HIV	HIV+ vs HIV-	CIN persistence	9	22
Liu 2018	HIV	HIV+ vs HIV-	HPV incidence	3	12%
Liu 2018	HIV	HIV+ vs HIV-	HPV incidence	2	8%
Liu_2018	HIV	HIV+ vs HIV-	HPV incidence	2	8%
Liu_2018	HIV	HIV+ vs HIV-	HPV incidence	2	3%
Liu 2018	HIV	HIV+ vs HIV-	HPV clearance	2	7%
Liu 2018	HIV	HIV+ vs HIV-	HPV clearance	2	7%
Liu 2018	HIV	HIV+ vs HIV-	HPV clearance	3	10%
Liu 2018	HIV	HIV+ vs HIV-	HPV clearance	3	1%
Liu 2018	HIV	HIV+ vs HIV-	HPV clearance	2	8%
Liu 2018	HIV	HIV+ vs HIV-	CIN incidence	2	8%
Looker 2018	HIV	HIV+ vs HIV-	HPV clearance	9	13%
Debeaudrap 2019	HIV	HIV+ vs HIV-	CIN persistence	15	29
Liu 2018	HIV	HIV+ vs HIV-	HPV clearance	2	<1%
Looker 2018	HIV	HIV+ vs HIV-	HPV clearance	7	<1%
Liu 2018	HIV+	CD4 <200 VS >500	HPV clearance	2	7%
Liu 2018	HIV+	CD4 200-500 VS >500	HPV clearance	2	4%
Kelly 2018	HIV+ on treatment	On ART vs no ART	CIN regression	10	16%
Kelly 2018	HIV+ on treatment	On ART vs no ART	CIN incidence	11	3%
Kelly 2018	HIV+ on treatment	On ART vs no ART	CIN progression	10	14%
Kelly 2018	HIV+ on treatment	On ART vs no ART	Invasive cervical cancer incidence	2	6%
Liu 2018	HIV+ on treatment	On ART vs no ART	CIN incidence	3	12%
Kelly 2018	HIV+ on treatment	On ART vs no ART	HPV prevalence	19	<1%
Kelly 2018	HIV+ on treatment	On ART vs no ART	Cervical cancer prevalence	7	<1%
Liu 2018	HIV+ on treatment	On ART vs no ART	CIN incidence	2	<1%
Liu 2018	HIV+ on treatment	On ART vs no ART	CIN regression	2	<1%
Allegreti_2015	IBD on immunosuppressive meds	Yes vs healthy controls	Cervical cancer incidence	5	16%
Li 2013	IVF	Yes vs no	Cervical cancer incidence	4	<1%

Appleby 2006	Parity	Per increase of 1 FTP	CIN incidence	5	<1%
Liu 2014	Pregnancy	Pregnant vs non pregnant	HPV incidence	14	<2%
Helm 2013	Retinoid use	Yes vs no	CIN regression	3	<1%
Helm 2013	Retinoid use	Yes vs no	CIN regression	2	<1%
Helm 2013	Retinoid use	Yes vs no	CIN regression	2	<1%
Simon 2015	Rheumatoid arthritis	Yes vs general population	Cervical cancer incidence	15	<1%
Liu 2015	Sexual partners	Multiple vs few partners	CIN incidence	2	<1%
Kaderli 2014	Smoking	Yes vs no	HPV incidence	10	16%
Appleby 2005	Smoking	Current vs Never	Cervical cancer incidence	4	16%
Appleby 2005	Smoking	Past vs never smoker	Cervical cancer incidence	4	3%
Kaderli 2014	Smoking	Yes vs no	HPV prevalence	5	<1%
Grulich_2007	Transplant recipient	Yes vs no	Cervical cancer incidence	3	9%
Brusselsaers 2019	Vaginal dysbiosis	Yes vs no	HPV persistence	7	3%
Tamarelle 2018	VMB	LL-VMB vs HL-VMB	HPV incidence	4	9%
Tamarelle 2018	VMB	LL-VMB vs HL-VMB	HPV incidence	2	<1%
			Progression from normal to		
Brusselsaers 2019	Vaginal dysbiosis	Yes vs no	ASCUS+	9	16%
Brusselsaers 2019	Vaginal dysbiosis	Yes vs no	HPV incidence	4	15%

^a Number of studies

Abbreviations FTP: full term pregnancy; CIN: cervical intraepithelial neoplasia; HPV: human papilloma virus; LP: lactobacillus predominant; LIP: Lactobacillus iners predominant; LCP: Lactobacillus crispatus predominant; CSTs: community state types; Chlamydia tr: Chlamydia trachomatis; COCP: combined oral contraceptive pill; ys: years; GDM: gestational diabetes mellitus; HIV: human immunodeficiency virus; ART: antiretroviral treatment; IBD: inflammatory bowel disease; meds: medications; IVF: in vitro fertilization; VMB: vaginal microbiota; LL-VMB: Low lactobacillus vaginal microbiota; HL-VMB: high lactobacillus vaginal microbiota.

Appendix 3d: Details of evidence grading for meta-analysis of risk factors for HPV, cervical precancer and cancer outcomes – only cohort studies included*

Exposure	Exposure contrast	Outcome	N*	Sample size Cases/cohort	Largest study [#]	Random effects summary RR (95% CI) [*]	Random P-value	95% Prediction interval	Egger's P [∞]	I ² (%)	Excess significance [§] O/E ^a	P-value ^j
Strong evidence												
HIV	HIV+ vs HIV-	HR HPV incidence	11	2323/8668	2.35(2.03-2.72)	2.2(1.89-2.54)	3.01E-26	1.63-2.97	0.77	22	10/9.7	0.77
IBD on immunosuppressant	Yes vs healthy controls	Cervical high-grade dysplasia/cancer	5	10829/244724	1.35(1.28-1.43)	1.33(1.27-1.39)	7.78E-37	1.24-1.43	0.29	0	3/3.5	NP
Vaginal dysbiosis	Yes vs no	Progression from normal to ASCUS+	9	27405/460746	1.44(1.22-1.71)	1.59(1.40-1.81)	5.34E-12	1.20- 2.12	0.63	26	5/6.8	NP
Highly suggestive												
HIV	Positive vs negative HIV+, CD4>200	HPV clearance	15	2977/7448	0.67(0.56-0.81)	0.53(0.43-0.64)	4.32E-10	0.26-1.06	0.37	73	8/7.4	0.74
HIV	HIV+ vs HIV-	HPV incidence	5	1151/2335	3.61(3.01-4.32)	3.09(2.17-4.40)	3.75E-10	0.91-10.55	0.709	82	5/5	0.96
HIV	HIV+ vs HIV-	Cervical cancer incidence	6	1160/122692	5.2(3.81-6.93)	5.82(2.98-11.34)	2.34E-07	0.67-50.17	0.78	86	4/4.1	NP
Suggestive evidence												
Chlamydia tr.	Yes vs no	HPV incidence	6	2753/7426	1.2(1.09-1.32)	1.74(1.28-2.38)	4.94E-04	0.64-4.73	0.04	90	5/2.1	0.01
HIV	HIV+ vs HIV-	CIN regression (LSIL)	2	1968/4554	0.69(0.57-0.84)	0.68(0.56-0.81)	3.46E-05	NA	NA	0	1/1.9	NP
HIV	HIV+ vs HIV-	HPV incidence	15	1722/8342	1.35(0.99-1.84)	1.73(1.39-2.16)	1.01E-06	0.91-3.31	0.57	47	6/3.6	NP
HIV	HIV+ vs HIV-	Clearance of HR HPV	11	3581/9903	0.67(0.59-0.77)	0.66(0.55-0.79)	8.03E-06	0.37-1.20	0.40	77	9/6	0.07

HIV+	On ART vs not on ART	CIN regression (SIL)	10	3074/7953	1.30(1.00-1.70)	1.62(1.32-1.99)	4.41E-06	0.93-2.82	0.38	50	6/5.3	0.67
Smoking	Yes vs no	HPV incidence	10	9442/28187	1.20(1.00-1.30)	1.37(1.21-1.56)	1.04E-06	1.06-1.77	0.48	22	4/4.5	NP
Vaginal dysbiosis	Yes vs no	HPV incidence	4	1926/ 5280	1.24(1.04-1.47)	1.33(1.18-1.49)	2.24E-06	1.03-1.73	0.31	0	3/2	0.30
VMB	LL vs HL	HPV incidence	4	1661/6995	2.11(1.54-2.91)	1.60(1.22-2.10)	7.27E-04	0.75-3.39	0.39	51	3/5.4	NP
Bacterial vaginosis	Yes vs no	CIN prevalence	20	109573/1652848	1.19(1.13-1.26)	1.63(1.30-2.40)	2.43E-05	0.78-3.39	0.20	92	8/4.3	0.03
Weak evidence												
Bacterial vaginosis	Yes vs no	HPV prevalence	2	199/667	2.42(1.64-3.57)	1.94(1.08-3.47)	2.67E-02	NA	NA	51	1/1.8	NP
BMI	Highest vs lowest level	Cervical cancer mortality	4	420/3144461	1.15(0.79-1.70)	1.82(1.07-3.10)	2.75E-02	0.22-15.20	0.63	65	2/0.5	0.02
Chlamydia coinfection with HPV	Yes vs no	Cervical cancer incidence (CIN1+)	3	492/2528	3.23(2.39-4.35)	3.3(2.47-4.40)	6.42E-16	NA	NA	0	2/1.9	0.90
Chlamydia infection	Yes vs no	Cervical cancer incidence (CIN3+)	3	832/4305	2.21(1.84-2.65)	2.21(1.62-3.03)	6.43E-07	0.09-53.40	0.96	46	2/2.9	NP
Chlamydia infection	Yes vs no	Cervical cancer incidence (SCC)	2	500/3077	2.40(1.95-2.94)	2.33(1.90-2.87)	6.59E-16	NA	NA	0.9	1/1.8	NP
Chlamydia infection	Yes vs no	HPV incidence (HR)	2	912/2878	1.84(1.36-2.47)	2.07(1.57-2.72)	1.13E-02	NA	NA	73	2/1.9	0.88
COCP	<5 years user vs Never	Invasive cervical cancer incidence	4	470/17983	2.10(1.40-3.00)	1.82(1.40-2.35)	6.12E-06	1.03-3.20	0.23	0	2/3.5	0.03
COCP	5-9 years user vs Never	Invasive cervical cancer incidence	4	448/17910	3.10(2.10-4.50)	1.95(1.14-3.36)	1.51E-02	0.18-20.80	0.18	75	3/4	NP
COCP	>10 years user vs Never	Invasive cervical cancer incidence	3	322/16209	4.70(2.90-7.50)	3.02(1.56-5.83)	1.02E-03	0-6554	0.36	75	2/3	NP
Environmental tobacco smoke	Yes vs no	Cervical cancer incidence (CIN2+)	7	432/65031	0.90(0.65-1.24)	1.41(1.01-1.96)	4.32E-02	0.58-3.44	0.19	52	2/1.4	0.60
HIV	HIV+, CD4<=200 vs HIV-, HIV+, CD4>200	HPV incidence	3	703/1212	5.19(4.30-6.26)	5.76(3.65-9.08)	4.73E-14	0.04-844.24	0.75	62	3/3	1.00
HIV	vs HIV-	HR HPV incidence	4	327/758	1.41(0.89-2.24)	2.03(1.30-3.17)	1.78E-03	0.36-11.36	0.40	55	2/1.1	0.33
HIV	HIV+ vs HIV-	HPV incidence	3	528/1694	2.98(2.07-4.29)	2.64(2.05-3.42)	1.13E-13	0.50-13.94	0.96	0	2/3	NP

HIV	HIV+ vs HIV-	HR HPV incidence	2	263/1210	2.30(1.57-3.37)	2.35(1.64-3.37)	3.62E-06	NA	NA	0	1/1.8	NP
HIV	HIV+ vs HIV-	HPV 16 incidence	2	91/1211	3.09(1.39-6.86)	3.05(1.71-5.43)	1.55E-04	NA	NA	0	2/1.9	0.74
HIV	HIV+ vs HIV-	HPV 18 incidence	2	54/1211	3.19(1.17-8.70)	2.56(1.17-5.60)	1.92E-02	NA	NA	0	1/1.7	NP
HIV	HIV+ vs HIV-	Clearance** of any HPV type	2	424/696	0.46(0.34-0.62)	0.34(0.16-0.75)	7.18E-03	NA	NA	63	2/1.8	0.66
HIV	HIV+ vs HIV-	Clearance** of HR HPV	2	617/1992	0.60(0.47-0.76)	0.53(0.37-0.75)	3.86E-04	NA	NA	47	2/1.4	0.35
HIV	HIV+ vs HIV-	Clearance** of HR HPV	3	675/1053	0.75(0.59-0.96)	0.58(0.39-0.87)	7.83E-03	0.01-44.81	0.50	61	2/9.4	0.18
HIV	HIV+ vs HIV-	Clearance** of HPV 16	3	485/994	0.57(0.41-0.79)	0.64(0.44-0.93)	1.99E-02	0.02-20.10	0.37	30	1/2.2	NP
HIV	HIV+ vs HIV-	Clearance** of HPV 18	2	348/624	0.50(0.31-0.81)	0.48(0.32-0.72)	4.31E-04	NA	NA	0	2/1.8	0.59
HIV	HIV+ vs HIV-	CIN incidence (LSIL)	2	362/2744	4.00(2.61-6.13)	3.73(2.62-5.32)	3.46E-13	NA	NA	0	2/2	NP
HIV	HIV+ vs HIV- on ART vs no ART	Clearance of HPV 16	9	2283/4964	0.56(0.39-0.81)	0.67(0.50-0.90)	7.28E-03	0.28-1.61	0.25	60	4/5.5	NP
HIV+	HIV+ vs HIV- on ART vs no ART	CIN incidence (SIL)	11	1826/6475	0.62(0.42-0.91)	0.72(0.54-0.95)	2.15E-02	0.35-1.49	0.48	40	3/6.7	NP
HIV+	HIV+ vs HIV- on ART vs no ART	CIN progression (SIL)	10	3493/8568	0.66(0.54-0.81)	0.74(0.61-0.90)	2.63E-03	0.42-1.31	0.13	60	5/6.9	NP
HIV+	HIV+ vs HIV- on ART vs no ART	Invasive cervical cancer incidence	2	2340/411779	0.50(0.29-0.87)	0.40(0.18-0.87)	2.03E-02	NA	NA	32	2/1.9	0.73
HIV+	CD4 <200 VS >500	Clearance of HPV	2	812/2218	0.47(0.38-0.59)	0.46(0.37-0.57)	1.11E-12	NA	NA	0	2/1.9	0.91
HIV+	CD4 200-500 VS >500	Clearance of HPV	2	812/2218	0.76(0.64-0.9)	0.76(0.64-0.89)	1.01E-03	NA	NA	0	1/1.2	NP
HIV+	on ART vs no ART	CIN incidence (LSIL)	3	522/1604	0.66(0.47-0.92)	0.65(0.52-0.82)	2.78E-04	0.16-2.91	0.65	0	2/1.8	0.79
Pregnancy Rheumatoid arthritis	Pregnant vs non pregnant Yes vs general population	HPV incidence	14	1474/9518	0.87(0.66-1.14)	1.42(1.03-1.96)	3.33E-02	0.42-4.80	0.93	82	6/1.5	<0.01
	Current vs Never	Cervical cancer incidence	15	297/297887	0.86(0.84-0.89)	0.85(0.73-0.99)	4.73E-02	0.54-1.34	0.97	63	2/1	0.04
Smoking		Cervical cancer incidence (CIN3+)	4	798/2874	1.68(1.17-2.41)	1.85(1.47-2.34)	2.22E-07	1.11-3.09	0.84	0	2/3.6	NP

Smoking	Past vs never smoker	Cervical cancer incidence (CIN3+)	4	798/2873	1.83(1.19-2.82)	1.53(1.12-2.10)	8.10E-03	0.73-3.20	0.13	3	1/3.6	NP
Smoking	Yes vs no	HPV prevalence	5	4451/10853	1.10(1.00-1.40)	1.24(1.03-1.50)	2.63E-02	0.66-2.31	0.46	66	3/2.5	0.67
Transplant recipient	Yes vs no	Cervical cancer incidence	3	24/18800	2.50(1.33-4.27)	2.13(1.38-3.30)	7.09E-04	0.12-36.38	0.39	0	1/0.9	0.89
Vaginal dysbiosis	Yes vs no	HPV persistence	7	1719/4711	1.03(0.97-1.09)	1.14(1.01-1.28)	3.20E-02	0.86-1.50	0.23	44	3/0.4	<0.01

*Only meta-analyses meeting at least weak grade of evidence listed

* Number of studies

Relative risk and 95% confidence interval of largest study (smallest standard error) in each meta-analysis

¥ Random effects refer to summary risk ratio (95% confidence interval) using the random-effects model

|| P value of summary random effects estimate

∞ P-value from the Egger's regression asymmetry test

§ Expected number of statistically significant studies using the point estimate of the largest study (smallest standard error) as the plausible effect size

^α Observed/Expected number of statistically significant studies

^j P value of the excess statistical significance test

** Clearance of prevalent and newly detected

All statistical tests were two-sided

Abbreviations: HPV: human papilloma virus; HR HPV: high risk HPV; IBD: inflammatory bowel disease; HIV: human immunodeficiency virus; NP: Not Pertinent, because the estimated is larger than the observed, and there is no evidence of excess statistical significance based on the assumption made for the plausible effect size; CIN: cervical intraepithelial neoplasia; SIL: squamous intraepithelial lesion; HSIL high grade SIL; LSIL: low grade SIL; ART: antiretroviral treatment; VMB: vaginal microbiota; LL-VMB: Low lactobacillus vaginal microbiota; HL-VMB: high lactobacillus vaginal microbiota; Chlamydia Tr: Chlamydia trachomatis.; SCC: squamous cell carcinoma; COCP: combined oral contraceptive pill.

Appendix 3e: Details of evidence grading for meta-analyses of risk factors for HPV, pre-invasive and invasive cervical cancer incidence, progression, regression, or mortality - all study types included (sensitivity analysis).

Exposure	Exposure contrast	Outcome	N ^a	Sample size Cases/ Cohort ^b	Largest Study ^c	Random Summary RR (95% CI) ^d	Random P-value ^e	95% Prediction interval	Egger's P ^f	I ² (%)	Excess significance ^g O/E ^h	P-value ⁱ
Strong evidence												
Sexual health												
Chlamydia tr. infection	Yes vs no	Cervical cancer incidence (CIN1+)	18	3392/7365	2.44 (2.06-2.89)	2.19 (1.74-2.74)	1.03E-11	1.18-4.06	0.34	47	9/13.0	NP
Chlamydia tr. infection	Yes vs no	Cervical cancer incidence (SCC)	11	3198/8118	2.55 (2.15-3.03)	2.09 (1.79-2.44)	6.87E-21	1.48-2.95	0.27	32	5/9.6	NP
Chlamydia tr. coinfection with HPV	Yes vs no	Cervical cancer incidence (CIN1+)	6	1086/4116	3.23 (2.39-4.35)	4.37 (2.75-6.96)	4.59E-10	1.31-14.66	0.35	44	4/5.2	NP
Chlamydia tr. infection	Yes vs no	HR HPV incidence	15	5049/22082	1.84 (1.36-2.47)	2.32 (2.02-2.65)	3.45E-34	2.00-2.69	0.21	0	11/13.4	NP
Number of sexual partners	Multiple vs few	CIN incidence	26	5638/16346	1.94 (1.47-2.41)	1.97 (1.80-2.15)	<1E-100	1.79-2.16	0.43	0	14/22.6	NP
Trichomonas vaginalis infection	Yes vs no	Cervical cancer incidence (CIN1+)	15	7715/75168	1.67 (1.23-2.28)	2.09 (1.69-2.60)	2.30E-11	1.22-3.60	0.93	34	7/9.5	NP
Contraception												
Oral contraceptive, medium and long duration >5 years	<8 years since last use vs never	Cervical cancer incidence (CIN3+)	4	5839/20573	2.10 (1.80-2.40)	2.13 (1.87-2.42)	4.37E-31	1.59-2.86	0.45	1	4/4.0	NP
Immunocompromise												

HIV infection	HIV+ vs HIV -	HR HPV incidence	11	2323/6345	2.35 (2.03-2.72)	2.20 (1.90-2.54)	3.01E-26	1.63-2.97	0.77	22	10/9.7	0.77
IBD on immunosuppression	Yes vs healthy controls	Cervical cancer incidence (HSIL+)	8	30671/2418502	1.35 (1.28-1.43)	1.34 (1.23-1.46)	5.98E-11	1.12-1.61	0.36	34	4/5.0	NP
Vitamin supplementation												
Vaginal dysbiosis	Yes vs	Progression from normal to ASCUS+	9	27405/433341	1.44 (1.22-1.71)	1.59 (1.40-1.82)	5.34E-12	1.20-2.12	0.63	26	5/6.8	NP
Vegetable intake	Highest vs lowest	Cervical cancer incidence	11	2421/32719	0.6 (0.47-0.76)	0.57(0.5-0.66)	1.35E-15	0.49-0.67	0.36	0	4/8.98	NP
Highly suggestive evidence												
Sexual health												
Chlamydia tr. infection	Yes vs no	HPV incidence	40	15642/59248	1.20 (1.09-1.32)	2.12 (1.80-2.49)	3.95E-19	0.90-5.00	<0.01	83	28/11.4	<0.01
Number of sexual partners	Multiple partners vs few partners	Cervical cancer incidence	24	5878/16319	1.56 (1.28-1.84)	1.99 (1.67-2.38)	1.96E-14	1.11-3.58	0.32	54	13/16.9	NP
Immunocompromise												
HIV infection	HIV+ vs HIV -	Clearance of HPV	15	2977/4471	0.67 (0.56-0.81)	0.53 (0.43-0.64)	4.32E-10	0.26-1.06	0.37	73	8/7.4	0.74
HIV infection	HIV+ vs HIV -	Treatment failure for any grade lesions	18	1431/3434	3.20 (2.40-4.30)	5.34 (4.21-6.77)	<1E-100	2.62-10.85	<0.01	44	17/16.1	0.49
HIV infection	HIV+ vs HIV -	Cervical cancer incidence	6	1160/12269	5.2 (3.81-6.93)	5.81(3-11)	2.34E-07	0.67-50.17	0.78	86	4/4.08	NP
HIV infection	HIV+ with CD4>200 vs HIV-	HPV incidence	5	1151/2335	3.61(3.01-4.32)	3.09(2.17-4.4)	3.75E-10	0.91-10.55	0.71	83	5/5	0.96
Smoking												
Smoking	Ever vs never	Cervical cancer incidence (CIN3+)	35	4749/11844	1.60 (1.40-1.90)	1.78 (1.55-2.04)	2.15E-16	0.85-3.71	0.24	83	22/17.8	0.16

Smoking	Current vs never	CIN incidence	27	2018/7088	2.30 (1.70-3.00)	2.21 (1.89-2.58)	2.88E-23	1.19-4.09	0.60	56	19/17.0	0.43
Smoking	Current vs never	Cervical cancer incidence (CIN3+)	19	10814/28619	2.26 (1.88-2.72)	1.69 (1.40-2.02)	2.10E-08	0.81-3.51	0.73	78	11/18.8	NP
Contraception												
Oral contraceptives	Long duration >10 years vs never	Invasive cervical cancer incidence	13	5615/23166	2.20 (1.90-2.70)	2.14 (1.66-2.76)	3.39E-09	0.96-4.80	0.99	65	8/11.5	NP
COC current users	Risk per year of use	Cervical cancer incidence	18	1105/3546	1.09 (1.07-1.11)	1.06 (1.04-1.08)	2.50E-10	1.01-1.11	0.057	32	6/1.1	<0.01
Pregnancy												
Age in pregnancy	Per 1-year decrease in age at FTP	Cervical cancer incidence	17	9871/32095	1.07 (1.05-1.09)	1.07 (1.05-1.08)	1.92E-19	1.03-1.10	0.98	25	7/1.9	<0.01
Parity	Per increase of 1 FTP	Cervical cancer incidence	17	9764/25297	1.08 (1.05-1.10)	1.11 (1.09-1.14)	1.22E-16	1.04-1.19	0.64	41	11/2.1	<0.01
Vitamin supplementation												
Vitamin C intake	By increased intake of 50mg/d	Cervical cancer incidence (CIN1+)	9	2405/7039	0.94 (0.91-0.98)	0.91(0.89-0.94)	1.09E-09	0.88-0.95	0.01	3	5/0.7	<0.01
Serum selenium levels*	Highest vs lowest	Cervical cancer incidence	21	1372/2602	-1.08 (-1.42- -0.75)	-4.84 (-5.99- -3.68)	2.14E-16	NA	<0.01	99	2-13.4	<0.01
Suggestive evidence												
Sexual health												
Bacterial Vaginosis	Yes vs no	CIN incidence	20	110722/1544804	1.19 (1.13-1.26)	1.50 (1.24-1.82)	3.40E-05	0.73-3.07	0.27	89	11/6.0	0.01
Candida albicans	Yes vs no	HPV incidence	5	1380/8860	0.48(0.33-0.71)	0.63(0.48-0.82)	9.00E-04	0.39-1.02	0.68	3	1/4.2	NP

Vaginal douching	Yes vs no	Cervical cancer incidence (CIN3+)	12	1896/5805	1.40 (1.10-1.90)	1.45 (1.19-1.77)	2.50E-04	0.81-2.61	0.12	51	5/4.5	0.75
Vaginal douching	>= 1 time/week vs less	Cervical cancer incidence (CIN3+)	5	1928/6290	1.50 (1.00-2.40)	2.06 (1.34-3.15)	8.80E-04	0.51-8.25	0.13	63	3/4.3	NP
Vaginal dysbiosis	Yes vs no	HPV incidence	4	1926/3354	1.24 (1.04-1.47)	1.33 (1.18-1.50)	2.24E-06	1.03-1.73	0.31	0	3/2.0	0.30
VMB, less than 40 years old	LL-VMB vs HL-VMB	HPV incidence	7	1772/5545	2.11 (1.54-2.91)	1.69 (1.31-2.18)	5.30E-05	0.85-3.37	0.54	51	4/6.3	NP
Immunocompromise												
HIV infection	Yes vs no	Cervical cancer incidence	9	1184/141492	5.20 (3.81-6.93)	3.98 (2.24-7.05)	2.32E-06	0.59-26.73	0.30	86	5/6.1	NP
HIV infection	HIV+ vs HIV -	Clearance of prevalent and newly detected HPV (any)	3	1007/2663	0.46 (0.39-0.54)	0.47 (0.33-0.68)	5.80E-05	0.01-39.10	0.65	87	3/2.98	0.90
HIV infection	HIV+ vs HIV -	LSIL regression	2	1968/2586	0.69 (0.57-0.84)	0.67 (0.56-0.81)	3.50E-05	NA	NA	0	1/1.9	NP
HIV infection	HIV+ vs HIV -	HPV incidence	15	1722/6620	1.35 (0.99-1.84)	1.73 (1.39-2.16)	1.01E-06	0.91-3.31	0.57	47	6/3.6	0.16
HIV infection	HIV+ vs HIV -	Clearance of HR HPV	11	3581/6322	0.67 (0.59-0.77)	0.66 (0.55-0.79)	8.03E-06	0.37-1.20	0.40	77	9/6.0	0.07
HIV+ treatment	ART vs no ART	CIN regression (SIL)	10	3074/4879	1.30 (1.00-1.70)	1.62 (1.32-1.99)	4.41E-06	0.93-2.83	0.38	49	6/5.3	0.67
HIV+ treatment	ART vs no ART	Cervical cancer incidence	3	2360/409519	0.50 (0.29-0.87)	0.43 (0.26-0.71)	8.80E-04	0.02-10.57	0.41	0	2/2.2	NP
Contraception												

IUD device use	Any vs no	Invasive cervical cancer incidence	16	4945/12482	0.89 (0.73-1.08)	0.64 (0.53-0.77)	1.91E-06	0.38-1.07	0.03	42	5/2.2	0.04
IUD device use	Use vs no use	Cervical cancer incidence	8	2205/4419	0.45 (0.30-0.67)	0.49 (0.34-0.70)	9.60E-05	0.21-1.13	0.67	34	3/7.6	NP
Oral contraceptives	Medium duration 5-9 years vs never	Invasive cervical cancer incidence	21	7930/30007	1.70 (1.50-2.00)	1.50 (1.27-1.77)	1.15E-06	0.84-2.69	0.22	58	9/16.0	NP
Smoking												
Environmental tobacco smoke	Yes vs no	Cervical cancer incidence (CIN2+)	17	2039/79240	1.30 (1.07-1.59)	1.60 (1.32-1.93)	1.90E-06	0.84-3.03	0.11	59	7/5.3	0.37
Passive smoking	Yes vs no	Cervical cancer incidence (CIN1+)	16	2230/7284	1.03 (0.99-1.07)	1.68 (1.32-2.15)	2.80E-05	0.67-4.21	<0.01	82	9/0.8	<0.01
Smoking	Current vs never	Cervical cancer incidence (CIN3+)	24	2702/9258	1.10 (0.90-1.40)	2.04 (1.70-2.44)	8.5E-15	0.88-4.75	0.67	84	16/2.6	<0.01
Smoking	Ever vs never	CIN incidence	25	2243/8384	1.30 (1.00-1.70)	1.96 (1.69-2.27)	2.65E-19	1.10-3.49	0.05	57	19/4.6	<0.01
Smoking	Past smoker vs never	Cervical cancer incidence (CIN3+)	19	10814/28619	1.29 (1.04-1.60)	1.21 (1.09-1.34)	3.20E-04	1.08-1.35	0.80	0	3/11.1	NP
Smoking	Yes vs no	HPV incidence	10	9442/18745	1.20 (1.00-1.30)	1.37 (1.21-1.56)	1.04E-06	1.06-1.77	0.48	22	4/4.5	NP
Vitamin supplementation												
Vitamin A intake	Highest vs lowest	Invasive cervical cancer incidence	22	3418/10478	0.95 (0.74-1.22)	0.59 (0.49-0.72)	1.51E-07	0.25-1.39	0.38	78	12/1.3	<0.01
Vitamin C intake	Highest vs lowest	Cervical cancer incidence (CIN1+)	12	3711/307410	0.98 (0.81-1.19)	0.58(0.46-0.74)	7.86E-06	0.27-1.26	0.02	70	6/0.6	<0.01

Vitamin E supplement intake	Highest vs lowest	Cervical cancer incidence	15	3815/10473	0.35 (0.28-0.44)	0.58 (0.47-0.72)	1.15E-06	0.25-1.37	0.51	83	11/14.9	NP
Vitamin E supplement intake	Highest vs lowest	Invasive cervical cancer incidence	9	1321/4177	0.48 (0.38-0.61)	0.53 (0.39-0.73)	7.30E-05	0.19-1.50	0.77	78	6/8.0	NP
Vitamin E supplement intake	Highest vs lowest	CIN incidence	17	2494/6296	0.66 (0.51-0.87)	0.56 (0.45-0.71)	1.06E-06	0.22-1.41	0.36	76	11/8.6	0.24
Fruit intake	Highest vs lowest	Cervical cancer incidence	6	1423/32346	0.76 (0.62-0.93)	0.79 (0.67-0.92)	3.00E-03	0.63-0.99	0.65	0	1/3.04	NP
Retinol intake	Highest vs lowest	Cervical cancer incidence	19	2925/9034	0.95 (0.74-1.22)	0.61 (0.49-0.76)	8.40E-06	0.25-1.50	0.74	79	10/1.1	<0.01
Weak evidence												
Immunocompromise												
EBV infection	Yes vs no	CIN1 incidence	6	52/193	2.69 (1.22-5.91)	2.32 (1.18-4.54)	0.01	0.58-9.19	0.95	18	2/1.3	0.50
EBV infection	Yes vs no	CIN2/3 incidence	7	79/226	6.30 (2.94-13.54)	2.35 (1.06-5.22)	0.04	0.24-23.47	0.11	59	3/3.6	NP
EBV infection	Yes vs no	Cervical cancer incidence	13	166/354	0.20 (0.08-0.48)	4.05 (1.78-9.24)	8.70E-04	0.20-80.35	0.08	78	10/5.9	0.02
HIV infection	HIV+ vs HIV -	Clearance of HPV 16	9	2283/2681	0.56 (0.39-0.81)	0.67 (0.50-0.90)	7.30E-03	0.28-1.61	0.25	60	4/5.5	NP
HIV infection	HIV+ vs HIV -	Treatment failure for high grade lesions	10	349/2339	3.50 (2.00-6.10)	2.66 (2.00-3.54)	2.14E-11	1.90-3.73	0.36	0	4/8.5	NP
HIV infection	HIV+ vs HIV -	LSIL incidence	2	362/2382	4.00 (2.61-6.13)	3.73 (2.62-5.32)	3.46E-13	NA	NA	0	2/2.0	NP
HIV infection	HIV+ vs HIV -	HPV incidence	3	528/1166	2.98 (2.07-4.29)	2.64 (2.04-3.42)	1.13E-13	0.50-13.94	0.96	0	2/3.0	NP

HIV infection	HIV+ vs HIV -	HR HPV incidence	2	263/947	2.30 (1.57-3.37)	2.35 (1.64-3.37)	3.62E-06	NA	NA	0	1/1.8	NP
HIV infection	HIV+ vs HIV -	HPV 16 incidence	2	91/973	3.09 (1.39-6.86)	3.05 (1.71-5.43)	1.50E-04	NA	NA	0	2/1.9	0.74
HIV infection	HIV+ vs HIV -	HPV 18 incidence	2	54/996	3.19 (1.17-8.70)	2.55 (1.17-5.60)	0.02	NA	NA	0	1/1.7	NP
HIV infection	HIV+ vs HIV -	Clearance of newly detected HPV	2	569/3593	0.67 (0.56-0.81)	0.74 (0.59-0.92)	8.20E-03	NA	NA	47	1/2.0	NP
HIV infection	HIV+ vs HIV -	Clearance of prevalent and newly detected HPV (Any HPV)	2	424/820	0.46 (0.34-0.62)	0.34 (0.16-0.75)	7.20E-03	NA	NA	63	2/1.8	0.66
HIV infection	HIV+ vs HIV -	Clearance of prevalent and newly detected HPV (HR HPV)	2	617/1375	0.60 (0.47-0.76)	0.53 (0.37-0.75)	3.86E-04	NA	NA	47	2/1.4	0.35
HIV infection	HIV+ vs HIV -	Clearance of prevalent and newly detected HPV (HR HPV)	3	675/960	0.75 (0.59-0.96)	0.58 (0.39-0.87)	7.80E-03	0.01-44.81	0.50	61	2/0.9	0.18
HIV infection	HIV+ vs HIV -	Clearance of prevalent and newly detected HPV (HPV 16)	3	485/509	0.57 (0.41-0.79)	0.64 (0.44-0.93)	0.02	0.02-10.10	0.37	30	1/2.2	NP
HIV infection	HIV+ vs HIV -	Clearance of prevalent and newly detected HPV (HPV 18)	2	348/1282	0.50 (0.31-0.81)	0.48 (0.32-0.72)	4.30E-04	NA	NA	0	2/1.8	0.59
HIV+	ART vs no ART	HR HPV prevalence	20	4163/4592	0.83 (0.74-0.94)	0.82 (0.68-0.99)	0.04	0.41-1.64	0.37	71	4/3.4	0.72
HIV+	ART vs no ART	CIN incidence	12	2190/6100	0.62 (0.42-0.91)	0.70 (0.55-0.90)	4.30E-03	0.39-1.28	0.43	34	4/7.6	NP

HIV+	ART vs no ART	SIL progression	10	3493/5075	0.66 (0.54-0.81)	0.74 (0.61-0.90)	2.60E-03	0.42-1.31	0.13	60	5/6.9	NP
HIV+	ART vs no SRT	LSIL incidence	3	522/1082	0.66 (0.47-0.92)	0.65 (0.52-0.82)	2.80E-04	0.15-2.91	0.65	0	2/1.8	0.79
HIV+, CD4 count	CD4 <200 VS >500	Clearance of HPV	2	812/1406	0.47 (0.38-0.59)	0.46 (0.37-0.57)	1.11E-12	NA	NA	0	2/1.99	0.91
HIV+, CD4 count	CD4 200-500 VS >500	Clearance of HPV	2	812/1406	0.76 (0.64-0.90)	0.76 (0.64-0.89)	1.00E-03	NA	NA	0	1/1.2	NP
HIV+, CD4 count	CD4 <200 VS >200	Clearance of HPV	1	764/1320	0.57 (0.39-0.83)	0.57 (0.39-0.83)	3.50E-03	NA	NA	100	1/0.9	0.73
HIV infection	HIV+ with CD4>200 vs HIV-	HPV incidence	3	703/1212	5.19(4.3-6.26)	5.76(3.65-9.1)	4.73E-14	0.04-844.24	0.75	62	3/3	0.99
HIV infection	HIV+ with CD4<=200 vs HIV-	HPV incidence	4	327/758	1.41(0.89-2.24)	1.94(1.45-2.6)	1.80E-03	0.36-11.36	0.4	55	2/1.12	0.30
SLE	Yes vs healthy controls	HSIL incidence	7	78/11814	25.45 (9.53-67.98)	9.01 (4.19-19.41)	1.92E-08	1.80-45.26	0.25	23	3/6.8	NP
Rheumatoid arthritis	Yes vs general population	Cervical cancer incidence	15	297/297590	0.86 (0.84-0.89)	0.85 (0.73-1.00)	0.05	0.54-1.34	0.97	63	3/1.0	0.04
Transplant recipient	Yes vs no	Cervical cancer incidence	3	24/188000	2.5(1.33-4.2)	2.31(1.4-3.3)	7.00E-04	0.12-36.68	0.39	0	1/0	0.89
Sexual health												
Bacterial Vaginosis	Yes vs no	HPV infection	12	7921/14883	1.28 (1.16-1.41)	1.43 (1.11-1.84)	5.20E-03	0.69-2.98	0.39	60	3/4.0	NP
Cervicovaginal lactobacilli	LP CSTs vs non LP CST	HR HPV incidence	9	415/890	0.78 (0.45-1.36)	0.64 (0.47-0.88)	5.40E-03	0.40-1.01	0.53	5	1/0.9	0.86

Cervicovaginal lactobacilli	LP CSTs vs non LP CST	CIN incidence	6	264/543	0.70 (0.35-1.38)	0.56 (0.35-0.90)	0.02	0.29-1.09	0.38	0	0/0.7	NP
Cervicovaginal lactobacilli	LP CSTs vs non LP CST	Cervical cancer incidence	3	23/111	0.18 (0.04-0.78)	0.14 (0.04-0.45)	8.90E-04	0-250.3	0.33	0	2/1.8	0.80
Cervicovaginal lactobacilli	LIP CST III vs non LIP CST III	Cervical cancer incidence	2	13/50	0.13 (0.01-2.75)	0.13 (0.02-0.95)	0.04	NA	NA	0	0/1.3	NP
Cervicovaginal lactobacilli	LCP CST I vs non LCP CST I	HR HPV incidence	8	329/762	0.58 (0.24-1.44)	0.53 (0.31-0.90)	0.02	0.25-1.11	0.35	3	1/2.1	NP
Cervicovaginal lactobacilli	LCP CST I vs non LCP CST I	CIN incidence	5	226/454	0.44 (0.19-1.03)	0.51 (0.29-0.91)	0.02	0.20-1.29	0.55	0	0/1.7	NP
Chlamydia tr. infection	Yes vs no	Cervical cancer incidence (CIN3+)	3	832/3473	2.21 (1.84-2.65)	2.21 (1.62-3.03)	6.43E-07	0.09-53.54	0.96	46	2/2.9	NP
Chlamydia tr. infection	Yes vs no	Cervical cancer incidence (CIN1+)	11	3321/9111	1.90 (0.82-4.29)	1.76 (1.03-3.01)	0.04	0.95-3.27	0.17	0	0/9.1	NP
Chlamydia tr. coinfection with HPV	Yes vs no	Cervical cancer incidence (CIN1+)	6	332/4686	3.23 (2.39-4.35)	4.37 (2.75-6.96)	4.59E-10	1.31-14.66	0.35	44	4/4.5	NP
Chlamydia tr. infection	Yes vs no	Cervical cancer incidence (ACC)	4	262/1848	1.46 (0.96-2.23)	1.60 (1.19-2.14)	1.70E-03	0.84-3.04	0.12	0	2/1.3	0.43
HSV type 2 infection	Yes vs no	Cervical cancer incidence (CIN3+)	20	3337/21251	1.10 (0.80-1.40)	1.21 (1.04-1.41)	0.02	1.03-1.43	0.43	0	1/1.7	NP
M.genitalium infection	Yes vs no	HR HPV incidence	5	1355/3337	1.57 (1.03-2.40)	1.55 (1.00-2.40)	0.05	0.56-4.26	0.66	19	1/3.9	NP
M.hominis infection	Yes vs no	Cervical cancer incidence (CIN1+)	6	1412/2037	1.34 (0.84-2.16)	1.48 (1.10-1.98)	9.10E-03	0.98-2.24	0.97	0	1/1.4	NP
U.urealyticum infection	Yes vs no	HPV incidence	8	2388/7071	1.38 (1.21-1.56)	1.57 (1.05-2.34)	0.03	0.48-5.09	0.42	80	5/3.5	0.28

U.parvum infection	Yes vs no	HPV incidence	3	187/692	2.54 (1.48-4.38)	3.03 (1.75-5.26)	8.20E-05	0.01-1011	0.88	55	2/2.7	NP
U.urealyticum infection	Yes vs no	HR HPV incidence	4	2148/6197	1.34 (1.17-1.54)	1.37 (1.05-1.80)	0.02	0.50-3.79	0.56	54	2/2.3	NP
U.urealyticum infection	Yes vs no	Cervical cancer incidence (CIN1+)	9	1865/2731	1.33 (0.90-1.98)	1.51 (1.14-2.01)	4.10E-03	0.77-2.97	0.49	36	4/2.0	0.11
Vaginal microbiota, all ages	LL-VMB vs HL-VMB	HPV incidence	13	4481/13118	2.42 (1.92-3.05)	1.53 (1.16-2.01)	2.30E-03	0.62-3.78	0.44	73	2/10.8	NP
Vaginal dysbiosis	Yes vs no	HPV incidence	7	1719/2992	1.03 (0.97-1.09)	1.14 (1.01-1.28)	0.03	0.86-1.50	0.23	44	3/0.4	<0.01
Vaginal douching	>= 20 years vs less	Cervical cancer incidence (CIN3+)	3	1244/4494	1.95 (1.19-3.03)	1.79 (1.23-2.61)	2.20E-03	0.12-25.87	0.27	6	2/3.0	NP
Vitamin supplementation												
Beta carotene	Highest intake (or serum level) vs lowest	Cervical cancer incidence (CIN1+)	9	1965/6047	0.89 (0.60-1.30)	0.67 (0.51-0.88)	3.80E-03	0.35-1.26	0.45	32	2/1.1	0.35
Carotenoid's intake	Highest vs lowest	Invasive cervical cancer incidence	8	980/2275	0.21 (0.15-0.30)	0.58 (0.37-0.92)	0.02	0.12-2.79	0.20	84	3/8.0	NP
Folate	Highest intake (or serum level) vs lowest	Cervical cancer incidence (CIN1+)	9	1850/5329	1.05 (0.70-1.60)	0.60 (0.41-0.88)	8.40E-03	0.19-1.83	0.09	60	3/0.5	<0.01
Lycopene	Highest intake (or serum level) vs lowest	Cervical cancer incidence (CIN1+)	8	694/2222	0.65 (0.44-0.98)	0.53 (0.37-0.75)	4.00E-04	0.28-1.02	0.04	4	3/2.7	0.80
Serum selenium levels	Highest vs lowest	Cervical cancer incidence	5	353/1206	0.58 (0.37-0.91)	0.55 (0.42-0.73)	2.20E-05	0.35-0.86	0.21	0	3/2.7	0.77

Serum iron levels	Highest vs lowest	Cervical cancer incidence	9	454/880	-0.51(-0.8- -0.22)	-1.45 (-1.98- -0.91)	1.02E-0.7	NA	0.11	93		
Serum zinc levels*	Highest vs lowest	Cervical cancer incidence	12	591/1544	-1.16 (-1.45- -0.87)	-5.34 (-6.84- -3.84)	3.19E-12	NA	<0.01	99	11/10.15	0.50
Serum copper levels*	Highest vs lowest	Cervical cancer incidence	14	747/1761	0.86 (0.58-1.14)	1.35 (0.99-2.59)	0.03	NA	0.72	98	13/12.86	0.89
Vitamin A levels	Highest vs lowest	Invasive cervical cancer incidence	9	761/2320	0.79 (0.54-1.14)	0.60 (0.41-0.89)	0.01	0.18-2.06	0.25	73	4/1.5	0.02
Pregnancy												
Pregnancy	Pregnant vs not pregnant	HPV incidence	14	1474/8044	0.87 (0.66-1.14)	1.42 (1.03-1.96)	0.03	0.42-4.80	0.93	82	6/1.5	<0.01
Age in pregnancy	Per one-year decrease in age at FTP	CIN3 incidence	15	3618/20466	1.04 (1.02-1.07)	1.04 (1.01-1.07)	0.02	0.94-1.14	0.86	59	4/0.9	<0.01
Contraception												
Condom use	Frequently vs rarely	Cervical cancer incidence	5	1648/8150	0.70 (0.50-1.20)	0.61 (0.38-0.98)	0.04	0.13-2.81	0.25	62	1/3.5	NP
Condom use	Ever vs never	CIN2/3 incidence	7	1491/5198	0.60 (0.40-0.90)	0.71 (0.51-0.97)	0.03	0.30-1.67	0.11	50	3/5.5	NP
OC, short duration <5 ys	<8 years since last use vs never	Cervical cancer incidence (CIN3+)	4	5660/20174	1.30 (1.10-1.50)	1.57 (1.16-2.14)	3.80E-03	0.47-5.30	0.30	61	2/2.5	NP
OC, medium and long duration >5 ys	>8 years since last use vs never	Cervical cancer incidence (CIN3+)	4	5520/20091	1.40 (1.00-1.90)	1.50 (1.04-2.15)	0.03	0.53-4.22	0.62	13	2/2.7	NP
OC, long duration >10 years	Yes vs never	Cervical cancer incidence (CIN2+)	3	1279/1415	1.50 (0.80-2.90)	2.55 (1.15-5.64)	0.02	0-9195	0.88	56	1/1.2	NP
Smoking												

Household Air Pollution	Yes vs no	CIN incidence	4	663/2410	3.46 (1.08-11.10)	6.46 (3.12-13.35)	4.85E-07	1.31-31.81	0.38	0	3/4.0	NP
Smoking	Yes vs no	HPV prevalence	5	4451/6402	1.20 (1.00-1.40)	1.24 (1.03-1.50)	0.03	0.66-2.31	0.46	66	3/1.0	0.02
Smoking	Ever vs never	CIN incidence	2	211/239	2.22 (0.49-10.10)	3.72 (1.23-11.24)	0.02	NA	NA	0	1/0.7	0.61
Smoking	Current vs never	Cervical cancer incidence (CIN3+)	19	798/2075	1.68(1.17-2.41)	1.85(1.47-2.33)	2.22E-0.7	1.11-3.09	0.84	0.71	2/3.36	NP
Anthropometric indices												
BMI	Highest vs lowest	Cervical cancer mortality	4	420/3144041	1.15 (0.79-1.70)	1.82 (1.07-3.10)	0.03	0.22-15.20	0.63	65	2/0.5	0.02
No significant association												
Immunocompromise												
HIV infection	HIV+ vs HIV -	Clearance of HPV 18	7	2170/2353	0.91 (0.62-1.33)	0.77 (0.52-1.15)	0.21	0.24-2.48	1.00	61	3/0.6	<0.01
HIV infection	HIV+ vs HIV -	Clearance of prevalent and newly detected HPV 16	2	105/1389	1.61 (0.74-3.51)	0.82 (0.21-3.20)	0.77	NA	NA	81	1/1.1	NP
HIV infection	HIV+ vs HIV -	Clearance of prevalent and newly detected HPV 18	2	74/1389	1.22 (0.85-1.76)	1.04 (0.58-1.86)	0.89	NA	NA	31	0/1.1	NP
HIV+	CD4 <350 VS >350	Clearance of HPV	1	319/486	0.77 (0.53-1.10)	0.77 (0.53-1.11)	0.16	NA	NA	100	0/1.0	NP
HIV+	ART vs no ART	LSIL incidence	2	358/896	0.55 (0.34-0.89)	0.70 (0.39-1.25)	0.23	NA	NA	48	1/1.8	NP

HIV+	ART vs no ART	Cervical cancer incidence (HSIL+)	14	1617/8910	1.01 (0.79-1.30)	0.91 (0.70-1.20)	0.51	0.40-2.08	0.28	57	3/0.7	0.01
HIV+	Yes vs no	LSIL regression	2	325/824	2.61 (1.75-3.89)	1.11 (0.16-7.61)	0.91	NA	NA	84	1/1.7	NP
HIV infection	HIV+ with CD4>200 vs HIV-	HR HPV incidence	2	152/334	1.26 (0.77-2.06)	1.3 (0.82-2.070)	0.26	NA	NA	NA	0/0.3	0.60
HIV infection	HIV+ with CD4<=200 vs HIV-	Clearance of HPV	4	299/468	1.49 (1.09-2.04)	1.186 (0.89 - 1.58)	0.24	0.51-2.77	0.137	0.51-2.77	0/1.19	0.84
HIV infection	HIV+ with CD4>200 vs HIV-	Clearance of HR HPV	2	153/214	1.33 (0.92-1.93)	1.18 (0.8-1.74)	0.40	NA	NA	NA	0/0.4	0.50
Genitourinary health												
Candida albicans	Yes vs no	CIN incidence	2	444/6459	0.8(0.32-1.97)	0.99(0.48-2.1)	0.99	NA	NA	NA	0/0.72	0.30
Cervicovaginal lactobacilli	LIP CST III vs non LIP CST III	HR HPV incidence	8	329/805	0.85 (0.48-1.51)	0.95 (0.68-1.33)	0.78	0.63-1.45	0.50	0	0/0.6	NP
Cervicovaginal lactobacilli	LIPCST III vs non LIP CST III	CIN incidence	5	226/454	1.25 (0.62-2.53)	1.01 (0.61-1.68)	0.96	0.44-2.31	0.71	0	0/0.4	NP
Cervicovaginal lactobacilli	LCPCST I vs non LCP CST I	Cervical cancer incidence	2	13/50	0.25 (0.02-2.65)	0.16 (0.03-1.00)	0.050	NA	NA	0	0/0.7	NP
M.hominis infection	Yes vs no	HPV incidence	3	154/514	1.52 (0.74-3.14)	1.65 (0.93-2.92)	0.08	0.04-66.38	0.77	0	0/0.8	NP
M.genitalium infection	Yes vs no	Cervical cancer incidence (CIN1+)	5	1243/2415	0.80 (0.37-1.72)	0.80 (0.43-1.51)	0.50	0.19-3.45	0.95	20	0/1.0	NP
Contraception												
Condom use	Frequently vs rarely	HPV incidence	6	1144/3292	1.50 (1.10-2.00)	0.94 (0.59-1.49)	0.78	0.21-4.24	0.102	76	2/3.2	NP

Condom use	Ever vs never	CIN incidence	4	1560/6898	1.80 (1.40-2.40)	1.31 (0.93-1.82)	0.12	0.34-5.03	0.57	62	1/3.8	NP
COCP, 2-9 years since last use	Risk per year of use	Cervical cancer incidence	18	1190/4044	1.05 (1.03-1.08)	1.02 (1.00-1.04)	0.060	0.96-1.08	0.21	40	4/1.0	<0.01
COCP, 10+ years since last use	Risk per year of use	Cervical cancer incidence	18	1043/4537	1.00 (0.96-1.04)	0.98 (0.95-1.01)	0.19	0.88-1.09	0.26	58	4/0.9	<0.01
Injectable contraceptives	<5 years of use vs never	Invasive cervical cancer incidence	3	3147/14427	1.10 (0.90-1.30)	0.90 (0.56-1.45)	0.66	0-218.60	0.61	73	1/0.9	0.91
Injectable contraceptives	>5 years of use vs never	Cervical cancer incidence (CIN3+)	2	2504/12372	1.20 (0.90-1.50)	1.49 (0.79-2.80)	0.21	NA	NA	55	1/1.6	NP
IUD device use	Use vs no use	HPV incidence	16	2093/15382	1.09 (0.80-1.47)	0.98 (0.86-1.12)	0.77	0.80-1.20	0.23	6	0/1.3	NP
OC, short duration <5 years	>8 years since last use vs never	Cervical cancer incidence (CIN3+)	4	5647/20369	1.20 (1.00-1.30)	0.98 (0.75-1.27)	0.87	0.36-2.67	<0.01	52	1/1.8	NP
Oral contraceptive	<5 years user vs never	Cervical cancer incidence (CIN3+)	5	1624/2544	0.70 (0.50-1.00)	0.95 (0.69-1.31)	0.75	0.45-2.03	0.09	22	1/1.7	NP
Oral contraceptive	5-9 years user vs never	Cervical cancer incidence (CIN2+)	5	1464/2132	1.20 (0.70-2.20)	1.32 (0.79-2.21)	0.28	0.30-5.93	0.76	49	1/0.6	0.59
Oral contraceptive	Ever vs never	Cervical cancer incidence (CIN3+)	16	7433/15619	0.68 (0.55-0.84)	1.12 (0.90-1.38)	0.31	0.48-2.62	0.35	83	7/10.9	NP
Oral contraceptives	<5 years user vs never	Invasive cervical cancer incidence	20	9149/32642	1.20 (1.10-1.30)	1.15 (1.00-1.32)	0.053	0.71-1.84	0.98	60	4/6.7	NP
Pregnancy												
Parity	Per increase of 1 full term pregnancy	CIN3 incidence	15	4620/25024	1.05 (1.01-1.06)	1.08 (1.00-1.16)	0.061	0.83-1.39	0.64	71	4/1.0	<0.01

IVF	Yes vs no	Cervical cancer incidence	4	33735/1420192	0.61 (0.52-0.71)	1.07 (0.45-2.55)	0.87	0.03-34.71	0.29	69	2/1.4	0.56
Smoking												
Smoking	Ever vs Never	Cervical cancer incidence	7	1401/1596	1.80 (0.81-4.04)	1.62 (0.91-2.89)	0.10	0.48-5.47	0.89	23	1/2.5	NP
Smoking	Past vs never	Cervical adenocarcinoma incidence	10	1417/16709	0.75 (0.53-1.07)	0.92 (0.75-1.34)	0.45	0.72-1.18	0.48	0	0/4.0	NP
Smoking	Current vs never	Cervical adenocarcinoma incidence	10	1417/15292	0.81 (0.58-1.13)	0.90 (0.72-1.12)	0.34	0.59-1.39	0.60	20	0/2.5	NP
Smoking	Past vs never	Cervical cancer incidence (CIN3+)	19	798/2075	1.83(1.19-2.82)	1.53(1.12-2.1)	0.08	0.73-3.20	0.13	0.73-3.2	1/3.61	NP
Anthropometric indices												
Height	Per 5 cm increase	Cervical cancer incidence	4	1182/989044	1.01 (0.92-1.11)	1.02 (0.87-1.20)	0.77	0.57-1.83	0.76	44	0/0.2	NP
BMI	Per 5KG/M2 increase	Cervical cancer incidence	9	5035/9680798	0.98 (0.95-1.02)	1.02 (0.97-1.07)	0.44	0.89-1.17	0.79	72	2/0.5	0.04
BMI	Per 5KG/M2 increase	Cervical cancer incidence	7	4837/7870866	0.98 (0.95-1.02)	1.01 (0.96-1.06)	0.67	0.87-1.17	0.96	68	1/0.4	0.36
BMI	Highest vs lowest	Cervical cancer incidence	7	4837/7870866	1.02 (0.80-1.31)	1.13 (0.93-1.38)	0.23	0.71-1.79	0.94	33	1/0.4	0.35
BMI	Per 5KG/M2 increase o	Cervical cancer mortality	3	307/3032453	1.04 (0.88-1.24)	1.02 (0.83-1.25)	0.84	0.11-9.52	0.86	65	0/0.2	NP
Medical history												
GDM	Yes vs no	Cervical cancer incidence	3	63694/1163875	0.9(0.65-1.26)	1.02(0.81-1.28)	0.84	0.23-4.49	0.04	0.04	0/3	NP
Vitamin supplementation												

Retinoid use	Yes vs no	Complete or partial regression of CIN2/3 at 3-12 months	3	186/314	0.92 (0.39-2.16)	0.98 (0.56-1.72)	0.95	0.03-36.66	0.16	0	0/0.2	NP
Retinoid use	Yes vs no	Complete regression of CIN2/3 at 9-27 months	2	160/246	0.71 (0.41-1.24)	0.79 (0.51-1.23)	0.29	NA	NA	0	0/0.5	NP
Retinoid use	Yes vs no	Complete regression of CIN2 at 9-27 months	2	116/144	0.50 (0.25-1.02)	0.81 (0.29-2.28)	0.69	NA	NA	69	0/1.1	NP
Vegetable intake	Highest vs lowest intake	Cervical dysplasia	5	1916/303706	0.98(0.77-1.25)	0.79(0.56-1.12)	0.19	0.32-1.95	0.19	31	0/0.27	0.59
Vegetable intake	Highest vs lowest intake	Cervical dysplasia	3	515	1.03(0.55-1.92)	1.14(0.74-1.76)	0.55	0.07-19.02	0.1	0	0/0.16	0.68
Vegetable intake	Highest vs lowest intake	Cervical cancer incidence	3	957/2967	0.97(0.97-0.71)	0.9(0.68-1.2)	0.47	0.15-5.44	0.23	0	0/0.17	0.67
Fruit intake	Highest vs lowest intake	Cervical dysplasia	3	1191/300924	1.06(0.85-1.32)	0.54(0.2-1.41)	0.21	0-5573	0.24	83	1/0.28	0.16
Fruit intake	Highest vs lowest intake	Cervical dysplasia	3	515/1317	0.97(0.93-1.02)	0.89(0.69-1.15)	0.38	0.07-10.64	0.13	21	0/0.16	0.68
Fruit intake	Highest vs lowest intake	Cervical cancer incidence	2	856/2720	0.86(0.61-1.22)	0.87(0.64-1.2)	0.4	NA	NA	NA	0/0.6	0.40

* Number of studies

^b Sum of all study cohorts in the meta-analysis

^c Relative risk and 95% confidence interval of largest study (smallest standard error) in each meta-analysis

^d Random effects refer to summary risk ratio (95% confidence interval) using the random-effects model

^e P value of summary random effects estimate

^f P value from the Egger's regression asymmetry test (P <0.10)

^g Expected number of statistically significant studies using the point estimate of the largest study (smallest standard error) as the plausible effect size

^h Observed/Expected number of statistically significant studies

ⁱP-value of the excess statistical significance test ($P < 0.10$)

All statistical tests were two-sided

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; CC, cervical cancer; CD4, cluster of differentiation 4; Chlamydia tr: Chlamydia trachomatis; CI, confidence interval; CIN: cervical intra-epithelial neoplasia; COC, combined oral contraceptive; CST, community state types; EBV, Epstein-Barr virus; FTP: full term pregnancy, HIV (+ or -), human immunodeficiency virus (positive or negative); HL-VMB, high lactobacillus vaginal microbiota; (HR) HPV, (high risk) human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; IBD, inflammatory bowel disease; ICC, invasive cervical cancer; IUD, intrauterine device; IVF, in vitro fertilisation; LL-VMB, low lactobacillus vaginal microbiota; LSIL, low-grade squamous intraepithelial lesion; LCP: Lactobacillus crispatus predominant; LIP: Lactobacillus iners predominant; LP: lactobacillus predominant; NA, not available; NP, not pertinent (because the estimated is larger than the observed, and there is no evidence of excess statistical significance based on the assumption made for the plausible effect size); OC: oral contraception; SCC, squamous cell carcinoma; SIL, squamous intraepithelial lesion; SLE, systemic lupus erythematosus, VMB: vaginal microbiota

Appendix 4. ICD9 and 10 codes from UK Biobank field IDs 40006 (ICD10), 40013 (ICD9) and linkage to the Hospital Episode Statistics used to identify cases and exclude controls

Inclusion criteria for cases

ICD9		ICD10	
180.0	Malignant neoplasm of endocervix	C53	
180.1	Malignant neoplasm of exocervix	C53.1	
180.8	Malignant neoplasm of other specified sites of cervix	C53.9	
180.9	Malignant neoplasm of cervix uteri, unspecified site	D06.0	CIS endocervix
233.1	Carcinoma in situ (CIN3)	D06.1	CIS ectocervix
		D06.7	CIS other parts of cervix
		D06.9	CIS cervix, unspecified

Exclusion criteria for controls (plus not a case)

ICD10	
N87.0	Mild dysplasia
N87.1	Moderate dysplasia
N87.2	Severe dysplasia

Abbreviations: ICD9-10: International classification of diseases versions 9 and 10

Appendix 5: Exposure-outcome pairs included in the main analysis, cohorts only.

Exposure	Exposure contrast	Outcome	MR
1. Age in pregnancy*	Per one-year decrease in age at full term pregnancy	CIN3	OK
2. Alcohol intake	Highest vs lowest	CC	OK
3. BMI	Highest vs lower	CC mortality	OK
4. Candida Albicans	Yes vs no	CIN incidence	Weak instrument
5. Chlamydia trachomatis	Yes vs no	CIN1+	No GWAS available
6. Contraception, oral	Short duration <5 years user vs Never	CIN3+	Weak instrument
7. Environmental tobacco smoke exposure	Increased vs lower	CIN2+ incidence	OK
8. Fruit intake	Highest vs lowest (no HPV adjustment)	Cervical dysplasia	Weak instrument
9. GDM	Yes vs no	cervical cancer	OK
10. Height	Height per 5 cm increase	CC	OK
11. HIV	positive vs negative	hrHPV incidence	No female-specific GWAS
12. HIV positive	On ART vs non-ART users	SIL-CIN regression	No female-specific GWAS
13. IBD on immunosuppression*	IBD on immunosuppression vs health controls	High grade dysplasia/cancer incidence	OK
14. IVF	yes vs no	CC	No GWAS available
15. Parity	Per increase of 1 full term pregnancy	CIN3	OK
16. Retinoid use	Yes vs no	complete regression of CIN2 at 9-27 months	No GWAS available
17. Sexual partners*	Multiple vs few partners	CIN	OK
18. Smoking*	Yes vs no	HPV incidence	OK
19. Pregnant	Yes vs no	HPV incidence	No GWAS available
20. Rheumatoid arthritis*	Yes vs no	CC incidence	OK
21. Vaginosis, BV	Yes vs no	CIN prevalence	No GWAS available
22. Vegetable intake	Highest vs lowest (no HPV adjustment)	cervical dysplasia	Weak instrument

Appendix 6. Replication lookup of UKB SNPS derived from conditional logistic regression in the FInnGen r5 public data.

Phenotype (UKB equivalent)	CHR	pos	alt	rsids	nearest_genes	pval	beta	OR	sebeta	maf
Malignant neoplasm of cervix uteri (ICC)	2	113230915	A	rs10175462	<i>PAX8</i>	0.01193	-0.0899	0.91402258	0.0358	0.4305
Malignant neoplasm of cervix uteri (ICC)	2	113235223	T	rs35724515	<i>PAX8</i>	0.01401	-0.088	0.91576088	0.0358	0.4304
Malignant neoplasm of cervix uteri (ICC)	5	1347013	T	rs27069	<i>CLPTMIL</i>	2.54E-07	-0.1864	0.82994155	0.0362	0.4156
Malignant neoplasm of cervix uteri (ICC)	6	32631294	A	rs9272050	<i>HLA-DQA1</i>	7.90E-08	-0.1951	0.82275238	0.0363	0.5592
Malignant neoplasm of cervix uteri (ICC)	12	109208011	G	rs117960705	<i>ACACB</i>	0.8257	-0.0325	0.96802245	0.1475	0.01498
Carcinoma in situ of cervix uteri (CIN3)	2	113230915	A	rs10175462	<i>PAX8</i>	0.06941	-0.1508	0.86001969	0.083	0.4305
Carcinoma in situ of cervix uteri (CIN3)	2	113235223	T	rs35724515	<i>PAX8</i>	0.07467	-0.1483	0.86217242	0.0832	0.4304
Carcinoma in situ of cervix uteri (CIN3)	5	1347013	T	rs27069	<i>CLPTMIL</i>	0.03929	-0.1728	0.84130586	0.0838	0.4156
Carcinoma in situ of cervix uteri (CIN3)	6	32631294	A	rs9272050	<i>HLA-DQA1</i>	0.0004878	-0.2932	0.74587295	0.0841	0.5592
Carcinoma in situ of cervix uteri (CIN3)	12	109208011	G	rs117960705	<i>ACACB</i>	0.3017	-0.3543	0.70166444	0.343	0.01498
Dysplasia of cervi uteri (Cervical dysplasia)	2	113230915	A	rs10175462	<i>PAX8</i>	0.0001735	-0.0865	0.91713555	0.023	0.4322
Dysplasia of cervi uteri (Cervical dysplasia)	2	113235223	T	rs35724515	<i>PAX8</i>	0.0002372	-0.0848	0.91869601	0.0231	0.432
Dysplasia of cervi uteri (Cervical dysplasia)	5	1347013	T	rs27069	<i>CLPTMIL</i>	0.0004168	-0.0823	0.92099562	0.0233	0.4133
Dysplasia of cervi uteri (Cervical dysplasia)	6	32631294	A	rs9272050	<i>HLA-DQA1</i>	5.33E-10	-0.1454	0.86467635	0.0234	0.559
Dysplasia of cervi uteri (Cervical dysplasia)	12	109208011	G	rs117960705	<i>ACACB</i>	0.1373	0.141	1.15142465	0.0949	0.01508

Abbreviations: CHR: chromosome

Appendix 7: Mendelian randomisation sensitivity analyses**A) Weighted-Median**

Exposure	OR	SE	CI-lower	CI-upper	P-value
Lifetime smoking index	1.992	0.557	1.152	3.445	1.36E-02
Number of sexual partners	1.945	0.399	1.301	2.906	1.17E-03
Rheumatoid arthritis	1.169	0.037	1.099	1.244	6.77E-07
Age at first pregnancy	0.813	0.087	0.659	1.004	5.44E-02
Systemic lupus erythematosus	0.986	0.016	0.954	1.019	3.94E-01
Inflammatory bowel disease	1.023	0.032	0.962	1.088	4.67E-01

B) MR-PRESSO

Exposure	Analysis	OR	SE	P-value
Lifetime smoking index	Raw	2.455734	0.50928208	3.02E-05
	Corrected	2.3140887	0.45967255	4.63E-05
Number of sexual partners	Raw	1.9476112	0.29985566	4.06E-05
	Corrected	NA	NA	NA
Rheumatoid arthritis	Raw	1.0982851	0.02744145	4.24E-04
	Corrected	1.0775831	0.02495215	2.17E-03
Age at first pregnancy	Raw	0.7995042	0.04995509	1.59E-02
	Corrected	NA	NA	NA
Systemic lupus erythematosus	Raw	0.9866571	0.01486467	3.77E-01
	Corrected	1.0009699	0.01101943	9.30E-01
Inflammatory bowel disease	Raw	0.9845036	0.0234237	5.13E-01
	Corrected	NA	NA	NA

Appendix 8: Search strategy for HPV DNA methylation review

With the following search criteria, studies were identified by two independent reviewers (SB, AM) and discrepancies resolved by a third author (IK) from MEDLINE, EMBASE and CENTRAL:

1. Uterine Cervical Neoplasms/
2. Cervical Intraepithelial Neoplasia/
3. CIN*.mp.
4. exp Uterine Cervical Dysplasia/
5. (cervi* adj5 (cancer* or tumor* or tumour* or malignan* or neoplas* or carcinoma* or adenocarcinoma* or precancer* or pre-cancer* or dysplasia or squamous or CIN* or HSIL or LSIL or ASCUS)).mp.
6. 1 or 2 or 3 or 4 or 5
7. exp papillomaviridae/
8. exp Papillomavirus Infections/
9. (HPV* or hpv* or human papilloma* or papilloma*).mp.
10. 7 or 8 or 9
11. exp methylation/
12. exp Epigenesis, Genetic/
13. Epigenomics/
14. (methylome* or epigenetic* or epigenome*).mp.
15. (bisulphite* or bisulfite* or pyrosequenc* or methylation* or mass spectrometry or mass-spectrometry or EpiTYPER).mp.
16. (next generation sequencing or NGS or high throughput sequencing or illumina array* or illumina sequenc* or solexa).mp.
17. (microsphere-based suspension array* or microsphere-based suspension array* or luminex Cmap or luminex C-map or luminex C map).mp.
18. (suspension adj5 (array* or technology*)).mp.
20. 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18
21. 6 and 10 and 20
22. (animals not (humans and animals)).sh.

23. 21 not 22

Appendix 9: Protocol for HPV DNA Methylation review

The full research protocol can be found at Open science Framework.⁴⁶⁹

Appendix 10: Characteristics of the studies for HPV DNA methylation review

Author, year	Country	Population	Material	Method (Index Test)	HPV subtype	CpG Sites	Sample (N)	Reference Standard	Outcome Measures
Badal 2003	New Mexico, Brazil	HPV16+ screening population	CEC (N), FFPE biopsy tissue (CIN, ICC)	MSRE	16	LCR and E2BS (11 CpGs)	81: N (25), CIN1 (13), CIN3 (10), ICC (33)	C (N) & H (CIN & ICC)	Proportion of cases methylated per grade
Bhattacharjee 2006	India	Married women attending family planning and cancer referral centre	CEC (N), biopsy tissue (ICC)	MSRE	16	LCR, E6	72: N (15), ICC (57)	C (N) & H (ICC)	Proportion of cases methylated per grade
Brandsma 2014	USA, Senegal	USA: HPV 16+ women attending screening, Senegal: never-before screened attending clinic	CEC (ND)	BS	16	E5, L1, L2 (12 CpGs)	33: CIN1 (16), CIN2 (7), CIN3 (5), ICC (5)	C & H (all)	Mean and Median percentage methylation per CpG site, Diagnostic accuracy of methylation in predicting CIN2, CIN3, ICC, $\geq$ CIN2
Brentnall 2014	England	Women with abnormal cytology referred to colposcopy	CEC (all)	Pyro	16, 18, 31	L1, L2, LCR	1493: $\leq$ CIN1 (937), CIN2/3 (556)	C (all) & H (clinically indicated)	Proportion of cases methylated per grade, Mean percentage methylation per CpG site, Diagnostic accuracy of methylation in predicting CIN2/3

Brentnall 2015	England	Women with abnormal cytology referred to colposcopy	CEC (CIN)	Pyro	33	L1, L2	1493: ≤CIN1 (937), CIN2/3 (556)	C (all) & H clinically indicated)	Proportion of cases methylated per grade, Diagnostic accuracy of methylation in predicting CIN2/3
Bryant 2014	Wales	HPV+ screening population and Cancers	CEC (N, Severe dyskaryosis) and tissue biopsy (ICC)	Pyro	16	LCR, L1, L2, E2	51: N (17), Severe dyskaryosis (20), ICC (24)	C (N/severe dyskaryosis) & H (ICC)	Median percentage methylation per CpG site, Diagnostic accuracy of methylation in predicting severe dyskaryosis* (*cancer not included)
Bryant 2015	Wales	HPV16+ attending colposcopy	CEC (N, CIN)	Pyro	16	E2, L1, L2 (12 CpGs)	234: Cytology - N (22), Borderline (63), Mild (54), Moderate (39), Severe (52), Glandular neoplasia (2), unknown (2). (Histopathology (200) - N (21), CIN1 (34), CIN2 (51), CIN3 (92), ACC in situ (2))	C (all) & H (N=200/234)	Median percentage methylation per CpG site, Diagnostic accuracy of methylation in predicting ≥CIN2 and ≥CIN3
Chaiwongkot 2013	Thailand, Germany	Women with abnormal cytology referred to hospital	FFPE (CIN, ICC)	Pyro	16	LCR (10 CpGs)	51: <CIN2/3 (18), ICC (33)	H (all)	Methylation levels for an individual HPV DNA molecule at multiple CpG Sites (methyl-haplotype), methylation levels in samples with integrated genomes vs. episomal
Chaiwongkot 2017	India	HPV+ screening cohort	Cervical swab (all)	Pyro	16	L1	101: Normal (15), CIN1 (21), CIN2 (29), CIN3(4) ICC (32)	C&H (all)	Mean percentage methylation per CpG site

Clarke 2018	USA	HPV+ screening population	CEC	NGS	12 HPV types	L1 and L2	659: 360 Normal and ASCUS, 299 CIN3 and ICC (AIS)	C & H (all)	Median percentage methylation per CpG site.
Ding 2009	Taiwan	HPV16+ with confirmed CIN and ICC	CEC (N, CIN), tissue biopsy (ICC)	BS	16	LCR	53: LSIL (17), HSIL (21), ICC (15)	C & H (all)	Proportion of cases methylated per grade
Dutta 2015	India	HPV16+ screening population and ICC referred to hospital	Cervical swabs (N, LSIL, HSIL) Fresh frozen tissue biopsy (ICC)	MSRE	16	LCR	611: N (89), LSIL (4), HSIL (24), ICC (98)	C (CIN) & H (ICC)	Proportion of cases methylated per grade
Frimer 2015	Costa Rica	HPV16+ screening population	CEC (all)	NGS	16	E6, E2, L1, L2 (21 CpGs)	65: N (21), CIN3 (31), ICC (13), longitudinal (13)	C (all) & H (ND)	Mean percentage methylation per CpG site, mean percentage methylation in HPV persistence vs. clearance
Gasperov 2015	Croatia	HPV 16+ screening population and confirmed ICC receiving surgical treatment	CEC (all)	MSP	16, 18, human	LCR, L1 (19 CpGs)	12 HPV 16+: N (4), LSIL (2) HSIL (4), ICC (2). 22 HPV18+: LSIL/CIN1 (9), HSIL/CIN2 (6), HSIL/CIN3 (6), ICC (1)	C (all) & H (ICC)	Proportion of cases methylated per grade
Hong 2008	China	HPV 16+ screening population and confirmed ICC referred to hospital	CEC (all)	Pyro	16	LCR	70: N (14), CIN1/2 (17), CIN3 (13), ICC (26)	C (N) & H (CIN, ICC)	Proportion of cases methylated per grade
Hsu 2017	Taiwan	HPV+ screening cohort	CEC (all)	Pyro	16, 18, 52, 58	L1	147: N (28), CIN1 (45), CIN2 (13), CIN3/CIS (61)	C & H (all)	Mean percentage methylation per CpG site

Hublarova 2009	Czech Republic	HPV 16 + screening population and ICC referred to hospital	CEC (all)	MSRE	16	LCR, E2BS	141: N (21), CIN1 (8), <CIN2/3 (89), ICC (23)	C & H (all)	Proportion of cases methylated per grade
Jacquín 2013	France	Not defined	CEC (all)	Pyro and HRM-PCR	16	LCR (E2BS1, E2BS2, SP1)	119: N (37), LSIL (30), HSIL (35), ICC (17)	C (all) & H (clinically indicated)	Mean percentage methylation per CpG site, Proportion of cases methylated per grade
Kalantari 2014	California	Consecutive patients attending colposcopy clinic	CEC (all)	BS	16, 18, 31, 45	L1, L2	63: N (6), ASCUS (9), LSIL/CIN1 (14), HSIL/<CIN2/3 (21), ICC (13)	C (all) & H (clinically indicated)	Proportion of cases methylated per grade (in triplicate), Diagnostic accuracy of methylation in predicting ICC
Kottaridi 2017	Greece, England	HPV+ women attending gynaecology clinics	CEC (all)	Pyro	16	L1 (12 CpGs)	150: N (12), CIN1 (23), CIN2 (45), CIN3 (61), ICC (9)	C & H (all)	Mean percentage methylation per CpG site, Diagnostic accuracy of methylation in predicting $\geq$ CIN2, $\geq$ CIN3 & ICC
Leung 2015	Hong Kong	HPV+ screening population	Vaginal swabs/Paraffin blocks (N, ASCUS, LSIL, HSIL) and FFPE (ICC)	Pyro	16, 18	LCR (E2BS1,2 & 4)	137: N (15), Low-grade CIN/ASCUS (28), High-grade CIN (42) ICC (52)	C & H (ND)	Mean percentage methylation per CpG site
Liu 2017	USA	Women with CIN or ICC. Otherwise not defined	CEC and Fresh Frozen Tissue	NGS and Pyrosequencing	13 HPV types	Whole genome	NGS 60: CIN1 (2), CIN2 (2), CIN3 (20), ICC (37). Pyro 111: (CIN3 (61), ICC (50))	C & H (all)	Mean percentage methylation per CpG site Diagnostic Accuracy of methylation in predicting ICC
Lorincz 2013	Wales	HPV16+ screening population	CEC (all)	Pyro	16	E6, L1, L2, LCR	Study 1 (association with disease grade) (82):	C (all) & H (clinically indicated)	Mean percentage methylation per CpG site, Mean percentage methylation in HPV persistence vs. clearance

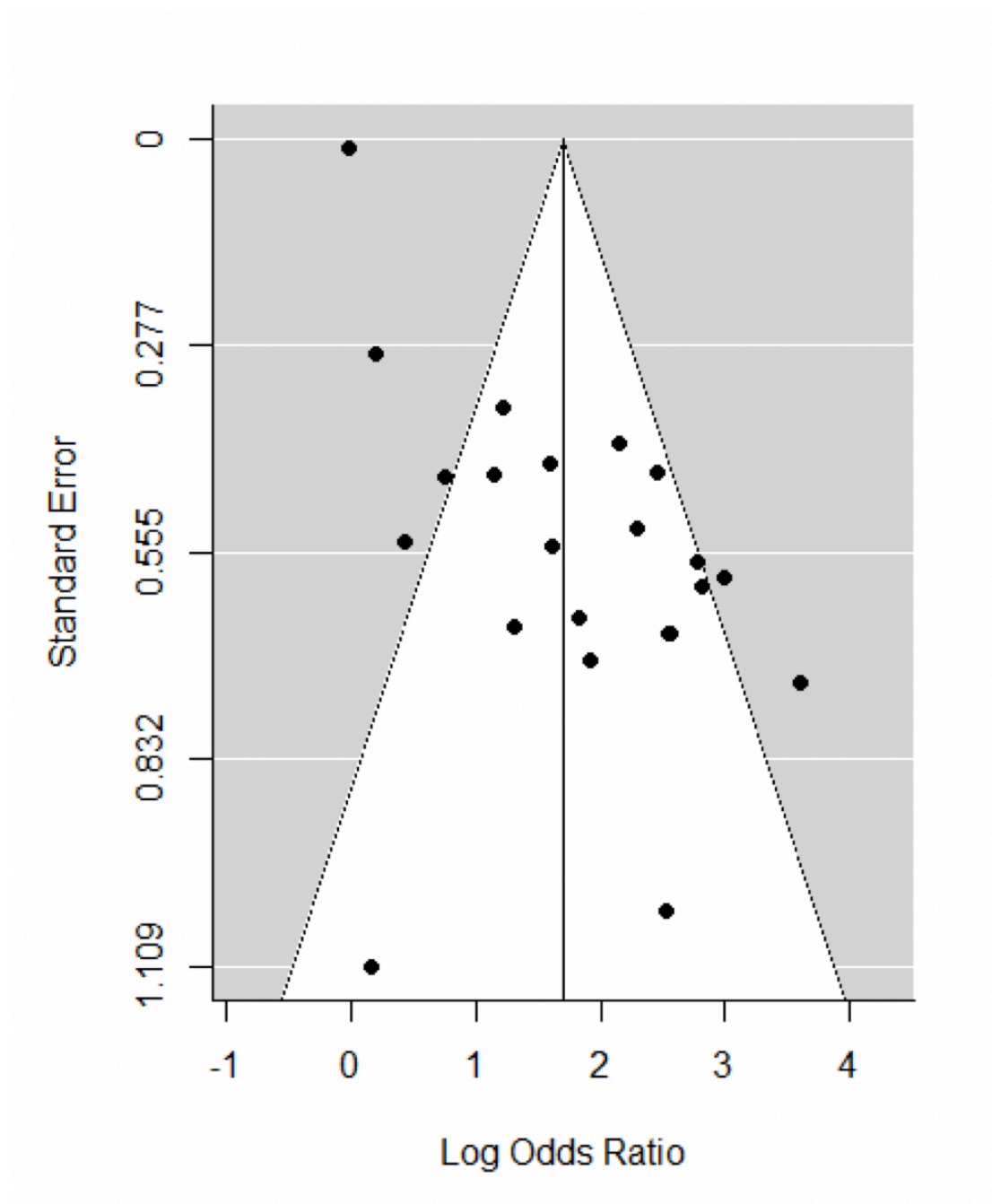
Lorincz 2016	England	HPV+ screening population	CEC (all)	Pyro	16, 18, 31, 33, human	L1, L2	<CIN (67), CIN2 (10), CIN3 (15). Study 2 (persistence): HPV persistence at 6-months (73) 99; HPV 16 (87): $\leq$ CIN2 (66) $\geq$ CIN2 (21)	C & H (all)	at 6 months, Diagnostic accuracy of predicting CIN2/3 & persistence of HPV16 at 6-months Diagnostic accuracy of methylation in predicting $\geq$ CIN2, $\geq$ CIN3 12 months from an abnormal smear
Louvanto 2015	Canada	Cases: Abnormal cytology referred to colposcopy Controls: matched age controls attending screening at same time period. 12 additional HPV positive CIN1 samples from a separate study	CEC (all)	Pyro	16	L1 (2 CpGs)	210: N (36), CIN1 (21), CIN2 (47), CIN3 (47), SCC (31), ADC (28)	C & H (all)	Methylation levels for an individual HPV DNA molecule at multiple CpG Sites (methyl-haplotype), Diagnostic accuracy of methylation in predicting $\geq$ CIN2
Marongiu 2014	England	HPV16+ screening population	CEC (all)	Pyro (16), BS (18, 31, 45)	16, 18, 31, 45	LCR, E6, L2, L1. HPV16 (20 CpGs) HPV18 (29 CpGs) HPV31 (4 CpGs) HPV45 (12 CpGs)	79 HPV16+, 18 HPV18+, 50 HPV31+, 18 HPV45+. (LSIL (67), HSIL (125))	C (all) & H (approx. 20% of all samples)	Median percentage methylation per CpG site, Diagnostic accuracy of methylation in predicting HSIL

Mirabello 2012	Costa Rica	HPV16+ screening population	Cervical swab (all)	Pyro	16	LCR, L1 (20 CpGs)	Study 1 (Diagnostic cohort) (99): HPV16 clearance <2 years (34), HPV16 persistence 2+ years (35), CIN3 (30). Study 2 (Pre-diagnostic cohort) (89): HPV16 clearance (34), HPV16 persistence (35), CIN3 (20)	C & H (clinically indicated)	Mean percentage methylation per CpG site, Mean percentage methylation in HPV persistence vs clearance
Mirabello 2013	Costa Rica	HPV16+ screening population	Cervical swab (all)	Pyro	16	LCR, E6, E7, L1 (20 CpGs)	149: N (92), ≥CIN2 (57).	C & H (clinically indicated)	Mean percentage methylation per CpG site
Mirabello 2015	USA	HPV 16+ screening population and confirmed ICC referred to hospital	CEC (all)	Pyro and NGS	16`	E6, E2, L2, L1 (18 CpGs)	99: N (40) CIN3 (59)	C (all) & H (CIN2/CIN3/ICC)	Median percentage methylation per CpG site, Diagnostic accuracy of methylation in predicting CIN2-3
Murakami 2013	Japan	HPV+ screening population	CEC (all)	BS	52, 58	HPV52: LCR, L1 (7 CpGs). HPV58: LCR, L1 (6 CpGs)	HPV52+ (54): <CIN1 (8), CIN1 (9), CIN2 (15), CIN3 (22) HPV58+ (32): <CIN1 (1), CIN1 (10), CIN2 (9), CIN3 (12). Persistence HPV52+ 12-months (15):	C (all) & H (clinically indicated)	Proportion of cases methylated per grade

Niyazi 2017	China	HPV+ women attending screening	CEC (all)	Pyro	16	L1 (13 CpGs)	CIN1 (6) CIN2 (9) 145: N (51) (transient HPV infection (32), persistent HPV infection at 12-months (21)), CIN 1 (21), CIN2-3 (33) ICC (38)	C & H (all)	Median percentage methylation per CpG site, Diagnostic accuracy of methylation in predicting $\geq$ CIN2 and persistent HPV infection, Median percentage methylation in HPV infection persistence vs clearance
Patel 2012	USA	HPV16+ screening population	CEC (all)	Pyro	16	LCR, E6, L1 (10 CpGs)	89: N (15), ASCUS (36), LSIL (24), HSIL (12), AGC (2)	C (all)	Proportion of cases methylated per grade
Piyathilake 2011	USA	HPV16+ screening population	CEC (all)	Pyro	16	LCR (6 CpGs)	75: $\leq$ CIN1 (45) $\geq$ CIN2 (30)	C & H (all)	Mean percentage methylation per CpG site
Piyathilake 2014	USA	HPV16+ screening population	CEC (all)	Pyro	16	LCR (6 CpGs)	309: $\leq$ CIN1 (225), CIN2 (34), CIN3 (50)	C (all) & H (clinically indicated)	Median percentage methylation per CpG site
Qiu 2015 (1)	China	Cytopathology samples sent to a university hospital	CEC (all)	MS-HRM	16	L1	114: CIN<2 (42) $\geq$ CIN2 (72)	C (all) & H (clinically indicated)	Proportion of samples methylated per disease grade, Diagnostic accuracy of methylation in predicting $\geq$ CIN2
Qiu 2015 (2)	China	Cytopathology samples sent to a university hospital	CEC (all)	MS-HRM	16	L1	81: N (16), CIN1 (16), CIN2 (16), CIN3 (26), ICC (7)	C & H (all)	Proportion of samples methylated per disease grade, Diagnostic accuracy of methylation in predicting $\geq$ CIN2
Simanaviciene 2015	Lithuania	Women with abnormal cytology or histology plus N controls from a	CEC (all)	BS	16, 18, 51	LCR, L1 (17 CpGs)	157: N (29), CIN1 (10), CIN2 (17), CIN3/CIS (70), ICC (31)	C & H (all)	Proportion of cases methylated per grade

		screening population.							
Snellenberg 2012	Netherla nds	HPV16+ screening population	CEC (N, CIN3) and FFPE (CIN3, ICC)	Luminex C-map	16	LCR (E2BS1, E2BS3, E2BS4)	65: N (17) CIN3 (19), ICC (29)	C (N, CIN3) & H (all)	Median percentage methylation per CpG Site
Sun 2011	USA	HPV16+ screening population	CEC (all)	Pyro and EpiTYP ER	16	LCR, L1	85: N/LSIL/CIN1 (46) CIN2 (12) CIN3/ICC (27)	C (N, LSIL) & H (CIN1, CIN2, CIN3, ICC)	Proportion of cases methylated per grade
Vasilijevic 2014	England	Women referred to colposcopy with mild dyskaryosis or worse	CEC (all)	Pyro	18, 31, 33	HPV18 (L2, L1, LCR, E6) HPV31 (L2, L1, LCR) HPV33 (L2, L1, LCR)	205 HPV18+, 206 HPV31+, 117HPV33+: ≤CIN1 (302) vs. ≥CIN2 (226)	C (all) & H (clinically indicated & all in persistence study arm samples)	Median percentage methylation per CpG site
Wang 2017	China	HPV+ women	CEC and Fresh Frozen Tissue	BS	16	L1 and LCR (18 CpGs)	122: N/LSIL (37), HSIL (35), ICC (37)	C & H (all)	Proportion of cases methylated per grade and Diagnostic accuracy of methylation in predicting HSIL (AUC only)
Wentzensen 2012	USA	HPV18, 31, 45+ women age >21 attending screening and women age <30 with ASCUS	CEC (all)	Pyro	18, 31, 45	Whole genome (focusing on L1, L2 and E2 but >1 site per gene)	188: ≤CIN1/ASCUS (96), CIN3 (92)	C & H (all)	Median percentage methylation per CpG Site
Xi 2011	USA	HPV16+ screening population	CEC (all)	BS	16	LCR (11 CpGs)	211: ≤CIN1 (117), ≥CIN2 (94)	C & H (all)	Median percentage methylation per CpG Site

Zhang 2019	China	Consecutive HPV16+ screening population	CEC (all)	Pyro	16	LCR (E2BS: 7 CpGs)	43: N(10), LSIL(15), HSIL/ICC (18),	C(N) & H(LSIL/H SIL/ICC)	Diagnostic accuracy of methylation in predicting HSIL+
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Appendix 11. Funnel plot of publication bias assessment and small study effects HPV DNA methylation review

Appendix 12a. Meta-analysis of proportion of HPV methylated samples for different genotypes and genes for $\leq$ CIN1/LSIL vs. $\geq$ CIN2/HSIL (where $\leq$ CIN1/LSIL: Normal/ASCUS/LSIL/CIN1 and $\geq$ CIN2/HSIL: CIN2/CIN3/HSIL/ICC). The grade was defined by histology; if this was not available, cytology was used.

Author, year	Cut-off	CpG	Study size	$\leq$ CIN1/LSIL % (N/N)	$\geq$ CIN2/HSIL % (N/N)
HPV16 L1				%	%
Brentnall 2014	>0%	L1 (bp6367, 6389)	556	90.1 (210/233)	97.8 (326/323)
Qiu 2015 (1)	>10%	L1 (bp5602, 5608, 5611, 5617)	114	40.5 (17/42)	91.6 (66/72)
Qiu 2015 (2)	>10%	L1 (bp7136, 7145)	81	34.3 (11/32)	89.8 (44/49)
Simanaviciene 2015	>0%	L1 (bp7136, 7145)	157	2.6 (1/39)	24.6 (29/118)
Gasparov 2015	>0%	L1 (bp7091, 7136, 7145, 7145)	16	30 (3/10)	33.3 (2/6)
Kalantari 2014	>0%	L1/L2 (bp 5602-7270)	63	13.8 (4/29)	50 (17/34)
Lorincz 2013	>0%	L1 (bp 6367,6389)	73	100 (48/48)	100 (25/25)
Wang 2017	>0%	L1 (bp 7089-7268)	100	16.6 (5/30)	50 (35/70)
Total N – n/N*			1160	299/463	534/697
Pooled Estimate (95% CI)				44.4 (16.0-74.1)	72.7 (47.8-92.2)
I^2 (95%CI)				97.61 (93.2-99.3)	97.2 (93.3-99.3)
τ^2 (95%CI)				0.18 (0.07-0.75)	0.11 (0.04-0.41)
HPV 16 L2					
Brentnall 2014	>0%	L2 (bp4238, 4259, 4275)	556	29.3 (68/233)	55 (178/323)
Kalantari 2014	>0%	L1/L2 (bp 5602-7270)	63	13.8 (4/29)	50 (17/34)
Lorincz 2013	>0%	L2 (bp 4238, 4247, 4259, 4268, 4275)	73	79 (38/48)	96 (24/25)
Total N – n/N			692	110/310	219/382
Pooled Estimate (95% CI)				40.1 (7-79.3)	61.8 (43.9-78.2)

I^2 (95%CI)				96.9 (88.1-99.9)	85.2 (39.6-99.7)
τ^2 (95%CI)				0.12 (0.03-5.12)	0.02 (0.00-1.04)
HPV16 LCR					
Badal 2003	>0%	5' LCR, Enh, E6 Prom	81	42.1 (16/38)	9.3 (4/43)
Bhattacharjee 2006	>0%	5'LCR (bp7289-7540)	72	4 (6/15)	54.4 (31/57)
Ding 2009	>0%	5'LCR, Enh, E6 Prom (bp7426-58)	53	5.9 (1/17)	41.7 (15/36)
Dutta 2015	>0%	Enh (bp7535-7694), E6 Prom	215	4.1 (41/93)	44.3 (54/122)
Hublarova 2009	>0%	E6 Prom (bp7851-7559)	141	81 (17/21)	35.8 (43/120)
Gasperov 2015	>0%	3'LCR, Enh, Prom (bp7091-58)	12	50 (3/6)	33.3 (2/6)
Simanaviciene 2015	>0%	5'LCR, Enh, Prom (bp7270-58)	157	2.7 (1/39)	23.7 (28/118)
Xi 2011	>0%	3'LCR, Enh, Prom (bp7535-58)	211	44.9 (53/117)	35.6 (34/94)
Snellenberg 2012	>0%	E2BS1 (bp 7370-7383)	65	11.8 (2/17)	64.6 (31/48)
Hong 2008	>0%	Enh, Prom (bp7676-58)	70	48.4 (15/31)	71.8 (28/39)
Lorincz 2013	>0%	Prom (bp31-58)	73	90 (43/48)	92 (23/25)
Wang 2017	>0%	3'L1, 5'LCR, Enh, Prom (bp7089-58)	101	8.8 (3/34)	32.8 (22/67)
Total N – n/N			1251	201/476	315/775
Pooled Estimate (95% CI)				37.5 (20.2-56.5)	44.5 (30.9-58.5)
I^2 (95%CI)				93.4 (86.4-97.7)	92.8 (85.1-97.6)
τ^2 (95%CI)				0.09 (0.05-1.04)	0.05 (0.02-0.16)
HPV 18 L1/L2					
Simanaviciene 2015	>0%	L1 (bp6916-7122)	21	18.2 (2/11)	80 (8/10)
Gasperov 2015	>0%	L1 (bp7017-7140)	21	100 (9/9)	92.3 (12/13)

Brentnall 2014	>0%	L2 (bp4256-4282)	201	70.9 (91/128)	84.1 (61/73)
Kalantari 2014	>0%	L1/L2 (bp5602-7270)	14	14.3 (1/7)	85.7 (6/7)
Total N – n/N			258	103/155	87/103
Pooled Estimate (95% CI)				52.6 (13.8-89.8)	86.0 (77.7-92.8)
I^2 (95%CI)				90.6 (67.7-99.4)	0.0 (0.0-75.4)
tau ² (95%CI)				0.14 (0.03-2.30)	0.00 (0.00-0.05)
HPV18 LCR					
Simanaviciene 2015	>0%	LCR (LCR5', Prom, Enh)	21	0 (0/13)	20 (2/8)
Total N – N/N			21	0/13	2/8
HPV 31					
Brentnall 2014	>0%	L1 (bp6352, 6364)	202	59.6 (62/104)	85.7 (84/98)
Kalantari 2014	>0%	L1/L2 (bp5518-5692)	15	0 (0/7)	50 (4/8)
Total N – n/N			217	62/111	88/106
Pooled Estimate (95% CI)				33.6 (0.0-87.1)	73.7 (34.4-99.3)
I^2 (95%CI)				89.6 (47.6-100)	89.6 (47.6-100)
tau ²				0.14 (0.01-100)	0.06 (0-78.5)
HPV 45 L1/L2					
Kalantari 2014	>0%	L1/L2 (bp4795-7135)	12	0 (0/1)	80 (8/10)
Total N – n/N			12	0/1	8/10
HPV 52 L1					
Murakami 2013	>0%	L1 (bp5606-7120)	54	15 (2.6/17)	44.7 (17/37)
Total N – n/N			54	2.6/17	17/37

HPV 52 LCR					
Murakami 2013	>0%	LCR (LCR5', Prom, Enh)	54	2.5 (0.4/17)	2.4 (0.9/37)
Total N – n/N			54	0.4/17	0.9/37
HPV 58 L1					
Murakami 2013	>0%	L1 (bp5606-7119)	41	12 (2.4/20)	51.1 (10.7/21)
Total N – n/N			41	2.4/20	10.7/21
HPV 58 LCR					
Murakami 2013	>0%	LCR (LCR5', Prom, Enh)	41	0 (0/20)	0 (0/21)
Total N – n/N			41	0/20	0/21

ASCUS: abnormal squamous cells of undetermined significance; Bp: base pair; CIN: cervical intraepithelial neoplasia; Enh: enhancer region; HSIL: high-grade squamous intraepithelial lesion; LCR: long control region; LSIL: low-grade squamous intraepithelial lesion; N: total number of samples; N: number of samples methylated; ICC: invasive cervical cancer Prom: promoter region; Q-test: Cochrane Q test.

Proportion of samples methylated at a CpG and/or gene was defined as: N methylated/ N methylated + N unmethylated

Where studies presented results for multiple CpG sites per gene, the mean result for the gene was meta-analysed.

Appendix 12b. Meta-analysis of proportion of HPV methylated samples for Normal vs ASCUS/CIN1/LSIL vs. CIN2/CIN3HSIL vs ICC. The grade was defined by histology; if this was not available, cytology was used.

Author, year	Cut-off	CpG	Study size (n)	Normal % (n/N)	CIN1/LSIL/ASCUS % (n/N)	CIN2/CIN3/HSIL % (n/N)	ICC % (n/N)
HPV16 L1							
Qiu 2015 (1)	>10%	L1 (bp5602, 5608, 5611, 5617)	114	5.9 (1/17)	64 (16/25)	90.2 (55/61)	100 (11/11)
Qiu 2015 (2)	>10%	L1 (bp7136, 7145)	81	6.3 (1/16)	62.5 (10/16)	88.1 (37/42)	100 (7/7)
Simanaviciene 2015	>0%	L1 (bp7136, 7145)	157	3.4 (1/29)	0 (0/10)	20.7 (18/87)	35.5 (11/31)
Gasperov 2014	>0%	L1 (bp7091, 7136, 7145, 7145)	12	75 (3/4)	0 (0/2)	0 (0/4)	100 (2/2)
Kalantari 2014	>0%	L1/L2 (bp 5602-7270)	63	8.3 (1/6)	13.0 (3/23)	33.3 (7/21)	83.1 (10/13)
Wang 2017	>0%	L1 (bp 7089-7268)	100	-	16.6 (5/30)*	34.3 (12/35)	65.7 (23/35)
Total N – n/N			527	7/72	34/106	129/250	64/99
Pooled Estimate (95% CI)				13.9 (0.3-36.8)	31.1 (6.2-62.1)	49.4 (20.0-79.0)	76.7 (53.6-94.7)
I ² (95%CI)				65.88 (0.00-96.98)	82.5 (49.4-97.7)	95.1 (86.8-99.2)	75.1 (52.1-93.3)
τ ² (95%CI)				0.04 (0.00-0.72)	0.08 (0.02-0.70)	0.12 (0.04-0.79)	75.5 (34.1-95.3)
Q-test (P-value)				10.53 (0.03)	24.56 (<0.001)	122.43 (<0.001)	21.49 (<0.001)
HPV 16 L2							
Kalantari 2014	>0%	L1/L2 (bp 5602-7270)	63	8.3 (1/6)	13.1 (3/23)	33.3 (7/21)	83.1 (10/13)
Total N – n/N			63	1/6	3/23	7/21	10/13
HPV16 LCR							
Badal 2003	>0%	5' LCR, Enh, E6 promoter (bp ND)	81	52 (13/25)	23 (3/13)	20 (2/10)	6.1 (2/33)
Bhattacharjee 2006	>0%	5'LCR (bp7289-7540)	72	40 (6/15)	-	-	54.4 (31/57)
Ding 2009	>0%	5'LCR, Enh, E6 promoter (bp7426-58)	53	-	5.9 (1/17)	33.3 (7/21)	53.3 (8/15)
Dutta 2015	>0%	Enh (bp7535-7694), E6 promoter	215	44.6 (40/89)	25 (1/4)	37.5 (9/24)	45.6 (45/98)
Hublarova 2009	>0%	E6 promoter (bp7851-7559)	141	81 (17/21)	62.5 (5/8)	31.5 (28/89)	43.4 (10/23)
Gasperov 2015	>0%	3'LCR, Enh, Promoter (bp7091-58)	12	47 (2/4)	50 (1/2)	25 (1/4)	43.4 (1/2)

Simanaviciene 2015	>0%	5'LCR, Enh, Promoter (bp7270-58)	157	4.3 (1/29)	0 (0/10)	21.8 (19/87)	29 (9/31)
Snellenberg 2012	>0%	E2BS1 (bp 7370-7383)	65	12 (2/17)	N/A	21.1 (4/19)	93 (27/29)
Hong 2008	>0%	Enh, Prom (bp7676-58)	70	71.4 (10/14)	29.4 (5/17)	46.2 (6/13)	84.6 (22/26)
Wang 2017	>0%	3'L1, 5'LCR, Enh, Promoter (bp 7089-58)	101	-	8.8 (3/34)*	18.2 (6/33)	47 (16/34)
Total N – n/N			967	91/214	19/105	82/300	171/348
Pooled Estimate (95% CI)				41.8 (21.0-64.1)	22.6 (7.1-42.0)	26.6 (20.7-32.7)	50.9(31.8-69.8)
I² (95%CI)				88.1 (71.2-97.0)	53.5 (0.0-89.9)	11.1 (0.0-71.3)	90.5 (78.5-97.0)
τ² (95%CI)				0.08 (0.03-0.34)	0.03 (0.00-0.22)	0.00 (0.00-0.00)	0.07 (0.03-0.24)
Q-test (P-value)				54.6 (<0.001)	12.46(0.05)	7.45 (0.45)	82.0 (<0.001)
HPV18 L1/L2							
Simanaviciene 2015	>0%	L1 (bp6916-7122)	21	12.5 (1/8)	33.3 (1/3)	50 (2/4)	100 (6/6)
Gasperov 2015	>0%	L1 (bp7017-7140)	22	-	100 (9/9)	91.7 (11/12)	100 (1/1)
Kalantari 2014	>0%	L1/L2 (bp5602-7270)	14	0 (0/2)	20 (1/5)	100 (2/2)	80 (4/5)
Total N – n/N			57	1/10	11/17	15/18	11/12
Pooled Estimate (95% CI)				10.5 (0.0-42.4)	56.8 (5.7-99.7)	83.6 (51.4-100)	91.4 (61.8-100)
I² (95%CI)				0.0 (0.0-98.9)	76.6 (19.9-99.3)	0.0 (0.0-69.1)	0.00 (0.00-91.9)
τ² (95%CI)				0.0 (0.00-4.53)	0.14 (0.01-6.38)	0.0 (0.00-0.03)	0.0 (0.0-0.59)
Q-test (P-value)				0.09 (0.77)	9.74 (0.01)	0.56 (0.91)	0.63 (0.73)
HPV18 LCR							
Simanaviciene 2015	>0%	LCR (LCR5', Prom, Enh)	21	0 (0/8)	0 (0/3)	0 (0/4)	22.2 (1.3/6)
Total N – n/N			21	0/8	0/3	0/4	1.3/6
HPV 31							
Kalantari 2014	>0%	L1/L2 (bp5518-5692)	15	0 (0/1)	0 (0/3)	0 (0/3)	48.6 (1.9/4)
Total N – n/N			15	0/1	0/3	0/3	1.9/4
HPV 45 L1/L2							
Kalantari 2014	>0%	L1/L2 (bp4795-7135)	12	0 (0/1)	0 (0/1)	75.9 (2.3/3)	94.4 (5.2/6)
Total N – n/N			12	0/1	0/1	2.3/3	5.2/6
HPV 52 L1							
Murakami 2013	>0%	L1 (bp5606-7120)	54	15 (2.6/17)**	34 (5.1/15)	52 (11.4/22)	-
Total N – n/N			54	2.6/17	5.1/15	11.4/22	
HPV 52 LCR							

Murakami 2013	>0%	LCR (LCR5', Prom, Enh)	54	2.5 (0.4/17)	1.3 (0.2/15)	3.2 (0.7/22)	-
Total N – n/N			54	0.4/17	0.2/15	0.7/22	
HPV 58							
Murakami 2013	>0%	L1 (bp5606-7119)	41	12 (2.4/20)**	38 (3.4/9)	61 (7.3/12)	-
Total N – n/N			41	2.4/20	3.4/9	7.3/12	
HPV 58							
Murakami 2013	>0%	LCR (LCR5', Prom, Enh)	41	0 (0/20)**	0 (0/9)	0 (0/12)	-
Total N – n/N			41	0/20	0/9	0/12	

ASCUS: abnormal squamous cells of undetermined significance; Bp: base pair; CIN: cervical intraepithelial neoplasia; Enh: enhancer region; HSIL: high-grade squamous intraepithelial lesion; ICC: invasive cervical carcinoma (squamous or adenocarcinoma; LCR: long control region; LSIL: low-grade squamous intraepithelial lesion; N: total number of samples; n: number of samples methylated; ND: not defined; Prom: promoter region; Q-test: Cochrane Q test.

* Normal & LSIL

** Cervicitis & CIN1

Appendix 12c. Sensitivity meta-analysis of proportion of HPV methylated samples for different genotypes and genes defined by histology alone for: A) $\leq$ CIN1 vs. $\geq$ CIN2; B) Normal vs. CIN1 vs. CIN2/CIN3 vs. ICC.

Author, year	Cut-off (+)	CpG	Total N	$\leq$ CIN1 vs. $\geq$ CIN2		Normal vs. CIN1 vs. CIN2/CIN3 vs ICC				
				$\leq$ CIN1 (n/N)	$\geq$ CIN2 (n/N)	Normal % (n/N)	CIN1 % (n/N)	CIN2 % (n/N)	CIN3 % (n/N)	ICC % (n/N)
HPV16 L1										
Qiu 2015 (1)	>10%	L1 (bp5602, 5608, 5611, 5617)	114	40.5 (17/42)	91.6 (66/72)	5.9 (1/17)	64 (16/25)	86.2 (25/29)	93.8 (30/32)	100 (11/11)
Qiu 2015 (2)	>10%	L1 (bp7136, 7145)	81	34.3 (11/32)	89.8 (44/49)	6.3 (1/16)	62.5 (10/16)	81.3 (13/16)	92.3 (24/26)	100 (7/7)
Simanaviciene 2015	>0%	L1 (bp7136, 7145)	157	2.6 (1/39)	24.6 (29/118)	3.4 (1/29)	0 (0/10)	5.9 (1/17)	24.3 (17/70)	35.5 (11/31)
Gasperov 2015	>0%	L1 (bp7091, 7136, 7145, 7145)	12	-	-	75 (3/4)	-	-	-	100 (2/2)
Kalantari 2014	>0%	L1/L2 (bp 5602-7270)	63	-	-	8.3 (1/6)	-	-	-	83.1 (10/13)
Wang 2017	>0%	L1 (bp 7089-7268)	100	-	50 (35/70)	-	-	-	-	65.7 (23/35)
Total N – n/N			527	29/113	209/309	8/72	26/51	39/62	71/128	64/99
Pooled Estimate (95% CI)				24.1 (5.3-50.0)	71.5 (24.8-99.8)	13.9 (0.3-36.8)	42.4 (6.9-82.6)	57.5 (6.9-99.3)	73.3 (24.1-100)	76.7 (53.6-94.7)
I² (95%CI)				89.4 (66.9-99.2)	97.98 (92.6-99.9)	65.88 (0.00-96.98)	88.2 (54.0-99.7)	94.9 (81.0-99.9)	96.7 (88-99.9)	70.4(24.4-92.1)
τ^2 (95%CI)				0.064 (0.015-98.7)	0.174 (0.04-6.97)	0.04 (0.00-0.72)	0.11 (0.02-5.20)	0.23 (0.05-7.58)	0.19 (0.05-7.58)	0.04 (0.01-0.21)
Q-test (P-value)				29.08 (<0.001)	120.6 (<0.001)	10.53 (0.03)	14.41(<0.001)	37.61 (<0.001)	74.31 (<0.001)	21.49 (<0.001)
HPV 16 L2										
Kalantari 2014	>0%	L1/L2 (bp 5602-7270)	63	-	-	8.3 (1/6)	-	-	-	83.1 (10/13)
Total N – n/N			63	-	-	1/6	-	-	-	10/13
HPV16 LCR										

Badal 2003	>0%	5' LCR, Enh, E6 Prom (bp ND)	81	42.1 (16/38)	9.3 (4/43)	52 (13/25)	23.1 (3/13)	-	20 (2/10)	6.1 (2/33)
Bhattacharjee 2006	>0%	5'LCR (bp7289- 7540)	72	4 (6/15)	54.4 (31/57)	40 (6/15)	-	-	-	54.4 (31/57)
Ding 2009	>0%	5'LCR, Enh, E6 Prom (bp7426-58)	53	-	-	-	-	-	-	53.3 (8/15)
Dutta 2015	>0%	Enh (bp7535- 7694), E6 Prom	215	-	-	44.6 (40/89)	-	-	-	45.6 (45/98)
Hublarova 2009	>0%	E6 Prom (bp7851- 7559)	141	81 (17/21)	35.8 (43/120)	81 (17/21)	62.5 (5/8)	31.5 (28/89)*	-	43.4 (10/23)
Gasperov 2015	>0%	3'LCR, Enh, Prom (bp7091-58)	12	-	-	47 (2/4)	-	-	-	43.4 (1/2)
Simanaviciene 2015	>0%	5'LCR, Enh, Prom (bp7270-58)	157	2.7 (1/39)	23.7 (28/118)	4.3 (1/29)	2.5 (0/10)	11.8 (2/17)	24 (17/70)	29 (9/31)
Snellenberg 2012	>0%	E2BS1 (bp 7370-7383)	65	11.8 (2/17)	64.6 (31/48)	12 (2/17)	-	-	21 (4/19)	93 (27/29)
Hong 2008	>0%	Enh, Prom (bp7676-58)	70	48.4 (15/31)	71.8 (28/39)	71.4 (10/14)	29.4 (5/17) (CIN1-2)	-	46.2 (6/13)	84.6 (22/26)
Wang 2017	>0%	3'L1, 5'LCR, Enh, Prom (bp 7089-58)	101	8.8 (3/34)	32.8 (22/67)	-	-	-	-	47 (16/34)
Total N – n/N			967	60/195	246/492	91/214	13/48	30/106	29/112	171/348
Pooled Estimate				35.4 (15.5-58.3)	36.3 (22.0-51.9)		28.4 (8.5- 53.0)	11.8 (0.3-32.3)	27.8 (21.5- 34.6)	51.6 (33.2- 69.8)
I² (95%CI)				92.8 (82.1-98.5)	92.3 (80.8-98.5)	88.1 (71.2-97.0)	61.4 (0.0_97.5)	N/A	0.0 (0.0-90.2)	89.4 (75.8- 96.7)
τ² (95%CI)				0.08 (0.03-0.44)	0.04 (0.01-0.22)	0.08 (0.03-0.34)	0.03 (0.00- 0.85)	N/A	0.00 (0.00- 0.07)	0.07 (0.02- 0.23)
Q-test (P-value)				71.67 (<0.001)	54.3 (<0.001)	54.6 (<0.001)	7.49 (0.06)	N/A	3.20 (0.53)	69.69 (<0.001)
HPV18 L1/L2										

Simanaviciene 2015	>0%	L1 (bp6916-7122)	21	18.2 (2/11)	80 (8/10)	12.5 (1/8)	33.3 (1/3)	0 (0/2)	100 (2/2)	100 (6/6)
Gasperov 2015	>0%	L1 (bp7017-7140)	22	-	-	-	-	-	-	100 (1/1)
Kalantari 2014	>0%	L1/L2 (bp5602-7270)	14	-	-	0 (0/2)	-	-	-	80 (4/5)
Total N – n/N			57	2/11	8/10	1/10	1/3	0/2	2/2	11/12
Pooled Estimate (95% CI)						10.5 (0.00-42.4)				91.4 (62.1-100)
I² (95%CI)						0.0 (0.0-98.9)				0.00 (0.00-91.9)
τ² (95%CI)						0.0 (0.00-4.53)				0.0 (0.0-0.59)
Q-test (P-value)						0.09 (0.77)				0.83 (0.73)
HPV18 LCR										
Simanaviciene 2015	>0%	LCR (LCR5', Enh, Prom)	21	0 (0/13)	20 (2/8)	0 (0/8)	0 (0/3)	0 (0/2)	16.7 (0.3/2)	22.2 (1.3/6)
Total N – n/N			21	0/13	2/8	0/8	0/3	0/2	0.3/2	1.3/6
HPV 31										
Kalantari 2014	>0%	L1/L2 (bp5518-5692)	15	-	-	0 (0/1)	-	-	-	48.6 (1.9/4)
Total N – n/N			15			0/1				1.9/4
HPV 45 L1/L2										
Kalantari 2014	>0%	L1/L2 (bp4795-7135)	12	-	-	0 (0/1)	-	-	-	94.4 (5.2/6)
Total N – n/N			12			0/1				5.2/6
HPV 52 L1										
Murakami 2013	>0%	L1 (bp5606-7120)	54	-	-	-	15 (2.6/17)**	34 (5.1/15)	52 (11.4/22)	-
Total N – n/N			54				2.6/17	5.1/15	11.4/22	
HPV 52 LCR										

Murakami 2013	>0%	LCR (LCR5', Prom, Enh)	54	-	-	2.5 (0.4/17)**	1.3 (0.2/15)	3.2 (7/22)	-
Total N – n/N			54			0.4/17	0.2/15	7/22	
HPV 58									
Murakami 2013	>0%	L1 (bp5606- 7119)	41	-	-	12 (2.4/20)**	38 (2.4/9)	61 (7.3/12)	-
Total N – n/N			41			2.4/20	2.4/9	7.3/12	
HPV 58									
Murakami 2013	>0%	LCR (LCR5', Prom, Enh)	41	-	-	0 (0/20)**	0 (0/9)	0 (0/12)	-
Total N – n/N			41			0/20	0/9	0/12	

ASCUS: abnormal squamous cells of undetermined significance; Bp: base pair; CIN1-3: cervical intraepithelial neoplasia grades 1 to 3; Enh: enhancer region HSIL: high-grade squamous intraepithelial lesions; ICC: invasive cervical carcinoma (squamous or adenocarcinoma; LCR: long control region; LSIL: low-grade squamous intraepithelial lesion; N: total number of samples; n: number of samples methylated; ND: not defined; Prom: promoter region; Q-test: Cochrane Q test.

* CIN2-3

** Cervicitis & CIN1

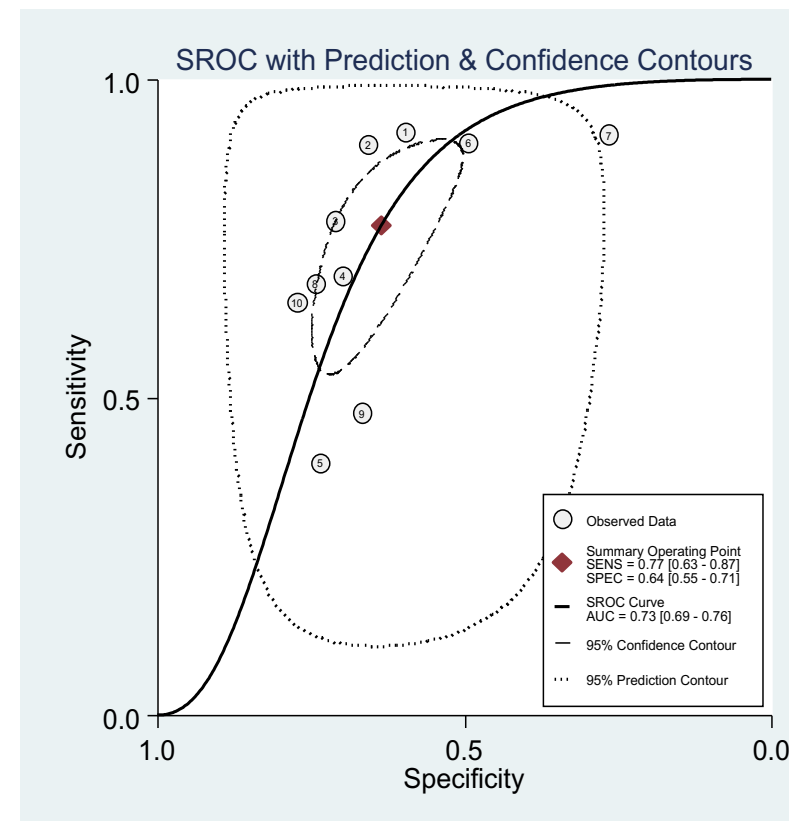
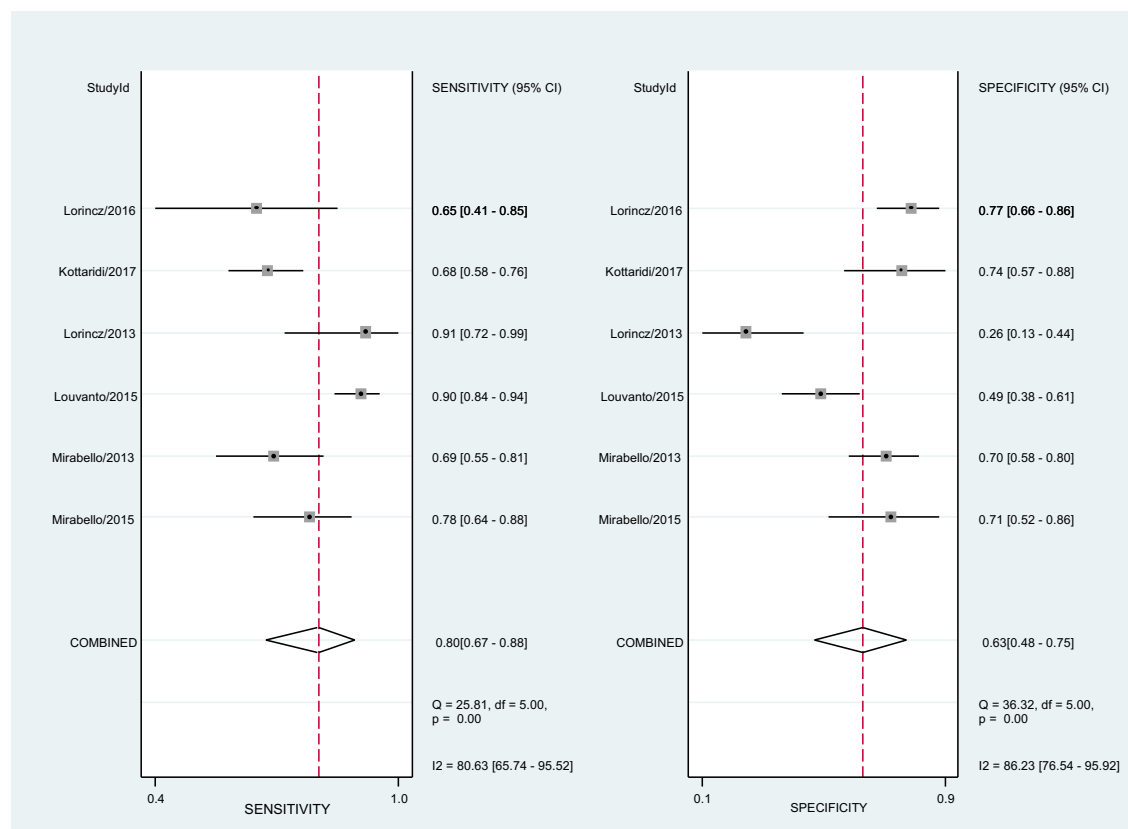
Appendix 12d. Sensitivity analysis of pooled difference in mean methylation level (MD) between $\leq$ CIN1/LSIL vs. $\geq$ CIN2/HSIL for HPV16 after exclusion of studies: with a low risk of bias only; used histology alone for reference; pyrosequencing only; exfoliated cervical cells only; where standard deviation was imputed.

Meta-analysis	Studies (n)	Mean Difference	CI_L	CI_H	τ^2	CI_L (τ^2)	CI_H (τ^2)	I ² (%)	CI_L (I ²)	CI_H (I ²)	Q-test	P-value
Main Analysis												
E1	1	4.03	-1.55	9.61	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E2	4	8.84	0.22	17.47	67.46	14.69	674.62	88.01	61.51	98.66	24.04	0.00
E5	1	12.65	2.86	22.44	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E6	3	2.45	0.49	4.42	0.53	0.00	100.00	13.38	0.00	96.67	2.50	0.29
E7	2	7.72	3.74	11.70	0.00	0.00	100.00	0.00	0.00	91.14	0.02	0.88
L1	12	11.29	6.46	16.13	63.36	27.19	196.15	92.97	85.01	97.61	124.39	0.00
L2	5	5.62	1.82	9.41	14.53	2.35	145.25	88.01	54.30	98.66	17.74	0.00
LCR	6	1.55	-0.28	3.38	3.55	0.38	33.52	77.14	26.61	96.96	16.14	0.01
Sensitivity Analyses												
Low ROB												
L1	10	8.89	4.49	13.29	41.70	15.51	140.57	90.65	78.29	97.03	84.26	0.00
L2	4	6.94	1.93	11.95	20.25	1.37	202.47	81.99	23.55	97.85	10.94	0.01
LCR	5	0.71	-0.34	1.77	0.47	0.00	6.14	32.92	0.00	86.53	4.89	0.30
Histology only												
L1	7	8.89	4.49	13.29	41.70	15.51	140.57	90.65	78.29	97.03	84.26	0.00
L2	2	6.94	1.93	11.95	20.25	1.37	202.47	81.99	23.55	97.85	10.94	0.01
LCR	1	0.71	-0.34	1.77	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Pyrosequencing only												
L1	7	8.89	4.49	13.29	41.70	15.51	140.57	90.65	78.29	97.03	84.26	0.00
L2	2	6.94	1.93	11.95	20.25	1.37	202.47	81.99	23.55	97.85	10.94	0.01
LCR	1	0.71	-0.34	1.77	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Exfoliated cervical cells only												
L1	10	9.58	4.47	14.70	58.35	22.73	211.27	92.88	83.57	97.93	93.83	0.00
L2	4	12.72	-4.16	29.60	366.79	128.01	3118.43	99.49	98.54	99.94	219.23	0.00
LCR	4	1.84	-1.23	4.91	7.71	1.15	100.00	80.40	38.05	98.16	15.86	0.00
Exclude SD imputed												
E1	1	4.03	-1.55	9.61	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E2	2	8.15	3.26	13.04	2.28	0.00	100.00	18.00	0.00	90.60	1.22	0.27
E5	1	12.65	2.86	22.44	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E6	2	3.15	-0.17	6.47	3.43	0.00	100.00	54.41	0.00	97.21	2.19	0.14
E7	2	7.72	3.74	11.70	0.00	0.00	100.00	0.00	0.00	91.14	0.02	0.88
L1	5	9.53	2.88	16.18	47.58	11.10	448.04	93.18	76.11	99.23	27.47	0.00

L2	4	6.19	0.63	11.76	27.61	4.21	276.11	93.78	69.72	99.34	16.19	0.00
LCR	2	1.21	0.47	1.96	0.00	0.00	21.06	0.00	0.00	93.81	0.02	0.90

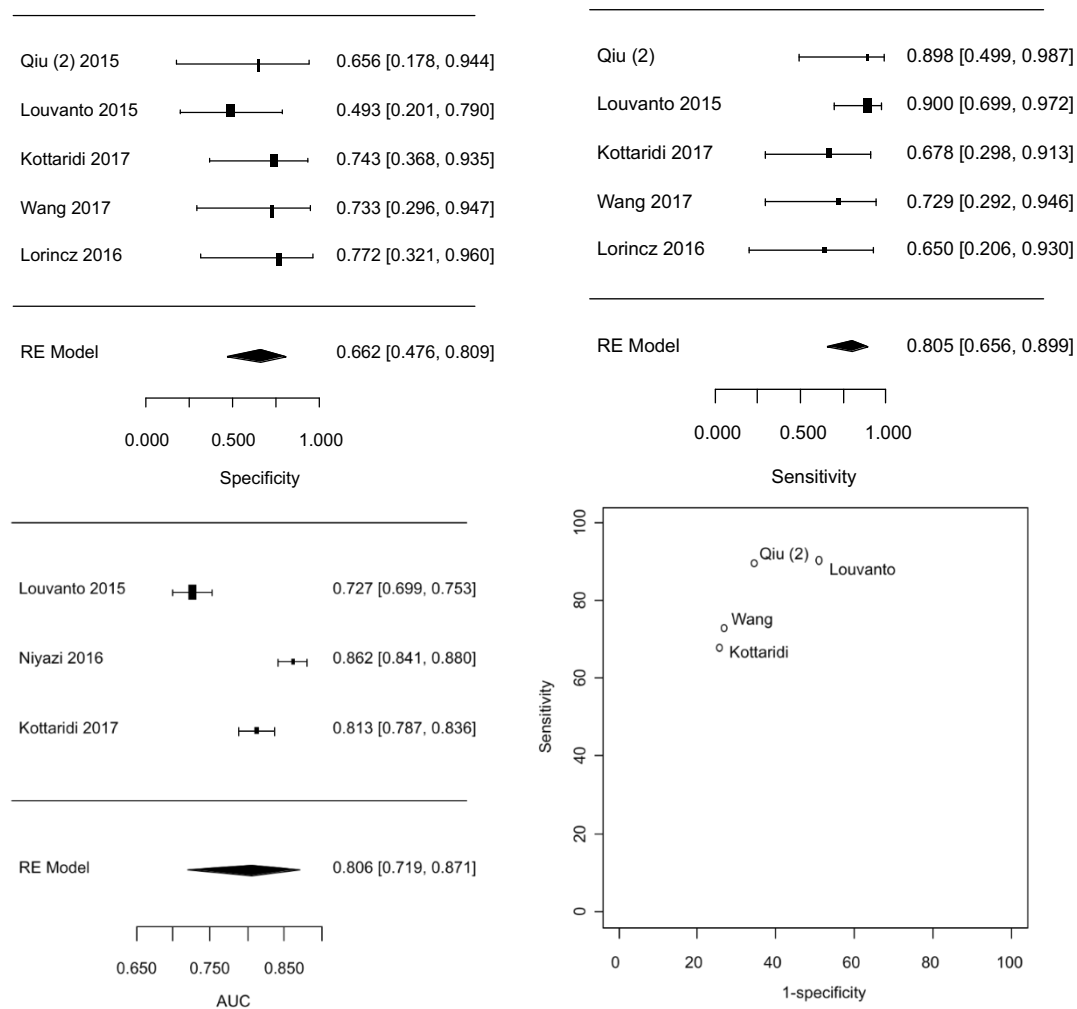
CEC: exfoliated cervical cells; CI L: confidence interval low; CI H: confidence interval high; CIN: cervical intraepithelial neoplasia; LCR: long control region; MD: Mean difference. Pyro: pyrosequencing; Q-test: Cochran Q test; RoGM: ratio of geometric means P-value: P-value of Cochran's Q; SE: standard error.

Appendix 13. Diagnostic accuracy of HPV16 L1/L2_in prediction of CIN2+: Sensitivity analyses restricted to studies with low Risk of Bias (bivariate model)



AUC: area under the curve; I^2 : heterogeneity; Q: Cochran Q test; SROC: summary receiver operating curve; 95%CI: 95% confidence interval

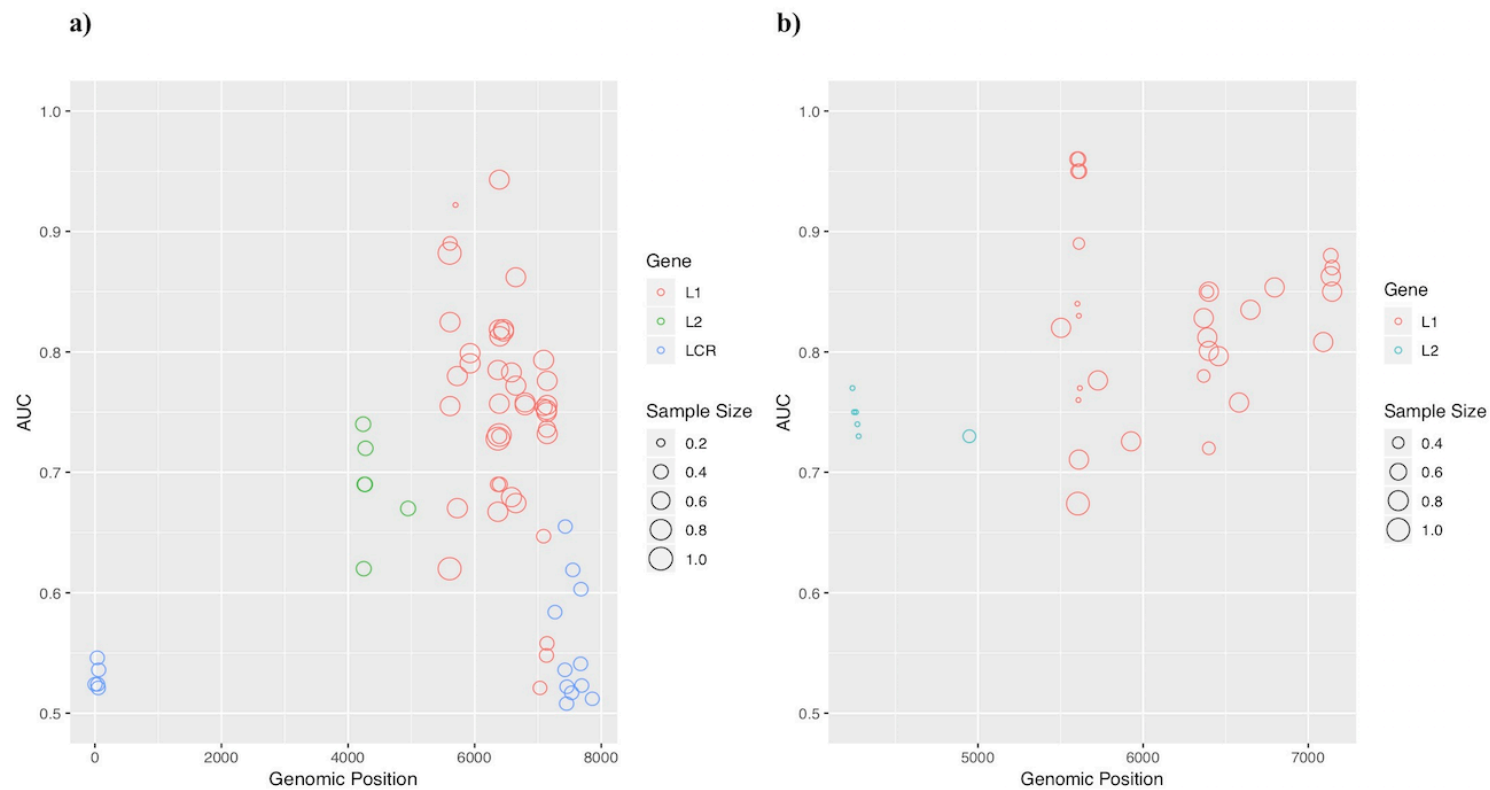
Appendix 14. Diagnostic accuracy of HPV16 L1/L2_in prediction of CIN2+: Sensitivity analyses restricted to Histology only samples (univariate model applied as Hessian matrix was not positive therefore between-study correlation of estimate -1 causing the determinant to be zero)



AUC: area under the curve; RE: random effects.

Appendix 15. AUC values for methylation in all HPV genotypes for prediction of a) CIN2+ and b) CIN3+

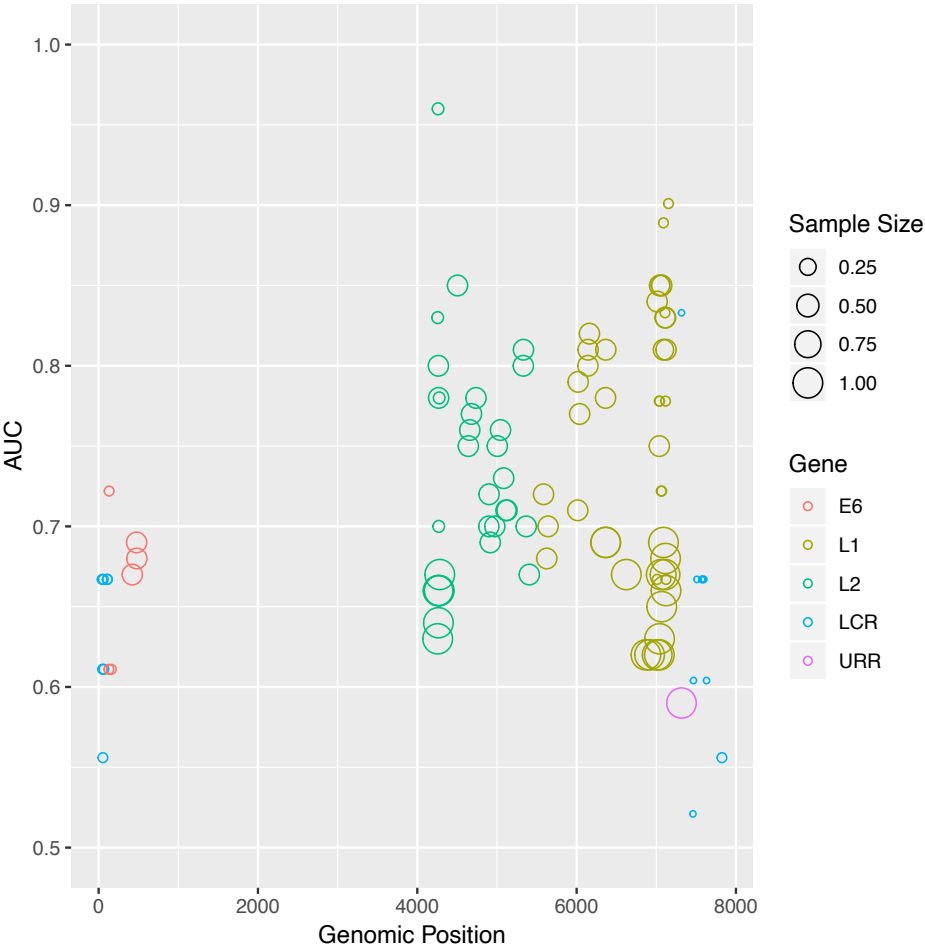
HPV16



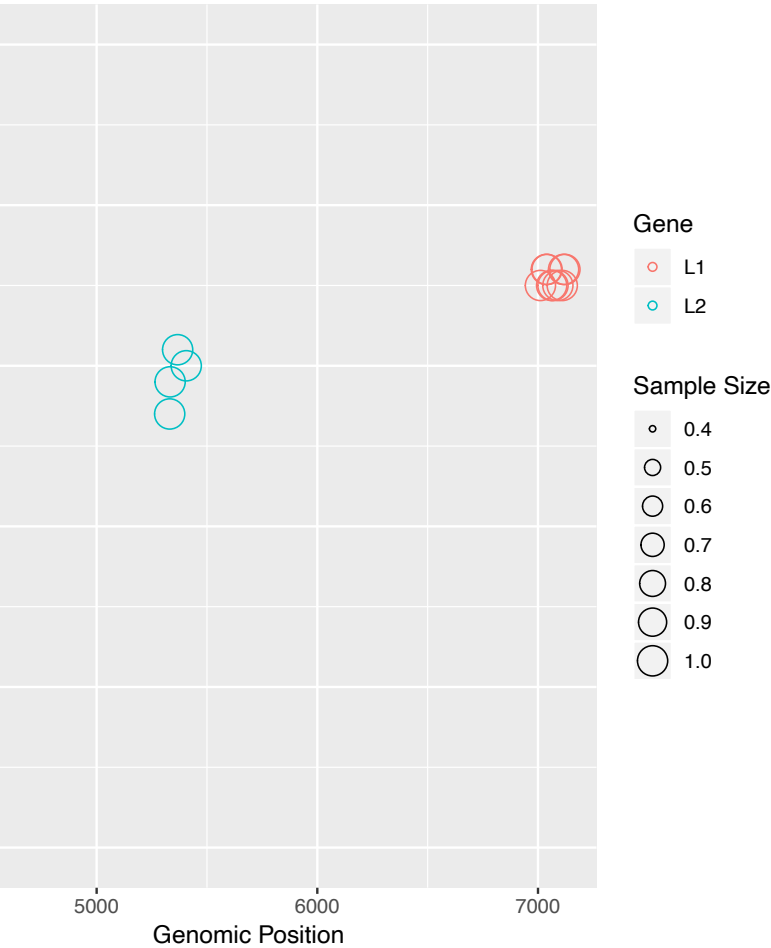
AUC: area under the curve; LCR: long control region

HPV18

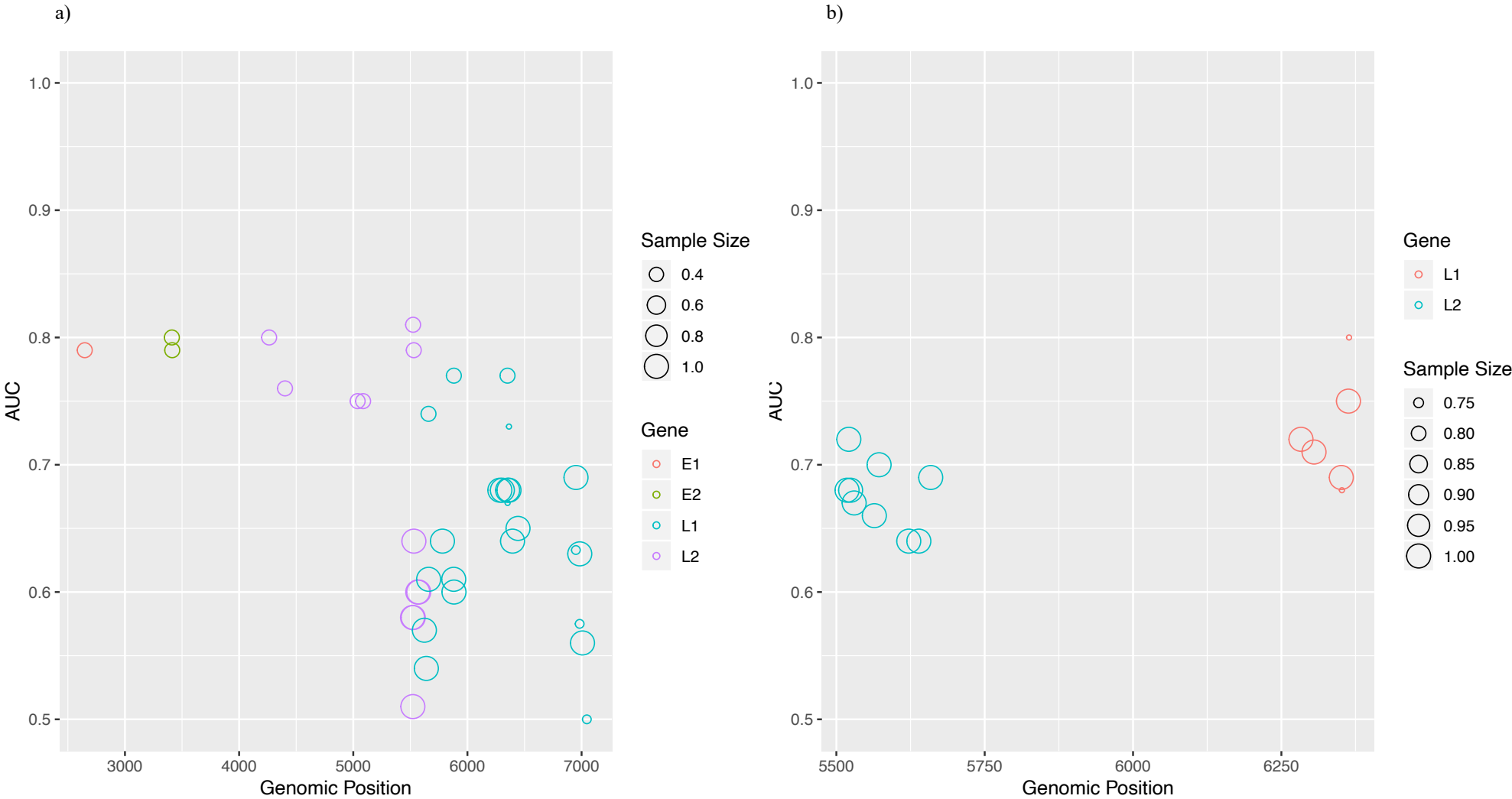
a)



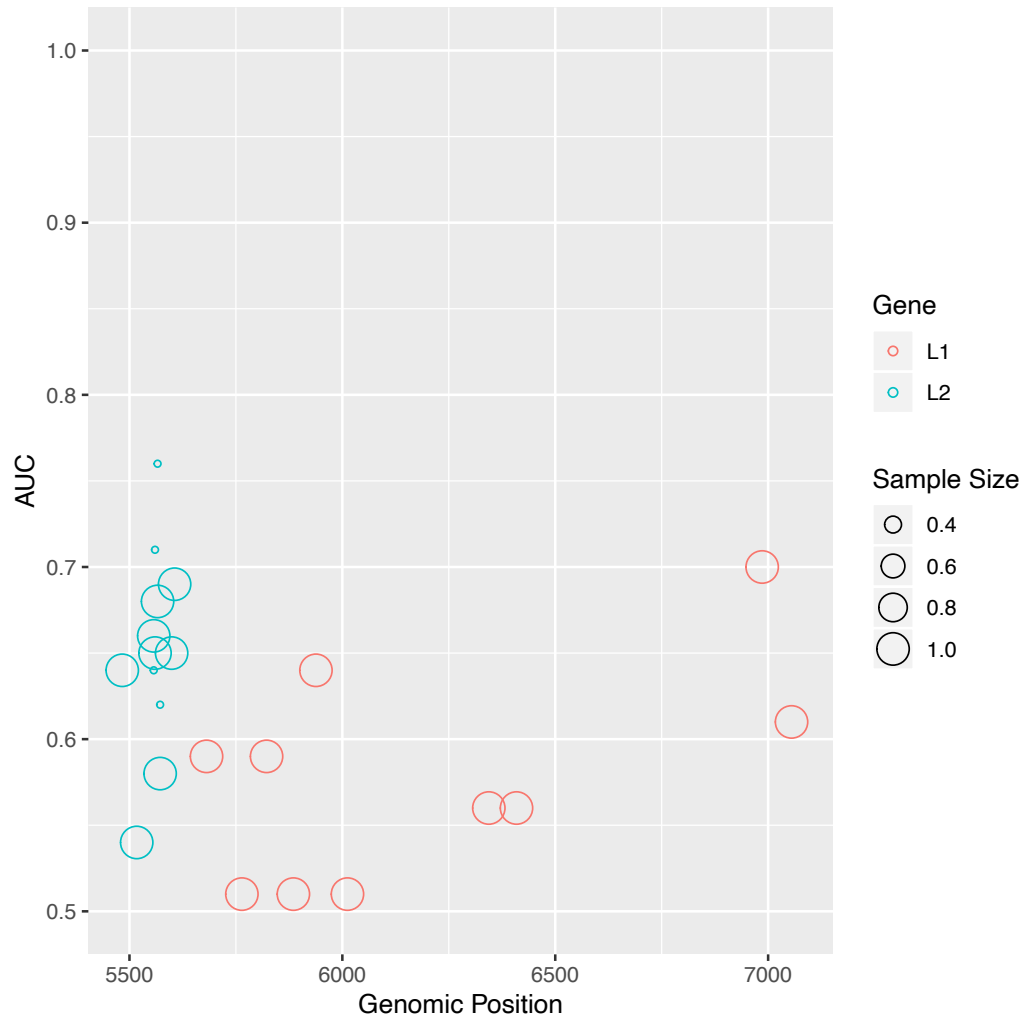
b)



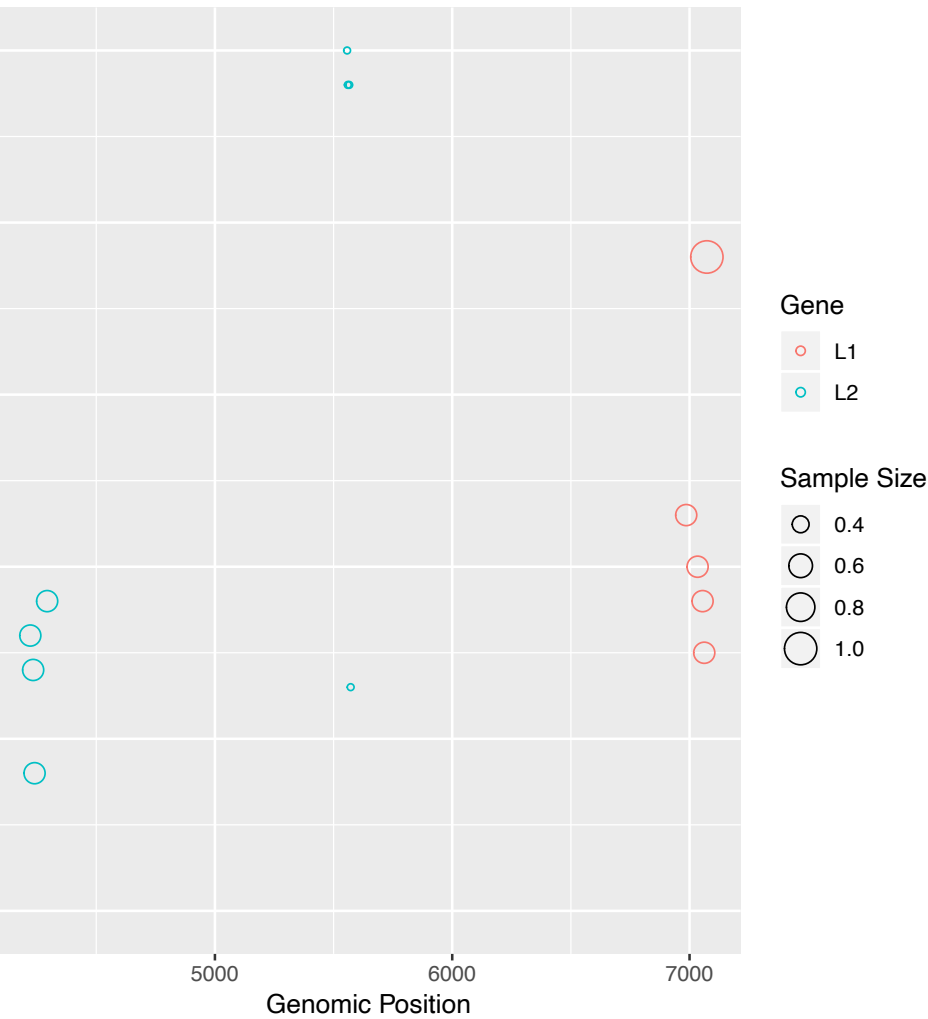
HPV31



HPV33
a)

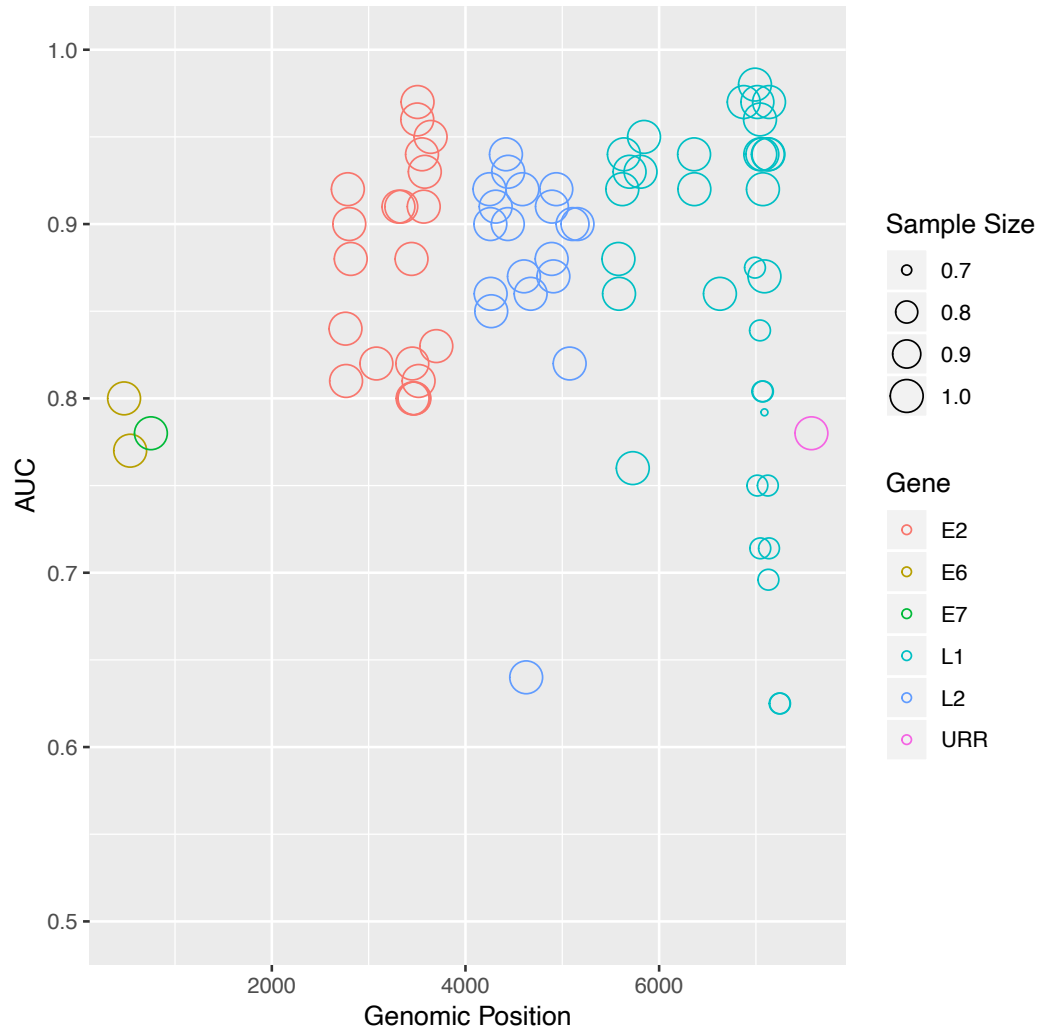


b)



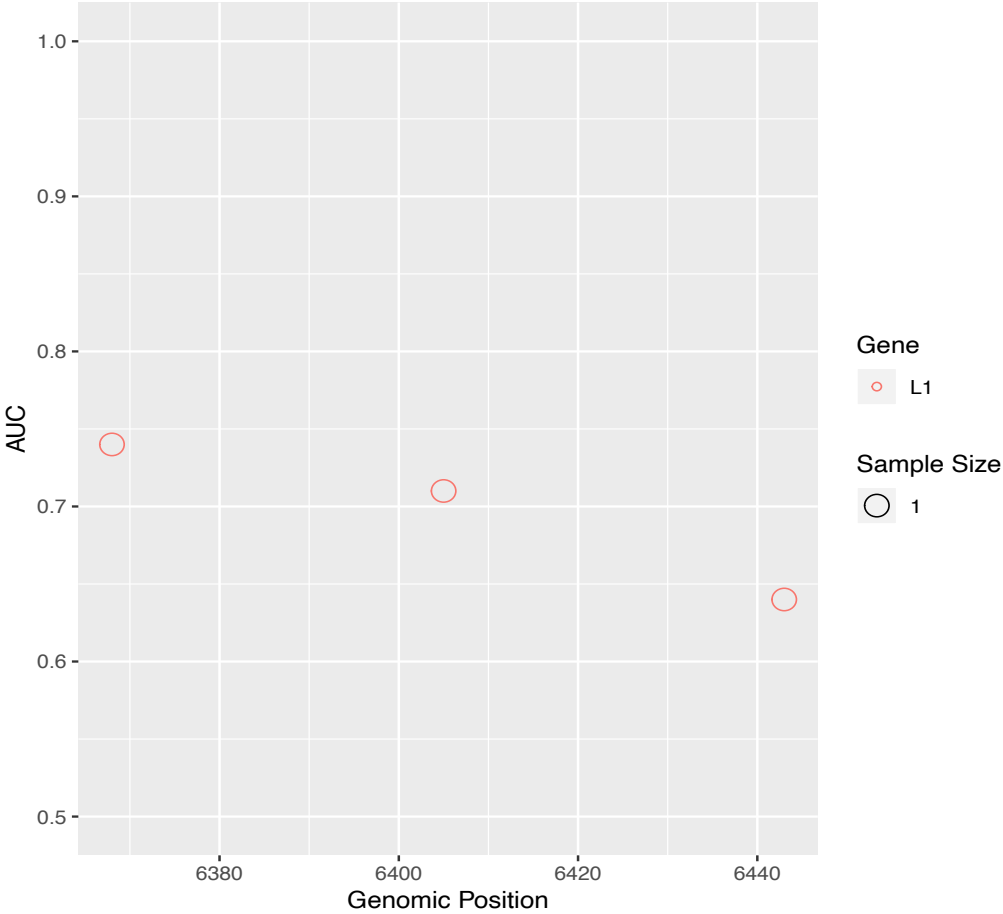
HPV 45

a)

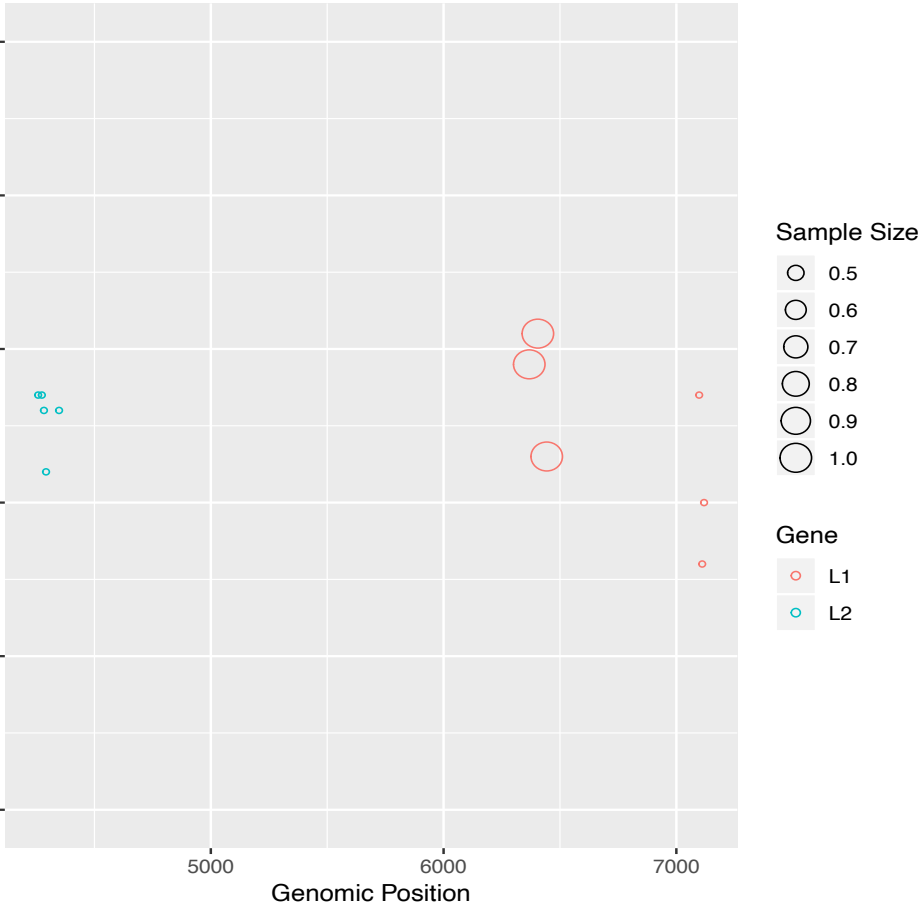


HPV 52

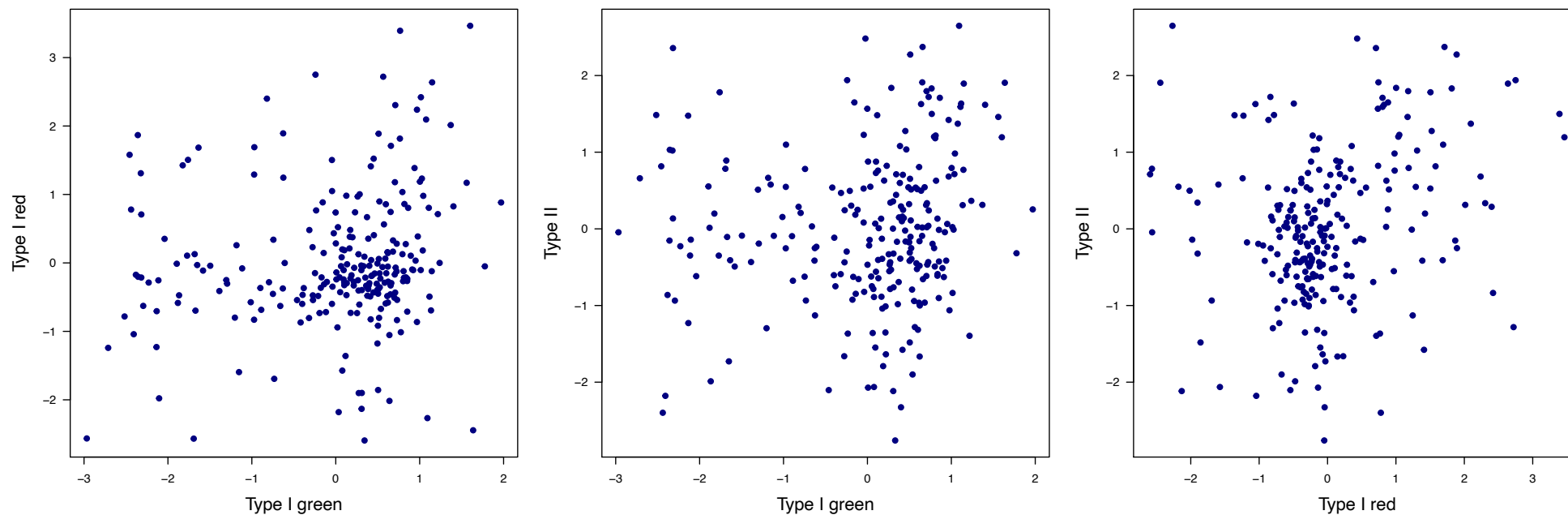
a)



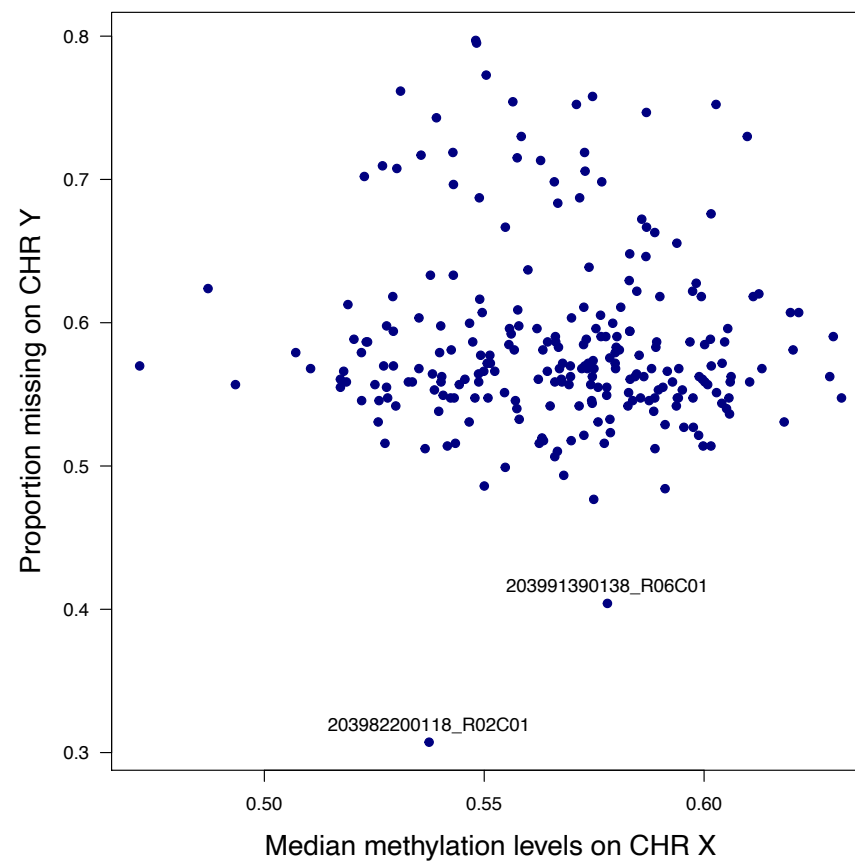
b)



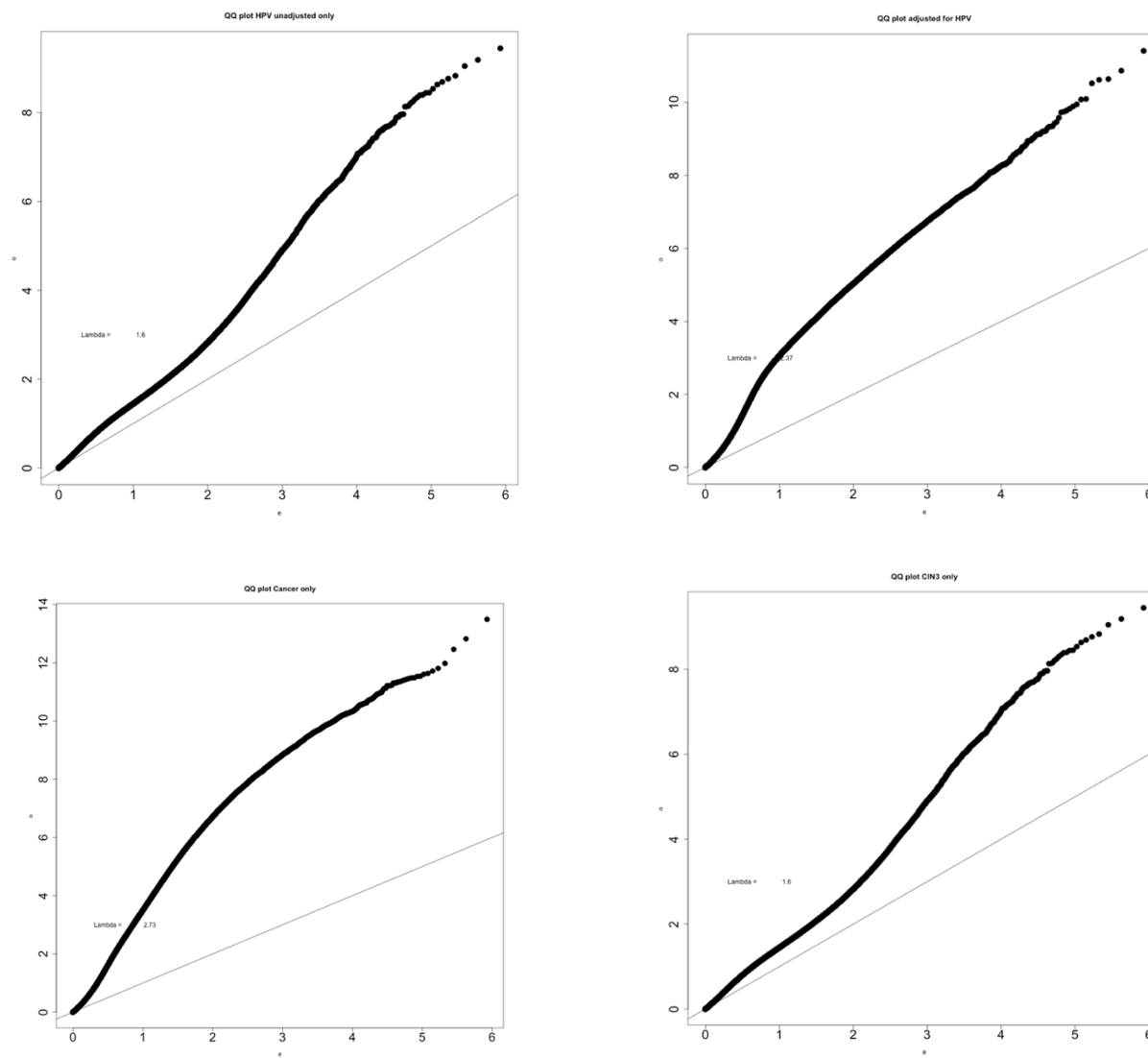
Appendix 16. Visualisation of technical metrics for illumina 850K probes. Samples are represented along three variables measuring the average signals along the three types of beads (type I green, type I red and type II). No outlying observations were observed



Appendix 17. Visualisation of the proportion of missing values on chromosome Y as a function of median methylation proportions on chromosome X. Women are expected to show higher proportions of missing values on chromosome Y and higher median proportions on chromosome X compared to Men.



Appendix 18. (a-d). Quantile-Quantile plots of EWAS logistic models a) unadjusted for HPV b) adjusted for HPV c) Cancer only d) CIN3 only



Appendix 19: Summary results for 409 CpG sites Bonferroni significant in main EWAS analysis, adjusted for HPV

CpG	CHR	BP	GENE	OR	OR 95%CI lower	OR 95%CI upper	COEF	SE	P Value	delta
cg16767801	20	21686548	<i>PAX1</i>	6.32	3.20	12.50	1.84	0.35	3.9009E-12	16.39%
cg07195011	5	11904114	CTNND2	7.05	3.25	15.30	1.95	0.40	1.3655E-11	18.38%
cg17213402	2	5813650		5.80	3.04	11.08	1.76	0.33	2.3096E-11	19.48%
cg27058257	19	30019529	VSTM2B	13.84	4.08	46.93	2.63	0.62	2.425E-11	18.77%
cg22085751	6	28921976		5.76	3.05	10.88	1.75	0.32	3.0224E-11	12.48%
cg11358689	11	131781260	NTM	5.40	2.93	9.97	1.69	0.31	8.1265E-11	24.88%
cg09252495	20	21503898		7.63	3.30	17.65	2.03	0.43	8.4004E-11	13.74%
cg00530925	10	23463094		7.66	3.42	17.15	2.04	0.41	1.1404E-10	13.13%
cg12178980	10	11206870	CUGBP2	14.77	4.14	52.72	2.69	0.65	1.3004E-10	9.33%
cg02409888	20	58568926	CDH26	6.68	3.33	13.40	1.90	0.35	1.496E-10	10.62%
cg09499856	5	41510325	PLCXD3	6.50	3.16	13.37	1.87	0.37	1.6784E-10	7.88%
cg14587524	19	38183262	ZNF781	6.77	3.17	14.48	1.91	0.39	1.819E-10	19.01%
cg09083627	13	88324597	SLITRK5	6.45	3.05	13.63	1.86	0.38	1.884E-10	13.91%
cg21183256	8	73163868		5.23	2.82	9.73	1.66	0.32	2.6508E-10	24.80%
cg07351192	3	5137773		7.53	3.37	16.81	2.02	0.41	3.4555E-10	12.11%
cg25260137	8	65282185		4.66	2.62	8.31	1.54	0.29	3.7587E-10	19.47%
cg13368756	5	11904127	CTNND2	5.86	2.87	12.00	1.77	0.37	4.5191E-10	20.69%
cg02688304	17	50236104	CA10	5.47	2.84	10.56	1.70	0.34	4.6009E-10	18.20%
cg01758512	6	96463902	FUT9	4.27	2.54	7.20	1.45	0.27	4.676E-10	20.68%
cg12989574	13	93879544	GPC6	9.27	3.53	24.34	2.23	0.49	5.1297E-10	11.48%
cg25832771	8	72756058	MSC	6.37	2.94	13.79	1.85	0.39	6.0599E-10	16.78%
cg13093774	8	22089400	PHYHIP	7.59	3.28	17.54	2.03	0.43	6.2214E-10	13.46%
cg09555914	19	58011309	ZNF773	11.52	3.60	36.83	2.44	0.59	6.2696E-10	13.37%

cg17403609	5	180075862	FLT4	7.66	3.31	17.74	2.04	0.43	6.6339E-10	15.26%
cg13912117	8	132054555		5.41	2.81	10.44	1.69	0.34	7.4124E-10	15.84%
cg19112357	3	13834712		6.48	3.15	13.31	1.87	0.37	7.4163E-10	14.87%
cg17509967	19	13617094	CACNA1A	7.31	3.15	16.97	1.99	0.43	7.5355E-10	15.92%
cg17644557	6	86159103	NT5E	6.70	3.15	14.24	1.90	0.38	7.6121E-10	11.54%
cg14739859	5	145758710		4.73	2.60	8.60	1.55	0.30	8.2525E-10	8.22%
cg07704959	3	40471662		4.36	2.54	7.50	1.47	0.28	9.0401E-10	16.39%
cg09582177	2	21451555		0.15	0.07	0.31	-1.88	0.37	9.628E-10	-9.39%
cg26530319	1	221060923		6.37	3.06	13.24	1.85	0.37	9.7775E-10	9.87%
cg23493016	10	106440778	SORCS3	7.25	3.19	16.48	1.98	0.42	1.0927E-09	10.69%
cg00414835	10	48439452	GDF10	5.88	3.00	11.55	1.77	0.34	1.1096E-09	8.89%
cg08448701	20	21686282	PAX1	5.01	2.66	9.44	1.61	0.32	1.138E-09	15.97%
cg01553433	19	17515486	BST2;MVB12A;BIS PR	0.20	0.11	0.36	-1.63	0.31	1.1382E-09	-19.30%
cg02440976	22	22863054	ZNF280B	5.03	2.64	9.62	1.62	0.33	1.1493E-09	9.31%
cg11671688	6	110301075	GPR6	4.75	2.62	8.59	1.56	0.30	1.3377E-09	13.13%
cg01650149	1	240255141	FMN2	4.73	2.60	8.62	1.55	0.31	1.4109E-09	19.84%
cg21294861	14	91125291	TTC7B	0.24	0.15	0.41	-1.41	0.26	1.5548E-09	-17.85%
cg02608002	19	17515778	BST2;MVB12A;BIS PR	0.21	0.12	0.38	-1.55	0.30	1.6079E-09	-20.92%
cg19839798	13	108519318	FAM155A	6.90	2.98	15.97	1.93	0.43	1.6423E-09	14.11%
cg02861380	1	75596758	LHX8	6.65	3.05	14.51	1.89	0.40	1.6631E-09	13.12%
cg15443663	16	48936072		4.19	2.46	7.14	1.43	0.27	1.716E-09	15.11%
cg10135483	6	87647527	HTR1E	5.14	2.66	9.93	1.64	0.34	1.9359E-09	16.28%
cg12753587	4	21950584	KCNIP4	4.84	2.62	8.95	1.58	0.31	1.9861E-09	13.85%
cg17233763	8	85095525	RALYL	4.71	2.56	8.66	1.55	0.31	2.2168E-09	18.21%
cg26337020	5	100239022	ST8SIA4	6.49	2.90	14.51	1.87	0.41	2.2266E-09	9.14%
cg18397073	19	42600278	POU2F2	7.40	3.16	17.33	2.00	0.43	2.253E-09	21.14%
cg19098763	1	50513661	ELAVL4	5.23	2.69	10.14	1.65	0.34	2.2809E-09	12.71%

cg02174225	11	133938941	JAM3	6.94	2.87	16.78	1.94	0.45	2.3409E-09	9.80%
cg14100362	5	3326444		4.74	2.59	8.70	1.56	0.31	2.3899E-09	18.54%
cg00849491	18	6510785	LINC01387	0.20	0.11	0.38	-1.60	0.32	2.569E-09	-10.42%
cg27317046	14	74968209	LTBP2	5.18	2.72	9.86	1.64	0.33	2.5719E-09	19.03%
cg25946752	8	50204309		0.22	0.12	0.39	-1.52	0.29	2.7126E-09	-14.40%
cg10530889	10	93805590		5.37	2.77	10.41	1.68	0.34	2.714E-09	11.51%
cg23214755	2	11607042	E2F6	0.22	0.13	0.39	-1.50	0.29	2.7634E-09	-26.60%
cg23322868	9	21993972	CDKN2B- AS1;CDKN2A	0.20	0.11	0.37	-1.59	0.31	2.8197E-09	-19.52%
cg15104158	3	192445388	FGF12	6.73	3.10	14.63	1.91	0.40	3.0139E-09	10.33%
cg00422488	9	100747767	ANP32B	0.22	0.12	0.39	-1.52	0.29	3.1902E-09	-11.04%
cg14745195	2	144011604	ARHGAP15	5.44	2.78	10.63	1.69	0.34	3.3853E-09	16.55%
cg00622837	6	166421992		5.90	2.79	12.47	1.77	0.38	3.4023E-09	11.72%
cg08958294	6	146350131	GRM1	5.57	2.72	11.41	1.72	0.37	3.4412E-09	8.08%
cg26978172	6	31696223	DDAH2	5.86	2.81	12.19	1.77	0.37	4.0337E-09	18.90%
cg05428701	11	129150329		0.23	0.13	0.40	-1.47	0.29	4.0837E-09	-18.24%
cg07624842	X	21958328	SMS	5.32	2.77	10.22	1.67	0.33	4.0871E-09	11.49%
cg01829277	12	101188532	ANO4	5.62	2.74	11.51	1.73	0.37	4.2324E-09	8.75%
cg09297468	6	87647376	HTR1E	6.69	2.98	15.02	1.90	0.41	4.3495E-09	11.81%
cg07709181	8	75545601	MIR2052HG	0.19	0.10	0.37	-1.64	0.33	4.4229E-09	-10.82%
cg05626764	15	26095764	ATP10A	7.62	2.90	19.98	2.03	0.49	4.5449E-09	12.64%
cg13097800	14	47104140		0.15	0.07	0.33	-1.92	0.41	4.5939E-09	-8.87%
cg25175900	12	22854001		4.13	2.41	7.09	1.42	0.28	4.6622E-09	6.16%
cg00000165	1	91194674		6.30	2.93	13.54	1.84	0.39	4.859E-09	11.28%
cg26047066	1	221069136		5.46	2.76	10.77	1.70	0.35	4.9945E-09	24.75%
cg18375860	1	237205409	RYR2	4.48	2.49	8.06	1.50	0.30	5.0034E-09	21.09%
cg04768713	19	53073334	ZNF701	12.76	3.71	43.92	2.55	0.63	5.0314E-09	11.30%
cg14166284	1	221055719	HLX	5.34	2.74	10.40	1.67	0.34	5.0332E-09	15.81%

cg22784954	5	5140646	ADAMTS16	4.15	2.35	7.32	1.42	0.29	5.0813E-09	17.85%
cg11750165	13	112722233	SOX1	5.49	2.68	11.25	1.70	0.37	5.0995E-09	13.11%
cg22288613	4	190962249		5.03	2.62	9.65	1.61	0.33	5.1569E-09	12.00%
cg06873316	11	20690682	NELL1	4.66	2.51	8.65	1.54	0.32	5.2593E-09	17.07%
cg22040301	11	2919798	SLC22A18AS	0.24	0.14	0.42	-1.43	0.28	5.3412E-09	-13.00%
cg12355110	19	57617754		5.40	2.72	10.74	1.69	0.35	5.4488E-09	12.23%
cg07035165	5	113698506	KCNN2	6.30	2.76	14.38	1.84	0.42	5.4841E-09	12.62%
cg07338917	13	112728964		6.00	2.84	12.65	1.79	0.38	5.5517E-09	7.20%
cg04402007	8	35093901	UNC5D	5.66	2.74	11.71	1.73	0.37	5.6008E-09	15.85%
cg14100973	4	87280816	MAPK10	4.80	2.56	9.01	1.57	0.32	5.6872E-09	19.08%
cg04996219	5	11904110	CTNND2	4.81	2.51	9.21	1.57	0.33	5.877E-09	22.39%
cg04597433	4	9783206	DRD5	5.12	2.69	9.74	1.63	0.33	6.025E-09	18.70%
cg01944624	19	58011140	ZNF773	6.76	2.71	16.86	1.91	0.47	6.0726E-09	19.37%
cg14035860	14	55707715		4.88	2.58	9.25	1.59	0.33	6.1374E-09	21.50%
cg21399832	4	117847410		4.77	2.52	9.04	1.56	0.33	6.1638E-09	16.48%
cg15685693	7	70597351	WBSCR17	4.19	2.41	7.29	1.43	0.28	6.2033E-09	18.02%
cg15617814	11	131780492	NTM	4.45	2.44	8.09	1.49	0.31	6.2042E-09	20.74%
cg00977827	1	149192743		5.76	2.64	12.55	1.75	0.40	6.7082E-09	16.23%
cg25001102	1	221068935		5.04	2.59	9.80	1.62	0.34	6.7726E-09	22.19%
cg02661372	1	181056912	IER5	0.26	0.15	0.43	-1.35	0.26	6.7997E-09	-11.13%
cg26755700	20	56689164		4.74	2.54	8.84	1.56	0.32	6.8655E-09	14.57%
cg14099514	5	41510519	PLCXD3	7.17	3.06	16.78	1.97	0.43	6.8752E-09	9.14%
cg08272731	1	75602167	LHX8	4.45	2.47	8.05	1.49	0.30	7.0525E-09	22.53%
cg05615464	15	54270557		8.47	3.31	21.68	2.14	0.48	7.08E-09	9.06%
cg07114310	7	51538921		4.34	2.41	7.82	1.47	0.30	7.1569E-09	11.04%
cg03502002	18	74962133	GALR1	4.96	2.58	9.55	1.60	0.33	7.2511E-09	20.18%
cg25784220	19	58609602	ZSCAN18	4.58	2.45	8.56	1.52	0.32	7.6095E-09	18.40%
cg12810297	1	199717229		4.72	2.52	8.84	1.55	0.32	7.6418E-09	13.22%

cg22001496	8	69243486		4.81	2.49	9.28	1.57	0.34	7.6843E-09	15.96%
cg10071824	13	28366759	GSX1	6.09	2.70	13.75	1.81	0.42	7.8435E-09	16.59%
cg13875518	20	21502218		9.51	3.38	26.73	2.25	0.53	7.9394E-09	9.85%
cg27615388	5	63257092	HTR1A	3.81	2.26	6.41	1.34	0.27	7.9487E-09	12.41%
cg20202112	2	74601685	DCTN1	4.82	2.55	9.11	1.57	0.32	8.0048E-09	15.02%
cg11548303	8	23567350		4.56	2.48	8.37	1.52	0.31	8.0419E-09	13.38%
cg07314384	2	132218291	RNU6-81P	6.15	2.82	13.44	1.82	0.40	8.123E-09	15.61%
cg01142635	4	6224117		4.87	2.55	9.28	1.58	0.33	8.1607E-09	11.59%
cg12354566	15	54270947		10.23	3.58	29.18	2.32	0.53	8.2642E-09	8.53%
cg23207710	3	192126960	FGF12	6.31	2.81	14.16	1.84	0.41	8.2704E-09	12.70%
cg25306915	8	35648588	UNC5D	0.18	0.09	0.37	-1.70	0.35	8.3226E-09	-12.88%
cg26469608	12	113913695		5.73	2.75	11.94	1.75	0.37	8.3329E-09	8.82%
cg08980987	12	25054905	BCAT1	4.78	2.57	8.86	1.56	0.32	8.3331E-09	16.73%
cg01866707	18	45862728		4.12	2.37	7.17	1.42	0.28	8.3441E-09	8.99%
cg06269753	8	72755871	MSC	6.67	2.87	15.53	1.90	0.43	8.3925E-09	12.50%
cg20890325	4	1239370	CTBP1	5.49	2.63	11.46	1.70	0.38	8.6849E-09	18.86%
cg11064255	18	5629890		6.76	2.71	16.82	1.91	0.47	8.8276E-09	15.68%
cg03357798	8	69243285		4.82	2.52	9.24	1.57	0.33	9.1753E-09	12.16%
cg12648074	5	140864474	PCDHGA4	7.33	3.03	17.69	1.99	0.45	9.4402E-09	13.57%
cg02882134	17	46942411	CALCOCO2	3.81	2.25	6.47	1.34	0.27	9.5509E-09	8.91%
cg21678377	2	115919009	DPP10	5.51	2.65	11.44	1.71	0.37	9.8109E-09	16.15%
cg26150462	10	11206868	CUGBP2	7.93	2.99	21.02	2.07	0.50	9.8514E-09	8.43%
cg21331821	19	58545149	ZSCAN1	4.16	2.32	7.48	1.43	0.30	9.9524E-09	9.20%
cg03109827	10	133110349	TCERG1L	4.16	2.37	7.33	1.43	0.29	1.0051E-08	16.17%
cg12664749	1	199717464		4.15	2.41	7.16	1.42	0.28	1.0146E-08	18.24%
cg18716164	19	30019647	VSTM2B	4.43	2.38	8.27	1.49	0.32	1.0157E-08	17.22%
cg00688962	4	21950567	KCNIP4	3.79	2.28	6.28	1.33	0.26	1.0311E-08	20.57%
cg12333537	18	26485722		0.25	0.15	0.43	-1.37	0.27	1.0791E-08	-15.47%

cg23934404	13	112758491		6.31	2.76	14.42	1.84	0.42	1.0839E-08	14.54%
cg11473001	11	131780499	NTM	4.11	2.33	7.23	1.41	0.29	1.0938E-08	15.32%
cg01445580	1	34631891	C1orf94;CSMD2	5.36	2.57	11.17	1.68	0.37	1.1243E-08	15.52%
cg22455450	19	53038972	ZNF808	8.88	3.20	24.63	2.18	0.52	1.127E-08	12.51%
cg15556502	11	43602845	MIR129-2	4.62	2.48	8.62	1.53	0.32	1.1304E-08	15.19%
cg01696784	2	235904501	SH3BP4	4.65	2.48	8.71	1.54	0.32	1.1517E-08	23.89%
cg24211976	15	60285744		4.81	2.59	8.94	1.57	0.32	1.1973E-08	29.64%
cg23591955	14	52294766		0.25	0.14	0.42	-1.40	0.28	1.2029E-08	-10.66%
cg16389285	15	26108399	ATP10A	4.40	2.36	8.23	1.48	0.32	1.21E-08	15.11%
cg12492087	15	42749885	ZFP106	4.34	2.41	7.80	1.47	0.30	1.221E-08	22.84%
cg26183190	6	33754751	LEMD2	4.12	2.30	7.37	1.42	0.30	1.2224E-08	10.34%
cg08396985	2	5896489		4.56	2.47	8.42	1.52	0.31	1.2305E-08	27.72%
cg04711162	19	30017511	VSTM2B	5.98	2.69	13.29	1.79	0.41	1.2519E-08	13.82%
cg06792417	3	137487857		7.04	2.96	16.76	1.95	0.44	1.2732E-08	9.72%
cg22446630	9	126360170	DENND1A	3.86	2.25	6.61	1.35	0.27	1.2909E-08	11.65%
cg23843400	11	10747348		0.26	0.15	0.44	-1.35	0.28	1.3119E-08	-5.42%
cg00116234	9	18474243	ADAMTSL1	6.53	2.84	15.01	1.88	0.42	1.3129E-08	11.43%
cg16927040	3	187388128	SST	5.33	2.62	10.84	1.67	0.36	1.3174E-08	14.12%
cg25514475	5	171693762	UBTD2	4.16	2.39	7.25	1.43	0.28	1.3221E-08	16.69%
cg08194879	6	123317569	CLVS2	3.73	2.21	6.29	1.32	0.27	1.3421E-08	13.87%
cg01972979	X	133680446	MGC16121;MIR503	4.53	2.47	8.31	1.51	0.31	1.3651E-08	20.11%
cg24426044	3	178254281	KCNMB2;KCNMB2-AS1	4.57	2.47	8.47	1.52	0.31	1.3818E-08	11.83%
cg03323696	5	59189120	PDE4D	4.64	2.51	8.60	1.53	0.31	1.3825E-08	12.54%
cg09868598	5	50674015		4.95	2.56	9.56	1.60	0.34	1.3959E-08	11.70%
cg25407557	12	132168981		3.90	2.29	6.67	1.36	0.27	1.4125E-08	6.90%
cg15160644	6	151335863	MTHFD1L	3.31	2.08	5.27	1.20	0.24	1.4373E-08	4.38%
cg16415863	19	17516339	BST2;MVB12A;BISPR	0.25	0.14	0.43	-1.40	0.28	1.4438E-08	-17.58%

cg00266009	8	85095275	RALYL	8.89	3.18	24.89	2.18	0.53	1.4532E-08	8.02%
cg04819716	7	153749318	DPP6	5.06	2.57	9.99	1.62	0.35	1.492E-08	16.35%
cg22376706	12	71314612	PTPRR	5.06	2.54	10.09	1.62	0.35	1.516E-08	12.76%
cg16587707	11	2920265	SLC22A18AS	0.20	0.11	0.39	-1.59	0.33	1.5325E-08	-8.78%
cg09040797	12	63544768	AVPR1A	4.54	2.44	8.46	1.51	0.32	1.5411E-08	17.97%
cg02292901	10	11001688		0.20	0.10	0.39	-1.62	0.35	1.5552E-08	-10.06%
cg19469068	8	69243293	C8orf34;C8orf34-AS1	3.79	2.22	6.49	1.33	0.27	1.5586E-08	19.37%
cg06051311	6	30131001	TRIM15	0.28	0.17	0.47	-1.26	0.26	1.6125E-08	-12.17%
cg26691307	5	113698769	KCNN2	7.25	3.13	16.79	1.98	0.43	1.6163E-08	7.61%
cg07488625	19	13617171	CACNA1A	4.61	2.35	9.05	1.53	0.34	1.6478E-08	13.28%
cg16393481	7	155607461		0.27	0.17	0.45	-1.29	0.25	1.6522E-08	-9.47%
cg06820905	8	69243752		5.53	2.56	11.94	1.71	0.39	1.6834E-08	11.39%
cg22370295	1	237205384	RYR2	4.41	2.43	7.99	1.48	0.30	1.6892E-08	20.80%
cg07369950	19	58609987	ZSCAN18	6.72	2.81	16.06	1.91	0.44	1.6997E-08	10.80%
cg14367229	13	28367741	GSX1	6.69	2.85	15.70	1.90	0.44	1.7019E-08	10.70%
cg05827312	13	28394966		5.42	2.66	11.03	1.69	0.36	1.7191E-08	10.36%
cg10124812	2	54087517	GPR75;LOC100302652	4.35	2.42	7.81	1.47	0.30	1.732E-08	13.50%
cg14060496	20	61638518	BHLHE23	4.07	2.28	7.25	1.40	0.29	1.7549E-08	17.25%
cg04436994	1	221055665	HLX	4.30	2.40	7.70	1.46	0.30	1.7654E-08	15.35%
cg20082563	5	10485736		0.25	0.15	0.43	-1.38	0.28	1.7954E-08	-17.76%
cg02721902	17	46825371		4.61	2.44	8.67	1.53	0.32	1.8169E-08	12.23%
cg09977449	17	50236098	CA10	5.00	2.54	9.84	1.61	0.35	1.8484E-08	15.45%
cg23688510	6	166581929	T	10.69	3.05	37.51	2.37	0.64	1.9116E-08	13.26%
cg08146483	1	75602412	LHX8	4.17	2.33	7.46	1.43	0.30	1.914E-08	14.81%
cg15669183	13	108518621	FAM155A	5.57	2.64	11.73	1.72	0.38	1.938E-08	12.08%
cg18881778	12	120241287	CIT	4.01	2.31	6.98	1.39	0.28	1.9493E-08	15.44%
cg20311863	1	91184126	BARHL2	4.93	2.48	9.80	1.60	0.35	1.959E-08	16.46%

cg18780257	3	192126023	FGF12	4.89	2.50	9.58	1.59	0.34	1.9885E-08	17.57%
cg27607773	2	28066049	RBKS	4.32	2.42	7.72	1.46	0.30	1.9909E-08	21.52%
cg22579471	5	55199404	IL31RA	0.26	0.15	0.44	-1.34	0.27	2.0031E-08	-11.13%
cg21236576	15	28371423	HERC2	3.86	2.24	6.64	1.35	0.28	2.0465E-08	10.96%
cg25095032	6	31696423	DDAH2	5.36	2.63	10.90	1.68	0.36	2.0617E-08	21.16%
cg07129725	19	56915266	ZNF583	4.99	2.44	10.18	1.61	0.36	2.0798E-08	7.46%
cg25570291	1	158985360	IFI16	0.27	0.16	0.45	-1.32	0.27	2.0935E-08	-14.22%
cg23615676	5	113697632	KCNN2	7.36	2.94	18.46	2.00	0.47	2.1028E-08	9.15%
cg12055940	11	71421279		4.08	2.32	7.19	1.41	0.29	2.1348E-08	5.71%
cg17165848	2	175462452	WIPF1	0.25	0.14	0.43	-1.40	0.28	2.1833E-08	-10.39%
cg07557260	8	132052870	ADCY8	4.77	2.47	9.21	1.56	0.34	2.2155E-08	12.37%
cg00757182	19	58011393	ZNF773	4.73	2.38	9.40	1.55	0.35	2.2294E-08	12.17%
cg13471424	1	149223500		5.54	2.63	11.70	1.71	0.38	2.2456E-08	13.08%
cg19717586	11	131781257	NTM	4.33	2.35	7.95	1.46	0.31	2.2561E-08	25.33%
cg23642130	19	30866365	ZNF536	4.26	2.35	7.72	1.45	0.30	2.2644E-08	18.13%
cg12785694	3	160122168	SMC4;MIR15B;MIR 16-2	0.24	0.14	0.43	-1.42	0.29	2.2794E-08	-20.56%
cg16023896	11	3250827	MRGPRE	0.16	0.07	0.35	-1.83	0.40	2.2811E-08	-7.38%
cg18191540	14	107281437		0.26	0.15	0.43	-1.36	0.27	2.2818E-08	-16.85%
cg20407119	11	56549867		0.24	0.14	0.42	-1.42	0.29	2.3048E-08	-9.71%
cg17393267	3	192127356	FGF12	8.74	2.72	28.08	2.17	0.60	2.3089E-08	13.21%
cg03881775	3	147111135	ZIC4	4.17	2.31	7.51	1.43	0.30	2.3182E-08	14.94%
cg10982443	14	20903611	KLHL33	5.04	2.54	9.99	1.62	0.35	2.3321E-08	23.49%
cg16055378	19	30015991	VSTM2B;LOC28439 5	4.05	2.29	7.17	1.40	0.29	2.3568E-08	15.70%
cg09876519	6	50675610		6.22	2.82	13.73	1.83	0.40	2.3591E-08	10.02%
cg12613383	1	242688681	PLD5	6.87	2.91	16.23	1.93	0.44	2.36E-08	8.96%
cg00688840	14	106363051		0.27	0.16	0.45	-1.32	0.27	2.3836E-08	-9.83%
cg06600429	5	160975956	GABRB2	6.75	2.89	15.79	1.91	0.43	2.398E-08	12.22%

cg05468843	11	117857990	IL10RA	0.26	0.15	0.45	-1.33	0.28	2.4006E-08	-11.26%
cg17535595	13	53422808	PCDH8	4.81	2.41	9.62	1.57	0.35	2.4113E-08	10.73%
cg08572394	5	76932996	OTP	5.37	2.60	11.11	1.68	0.37	2.4355E-08	14.30%
cg19723208	1	62996435	DOCK7	4.41	2.40	8.10	1.48	0.31	2.4367E-08	17.65%
cg04580344	6	127797022	C6orf174	3.81	2.21	6.58	1.34	0.28	2.4637E-08	7.38%
cg12553083	1	108492681	VAV3	0.26	0.15	0.45	-1.34	0.27	2.4793E-08	-15.73%
cg18115064	14	48143618	MDGA2	8.86	3.16	24.84	2.18	0.53	2.5082E-08	9.43%
cg10237903	8	139509075	FAM135B	3.84	2.22	6.63	1.34	0.28	2.5326E-08	19.70%
cg22489957	6	28921805		4.69	2.48	8.90	1.55	0.33	2.548E-08	9.78%
cg11877129	12	128752356	TMEM132C	6.61	2.83	15.44	1.89	0.43	2.5483E-08	10.19%
cg10778841	10	106401319	SORCS3	3.65	2.18	6.11	1.30	0.26	2.5714E-08	18.10%
cg22049569	19	54466538	CACNG8	4.17	2.30	7.54	1.43	0.30	2.6019E-08	10.13%
cg08790440	2	124782831	CNTNAP5	4.02	2.26	7.13	1.39	0.29	2.6052E-08	17.99%
cg09989037	1	44300942	ST3GAL3	0.29	0.18	0.47	-1.25	0.25	2.6088E-08	-10.64%
cg13849378	1	241520331	RGS7	4.08	2.26	7.38	1.41	0.30	2.6229E-08	21.03%
cg19928294	6	102943909		0.26	0.15	0.44	-1.37	0.28	2.639E-08	-10.73%
cg12746465	20	57782636	ZNF831	0.20	0.10	0.39	-1.60	0.33	2.655E-08	-9.19%
cg09170366	3	152374157		4.65	2.38	9.08	1.54	0.34	2.6615E-08	12.05%
cg12431123	12	103351432	ASCL1	7.05	2.87	17.33	1.95	0.46	2.6913E-08	13.94%
cg10182317	6	123317487	CLVS2	3.96	2.28	6.88	1.38	0.28	2.6919E-08	18.72%
cg23861668	11	20691126	NELL1	4.46	2.38	8.34	1.50	0.32	2.6957E-08	18.22%
cg07726288	1	248855278		3.87	2.24	6.68	1.35	0.28	2.7063E-08	7.48%
cg22457238	19	58570995	ZNF135	5.77	2.62	12.68	1.75	0.40	2.7075E-08	11.50%
cg20318143	19	17516370	BST2;MVB12A;BIS PR	0.26	0.15	0.44	-1.37	0.28	2.716E-08	-12.64%
cg22038124	15	51396175	TNFAIP8L3	4.15	2.32	7.42	1.42	0.30	2.7179E-08	14.60%
cg03732161	9	18473918	ADAMTSL1	4.70	2.44	9.05	1.55	0.33	2.7309E-08	12.52%
cg24238849	20	21686715	PAX1	5.80	2.68	12.51	1.76	0.39	2.7361E-08	9.97%

cg18729357	19	53496893	ZNF702P	5.12	2.53	10.35	1.63	0.36	2.7515E-08	11.86%
cg23034757	5	140787864	PCDHGA4	6.08	2.75	13.47	1.81	0.41	2.7726E-08	11.10%
cg14519115	5	41510650	PLCXD3	8.20	3.11	21.62	2.10	0.49	2.7879E-08	8.78%
cg08326559	7	103498178	RELN	3.97	2.25	6.99	1.38	0.29	2.8243E-08	12.53%
cg06735418	6	55911430		0.29	0.18	0.48	-1.23	0.25	2.8331E-08	-18.57%
cg10691901	9	74062074		5.49	2.60	11.60	1.70	0.38	2.883E-08	11.26%
cg15638709	10	110226387		4.08	2.30	7.22	1.41	0.29	2.9122E-08	18.95%
cg13445796	11	20181725	DBX1	4.41	2.35	8.26	1.48	0.32	2.933E-08	15.30%
cg12169233	19	11959578		8.79	2.81	27.49	2.17	0.58	2.9426E-08	11.94%
cg03192421	20	10643245	JAG1	3.94	2.25	6.90	1.37	0.29	2.9497E-08	17.01%
cg14323415	X	7909363		3.80	2.21	6.54	1.34	0.28	2.9564E-08	9.03%
cg09632273	6	31696229	DDAH2	4.45	2.40	8.25	1.49	0.31	2.9663E-08	21.21%
cg25282723	2	208174949		3.72	2.23	6.21	1.31	0.26	2.9909E-08	15.26%
cg10769146	1	214156644		6.75	2.86	15.95	1.91	0.44	2.9982E-08	6.43%
cg21093192	19	54412993		5.58	2.58	12.07	1.72	0.39	3.0107E-08	14.70%
cg05353133	1	229567914	ACTA1	4.78	2.46	9.29	1.57	0.34	3.0304E-08	9.09%
cg03313364	17	40440904	STAT5A	0.23	0.13	0.43	-1.45	0.31	3.036E-08	-10.84%
cg00038681	3	77517922	ROBO2	4.11	2.33	7.24	1.41	0.29	3.0644E-08	15.88%
cg19632836	13	112332213		4.01	2.28	7.06	1.39	0.29	3.0648E-08	20.07%
cg26549174	19	10381355	ICAM1	0.26	0.15	0.44	-1.35	0.27	3.0855E-08	-11.57%
cg02763101	19	56904945	ZNF582	5.15	2.41	10.97	1.64	0.39	3.0896E-08	21.50%
cg23095192	2	145271307	ZEB2	4.50	2.41	8.41	1.50	0.32	3.1146E-08	11.92%
cg27273868	6	162075441	PARK2	0.26	0.15	0.45	-1.35	0.28	3.1178E-08	-4.64%
cg22702328	17	50235965	CA10	4.76	2.45	9.25	1.56	0.34	3.1385E-08	15.64%
cg03684768	10	120000694		4.08	2.25	7.41	1.41	0.30	3.1472E-08	16.16%
cg23108778	1	7616070	CAMTA1	0.26	0.16	0.45	-1.33	0.27	3.1542E-08	-11.76%
cg05336115	20	983104	RSPO4	6.25	2.71	14.41	1.83	0.43	3.1665E-08	11.14%
cg16988262	1	15930761		0.27	0.15	0.46	-1.33	0.29	3.1833E-08	-14.64%

cg11338643	6	166580983	T	6.94	2.83	17.02	1.94	0.46	3.1908E-08	11.97%
cg12006284	11	32449638	WT1	4.25	2.34	7.70	1.45	0.30	3.2022E-08	11.44%
cg14972743	19	58609960	ZSCAN18	5.36	2.57	11.16	1.68	0.37	3.2058E-08	12.31%
cg11850773	18	904963	ADCYAP1	4.17	2.32	7.51	1.43	0.30	3.2094E-08	15.41%
cg10494860	20	62693951	TCEA2	4.20	2.36	7.48	1.44	0.29	3.2573E-08	14.53%
cg16451391	5	70741802		4.26	2.37	7.67	1.45	0.30	3.2634E-08	10.51%
cg07194250	X	133680463	MGC16121;MIR503	4.35	2.39	7.90	1.47	0.31	3.2971E-08	14.09%
cg05057720	14	38724675	CLEC14A	3.78	2.21	6.47	1.33	0.27	3.3084E-08	18.84%
cg24348495	20	62693971	TCEA2	4.58	2.45	8.59	1.52	0.32	3.3156E-08	21.23%
cg14323675	9	17907018		6.00	2.72	13.21	1.79	0.40	3.3246E-08	11.95%
cg21497056	7	70597354	WBSCR17	3.80	2.23	6.47	1.33	0.27	3.3429E-08	17.97%
cg07393736	3	172167810		5.59	2.64	11.83	1.72	0.38	3.3453E-08	14.05%
cg10676084	15	27017560	GABRB3	4.85	2.46	9.54	1.58	0.35	3.3525E-08	11.36%
cg16509658	5	160975347	GABRB2	4.63	2.29	9.35	1.53	0.36	3.359E-08	10.15%
cg22762091	8	132052843	ADCY8	3.97	2.26	6.97	1.38	0.29	3.3718E-08	16.63%
cg03307893	15	26108683	ATP10A	4.99	2.39	10.43	1.61	0.38	3.3889E-08	12.50%
cg20167366	6	30094980		4.77	2.44	9.34	1.56	0.34	3.4363E-08	13.97%
cg05181865	19	58570652	ZNF135	3.55	2.11	5.95	1.27	0.26	3.4392E-08	10.12%
cg04475027	12	128752040	TMEM132C	4.74	2.44	9.22	1.56	0.34	3.4462E-08	16.59%
cg08074851	2	47797590	KCNK12	5.03	2.46	10.25	1.61	0.36	3.4747E-08	14.99%
cg18072285	5	95808995	LOC101929710	0.28	0.17	0.46	-1.28	0.26	3.5514E-08	-10.23%
cg24621437	10	108924560	SORCS1	4.40	2.34	8.26	1.48	0.32	3.5597E-08	14.78%
cg03818930	6	39095898		0.26	0.16	0.45	-1.33	0.27	3.6059E-08	-8.34%
cg04513294	6	40919057	LOC101929555	3.73	2.17	6.42	1.32	0.28	3.6438E-08	14.04%
cg13999330	7	2472814	CHST12	6.08	2.62	14.06	1.80	0.43	3.6495E-08	12.10%
cg06330323	16	2132315	TSC2	4.95	2.46	9.96	1.60	0.36	3.6703E-08	23.04%
cg19058262	19	1231292	C19orf26	4.06	2.25	7.36	1.40	0.30	3.6723E-08	8.95%
cg01395723	5	113696634	KCNN2	5.07	2.46	10.43	1.62	0.37	3.6779E-08	14.28%

cg26245879	3	10375226	ATP2B2	0.27	0.16	0.45	-1.32	0.26	3.7217E-08	-7.56%
cg22976218	2	182544321	NEUROD1	6.16	2.78	13.62	1.82	0.41	3.7313E-08	11.39%
cg08851784	5	22212938	CDH12	4.32	2.36	7.90	1.46	0.31	3.747E-08	10.21%
cg07733830	7	63768483		3.88	2.24	6.70	1.35	0.28	3.7474E-08	14.87%
cg22047387	12	55413872	NEUROD4	5.42	2.58	11.40	1.69	0.38	3.7518E-08	10.55%
cg04471914	7	153749310	DPP6	4.08	2.29	7.29	1.41	0.30	3.7554E-08	18.02%
cg01718742	16	62070184	CDH8	4.09	2.30	7.25	1.41	0.29	3.7657E-08	13.60%
cg10119075	10	7709738	ITIH5	6.10	2.67	13.93	1.81	0.42	3.7847E-08	11.77%
cg14724613	7	86273429	GRM3	7.66	3.00	19.54	2.04	0.48	3.7941E-08	9.28%
cg14403099	X	51360708		3.61	2.15	6.07	1.28	0.26	3.8046E-08	7.34%
cg09840146	5	140705747		5.65	2.62	12.17	1.73	0.39	3.8081E-08	11.31%
cg13135595	18	77558846		4.88	2.35	10.14	1.59	0.37	3.8828E-08	10.43%
cg11227141	1	2003650	PRKCZ	3.36	2.06	5.49	1.21	0.25	3.8846E-08	8.79%
cg04094811	6	1393336	FOXF2	6.78	2.80	16.38	1.91	0.45	3.8896E-08	12.53%
cg00816164	3	20394477		0.24	0.13	0.43	-1.42	0.30	3.917E-08	-13.87%
cg14119999	13	108518445	FAM155A	6.37	2.72	14.88	1.85	0.43	3.9256E-08	12.52%
cg09577391	19	58515046	ZNF606	4.13	2.31	7.36	1.42	0.30	3.9361E-08	9.23%
cg16638540	19	58570468	ZNF135	4.93	2.49	9.77	1.59	0.35	3.9487E-08	12.32%
cg14445507	7	152890360		4.26	2.28	7.94	1.45	0.32	3.9489E-08	8.82%
cg19839825	19	7616011	PNPLA6	4.64	2.44	8.83	1.54	0.33	3.9505E-08	17.34%
cg11224418	21	35897663	RCAN1	3.92	2.25	6.80	1.37	0.28	3.9651E-08	19.35%
cg01485075	19	22817371	ZNF492	4.84	2.38	9.83	1.58	0.36	3.9666E-08	14.69%
cg06640533	16	58197401	CSNK2A2	3.18	2.00	5.05	1.16	0.24	3.9995E-08	4.86%
cg18210226	6	123317714	CLVS2	4.40	2.37	8.17	1.48	0.32	4.0132E-08	15.29%
cg26609631	13	28366814	GSX1	3.79	2.19	6.56	1.33	0.28	4.0183E-08	21.51%
cg22607266	1	199717290		3.83	2.24	6.55	1.34	0.27	4.032E-08	17.18%
cg16661508	12	79258427	SYT1	5.00	2.48	10.10	1.61	0.36	4.0362E-08	12.37%
cg09406615	3	165555103	BCHE	4.45	2.36	8.40	1.49	0.32	4.0585E-08	14.20%

cg23245720	X	133680549	MGC16121;MIR503	4.52	2.38	8.58	1.51	0.33	4.0615E-08	15.72%
cg03189837	5	59817962	PART1	0.27	0.16	0.46	-1.30	0.26	4.079E-08	-10.71%
cg00446413	7	153749206	DPP6	4.81	2.42	9.56	1.57	0.35	4.1447E-08	13.29%
cg07166360	3	137483071	SOX14	4.90	2.41	9.96	1.59	0.36	4.1468E-08	10.47%
cg11098290	17	63355141		0.21	0.11	0.41	-1.57	0.34	4.1505E-08	-6.76%
cg15148973	10	122333392	PPAPDC1A	0.25	0.14	0.44	-1.38	0.29	4.1509E-08	-12.35%
cg12641543	19	57615001		5.49	2.58	11.67	1.70	0.39	4.1563E-08	10.30%
cg04023663	7	7031200		3.54	2.14	5.86	1.26	0.26	4.1826E-08	9.31%
cg20450979	4	176923542	GPM6A	5.23	2.41	11.35	1.65	0.40	4.2242E-08	11.37%
cg01041222	4	158142863	GRIA2	5.21	2.56	10.62	1.65	0.36	4.2299E-08	7.54%
cg25830612	22	26242380	MYO18B	0.26	0.16	0.45	-1.33	0.27	4.2483E-08	-12.21%
cg12976081	3	147105899	ZIC4	6.16	2.71	14.00	1.82	0.42	4.2506E-08	10.03%
cg11465936	X	152647550		0.27	0.16	0.46	-1.30	0.26	4.3047E-08	-5.91%
cg19844799	20	39439001		0.22	0.12	0.41	-1.51	0.32	4.3171E-08	-8.12%
cg05923226	19	15121516	CCDC105	5.03	2.53	10.01	1.62	0.35	4.333E-08	9.23%
cg04467624	20	55799928	BMP7	3.84	2.23	6.60	1.35	0.28	4.359E-08	13.99%
cg23055735	11	70557464	SHANK2	3.94	2.27	6.85	1.37	0.28	4.4071E-08	14.90%
cg11595545	1	111217497	KCNA3	5.19	2.41	11.19	1.65	0.39	4.4293E-08	19.45%
cg22164298	4	114276834	ANK2	0.29	0.18	0.47	-1.25	0.25	4.4821E-08	-15.25%
cg00800512	19	58095518	ZIK1	4.72	2.37	9.40	1.55	0.35	4.5252E-08	13.38%
cg21660452	11	64397807	NRXN2	5.19	2.62	10.30	1.65	0.35	4.5984E-08	20.48%
cg22685409	11	30038692	KCNA4	4.98	2.46	10.10	1.61	0.36	4.6135E-08	10.60%
cg10746084	6	169654126	THBS2	4.19	2.30	7.63	1.43	0.31	4.6272E-08	6.61%
cg08620154	8	69244553		6.88	2.64	17.89	1.93	0.49	4.6299E-08	13.39%
cg27528660	8	72987924	TRPA1	4.48	2.37	8.46	1.50	0.32	4.6313E-08	13.97%
cg15489250	14	36994906		6.36	2.57	15.74	1.85	0.46	4.6451E-08	14.82%
cg11014373	2	105479054		5.08	2.48	10.40	1.62	0.37	4.6547E-08	13.70%
cg23878255	7	141645174	CLEC5A	0.27	0.16	0.46	-1.31	0.27	4.6607E-08	-20.64%

cg23664334	1	149288143	LOC388692	4.78	2.45	9.36	1.57	0.34	4.7172E-08	6.26%
cg02139965	5	169739749		0.27	0.16	0.46	-1.30	0.27	4.7362E-08	-14.84%
cg22478591	4	176923544	GPM6A	6.53	2.67	15.97	1.88	0.46	4.7363E-08	9.92%
cg23673107	19	11908712	ZNF491	4.05	2.25	7.29	1.40	0.30	4.7385E-08	7.23%
cg21130374	21	42734266	MX2	0.26	0.15	0.45	-1.33	0.27	4.7514E-08	-20.94%
cg09111484	12	80749651		0.24	0.13	0.44	-1.43	0.31	4.78E-08	-5.86%
cg14043737	20	61637672	BHLHE23	5.14	2.50	10.56	1.64	0.37	4.8021E-08	9.66%
cg25734490	12	103351188	ASCL1	5.55	2.49	12.34	1.71	0.41	4.8209E-08	8.10%
cg24520975	6	31651362		3.80	2.21	6.52	1.34	0.28	4.843E-08	13.61%
cg19822343	5	180527495		6.10	2.67	13.92	1.81	0.42	4.8738E-08	10.59%
cg14069287	4	169631613	PALLD	4.25	2.36	7.66	1.45	0.30	4.8776E-08	10.44%
cg26490272	8	72756153	MSC;MSC-AS1	4.11	2.22	7.62	1.41	0.31	4.8874E-08	19.56%
cg14023149	17	72199483	RPL38	6.05	2.72	13.43	1.80	0.41	4.9039E-08	9.08%
cg03239178	12	103358881		6.27	2.70	14.54	1.84	0.43	4.9293E-08	10.34%
cg10866298	8	9760869	LOC157627	14.33	3.71	55.27	2.66	0.69	4.9475E-08	11.03%
cg07537187	9	88669060	GOLM1	3.34	2.05	5.43	1.21	0.25	4.9541E-08	8.10%
cg12373617	5	113698140	KCNN2	7.23	2.65	19.69	1.98	0.51	4.9729E-08	16.95%
cg03075534	1	240161447		3.34	2.07	5.40	1.21	0.24	4.9775E-08	17.96%
cg16430072	5	113696863	KCNN2	15.62	3.37	72.47	2.75	0.78	4.9976E-08	11.54%
cg13209481	14	36973483	SFTA3	4.57	2.35	8.88	1.52	0.34	5.039E-08	15.46%
cg06801852	14	105622992	JAG2	3.67	2.17	6.19	1.30	0.27	5.0757E-08	11.54%
cg19010490	15	37388127	MEIS2	4.04	2.23	7.34	1.40	0.30	5.0925E-08	16.60%
cg08481464	10	77871618	C10orf11	3.97	2.26	6.98	1.38	0.29	5.1114E-08	14.34%
cg15499265	9	117157330	AKNA	3.81	2.23	6.52	1.34	0.27	5.1401E-08	9.95%
cg20405017	17	50235393	CA10	3.92	2.24	6.89	1.37	0.29	5.188E-08	19.45%
cg24124798	19	58400275	ZNF814	5.41	2.52	11.59	1.69	0.39	5.2512E-08	9.45%
cg16020747	8	85095394	RALYL	3.67	2.17	6.21	1.30	0.27	5.2602E-08	26.21%
cg26848718	11	32454975	WT1	5.03	2.49	10.16	1.62	0.36	5.2756E-08	7.17%

cg07362849	19	17516901	BISPR;BST2;MVB1 2A	0.27	0.16	0.46	-1.30	0.27	5.2825E-08	-11.91%
cg07837260	9	19788564		5.31	2.57	10.97	1.67	0.37	5.2849E-08	10.91%
cg08812936	3	178254213	KCNMB2	3.97	2.24	7.05	1.38	0.29	5.2893E-08	11.94%
cg11687330	11	132953239	OPCML	5.31	2.47	11.38	1.67	0.39	5.3558E-08	12.80%
cg15498379	19	58609981	ZSCAN18	5.58	2.55	12.23	1.72	0.40	5.3681E-08	13.12%
cg13065624	1	170253617	LOC284688	3.59	2.15	5.98	1.28	0.26	5.3799E-08	14.47%
cg16304950	18	5543945	EPB41L3	6.79	2.66	17.36	1.92	0.48	5.3971E-08	13.79%
cg13155726	20	55800007	BMP7	3.88	2.22	6.78	1.36	0.28	5.4024E-08	8.68%
cg04034967	7	1309108		3.80	2.19	6.60	1.34	0.28	5.4148E-08	18.08%
cg19590598	2	127782813		4.14	2.29	7.51	1.42	0.30	5.4365E-08	12.54%
cg10706013	21	44104950	PDE9A	0.27	0.16	0.46	-1.31	0.27	5.4429E-08	-16.34%
cg13239420	1	240161249		4.10	2.26	7.45	1.41	0.30	5.4558E-08	17.81%
cg08061720	16	77619380		0.26	0.15	0.45	-1.36	0.28	5.4761E-08	-13.95%
cg18063733	5	50265443		4.50	2.34	8.65	1.50	0.33	5.5228E-08	13.76%
cg16862704	6	155189348		3.82	2.13	6.88	1.34	0.30	5.6329E-08	16.80%
cg09515921	1	221057558	HLX	4.33	2.36	7.96	1.47	0.31	5.6334E-08	22.63%
cg20229496	19	35395800		4.34	2.33	8.05	1.47	0.32	5.6397E-08	8.44%
cg26155697	5	52150386	ITGA1	4.00	2.26	7.08	1.39	0.29	5.6551E-08	15.90%
cg21806580	5	18746010		0.24	0.14	0.43	-1.42	0.30	5.6701E-08	-13.19%
cg15665203	13	112282481		3.76	2.21	6.40	1.32	0.27	5.6735E-08	16.17%
cg01874697	10	106401517	SORCS3	4.94	2.44	10.00	1.60	0.36	5.7161E-08	13.41%
cg03701266	5	10649678	ANKRD33B	4.12	2.31	7.36	1.42	0.30	5.7261E-08	9.21%
cg25114913	6	96463959	FUT9	4.67	2.37	9.23	1.54	0.35	5.7465E-08	14.38%
cg18702971	22	29920546	THOC5	3.48	2.08	5.82	1.25	0.26	5.7477E-08	6.37%
cg19484548	14	100953108	WDR25	3.59	2.11	6.11	1.28	0.27	5.7826E-08	12.26%
cg11325267	3	137480853		4.43	2.33	8.43	1.49	0.33	5.7868E-08	10.27%
cg22269543	1	214156497	PROX1-AS1	6.14	2.57	14.69	1.82	0.45	5.8661E-08	9.81%

cg03045635	4	9783198	DRD5	3.95	2.25	6.91	1.37	0.29	5.8747E-08	17.47%
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Appendix 20. Prediction performance as measured by the sensitivity and specificity for logistic models including cg16767801 and/or cg23642047 as predictor(s), and comparing controls (N=114) to CIN3 cases only (A, N=73) or cancer cases only (B, N=54). Performance metrics are reported as the mean, 5th and 95th quantiles computed over N=1,000 subsamples for different proportions of the population predicted as cases.

A

cg16767801			cg23642047			cg16767801 + cg23642047		
Proportion	Sensitivity	Specificity	Proportion	Sensitivity	Specificity	Proportion	Sensitivity	Specificity
10%	0.95 [0.95-0.96]	0.13 [0.12-0.13]	10%	0.95 [0.93-0.97]	0.13 [0.11-0.14]	10%	1.00 [1.00-1.00]	0.16 [0.16-0.16]
20%	0.90 [0.88-0.90]	0.26 [0.25-0.26]	20%	0.91 [0.90-0.93]	0.27 [0.26-0.28]	20%	0.97 [0.95-0.97]	0.30 [0.29-0.31]
30%	0.85 [0.84-0.86]	0.40 [0.39-0.40]	30%	0.87 [0.85-0.88]	0.41 [0.39-0.41]	30%	0.93 [0.93-0.95]	0.45 [0.45-0.46]
40%	0.80 [0.78-0.82]	0.53 [0.52-0.54]	40%	0.78 [0.77-0.79]	0.52 [0.51-0.53]	40%	0.90 [0.89-0.90]	0.59 [0.59-0.60]
50%	0.76 [0.74-0.77]	0.67 [0.66-0.68]	50%	0.70 [0.70-0.70]	0.63 [0.63-0.63]	50%	0.83 [0.82-0.84]	0.71 [0.71-0.72]
60%	0.72 [0.70-0.73]	0.80 [0.79-0.81]	60%	0.62 [0.62-0.63]	0.74 [0.74-0.75]	60%	0.74 [0.73-0.75]	0.82 [0.81-0.82]
70%	0.62 [0.62-0.63]	0.91 [0.90-0.91]	70%	0.47 [0.45-0.48]	0.81 [0.80-0.82]	70%	0.67 [0.66-0.68]	0.94 [0.93-0.95]
80%	0.49 [0.49-0.49]	0.99 [0.99-0.99]	80%	0.36 [0.34-0.37]	0.91 [0.89-0.91]	80%	0.48 [0.47-0.48]	0.98 [0.97-0.98]
90%	0.23 [0.23-0.23]	0.99 [0.99-0.99]	90%	0.20 [0.18-0.22]	0.97 [0.96-0.98]	90%	0.25 [0.25-0.25]	1.00 [1.00-1.00]

B

cg16767801			cg23642047			cg16767801 + cg23642047		
Proportion	Sensitivity	Specificity	Proportion	Sensitivity	Specificity	Proportion	Sensitivity	Specificity
10%	1.00 [1.00-1.00]	0.14 [0.14-0.14]	10%	0.96 [0.94-0.98]	0.12 [0.11-0.13]	10%	1.00 [0.98-1.00]	0.14 [0.13-0.14]
20%	0.97 [0.94-0.98]	0.27 [0.26-0.28]	20%	0.92 [0.91-0.93]	0.25 [0.25-0.25]	20%	0.99 [0.96-1.00]	0.28 [0.27-0.29]
30%	0.93 [0.91-0.94]	0.40 [0.39-0.41]	30%	0.90 [0.89-0.91]	0.39 [0.39-0.39]	30%	0.97 [0.96-0.98]	0.42 [0.42-0.43]
40%	0.91 [0.91-0.91]	0.54 [0.54-0.54]	40%	0.85 [0.81-0.87]	0.51 [0.50-0.53]	40%	0.96 [0.96-0.96]	0.57 [0.57-0.57]
50%	0.90 [0.89-0.91]	0.69 [0.68-0.69]	50%	0.76 [0.74-0.78]	0.62 [0.61-0.63]	50%	0.96 [0.96-0.96]	0.72 [0.72-0.72]
60%	0.85 [0.83-0.87]	0.82 [0.81-0.82]	60%	0.66 [0.65-0.67]	0.73 [0.72-0.73]	60%	0.96 [0.96-0.96]	0.87 [0.87-0.87]
70%	0.81 [0.81-0.81]	0.95 [0.95-0.95]	70%	0.57 [0.56-0.57]	0.83 [0.82-0.83]	70%	0.88 [0.87-0.89]	0.98 [0.97-0.98]
80%	0.61 [0.59-0.61]	1.00 [0.99-1.00]	80%	0.46 [0.44-0.48]	0.93 [0.92-0.94]	80%	0.61 [0.61-0.61]	1.00 [1.00-1.00]
90%	0.30 [0.30-0.30]	1.00 [1.00-1.00]	90%	0.30 [0.30-0.30]	1.00 [1.00-1.00]	90%	0.30 [0.30-0.30]	1.00 [1.00-1.00]

Appendix 21: Protocol for DTA meta-analysis of human and HPV DNA methylation in the triage of HPV positive women

TITLE: Diagnostic test accuracy of human and HPV DNA methylation in the triage of HPV positive women

Background

Cervical cancer continues to be the fourth most common cancer in women globally, with an estimated 570,000 cases and 311,000 deaths in 2018 ^{470,471}. Almost 99% of cervical cancers are caused by infection with human papillomavirus (HPV). More than 100 strains of HPV have been identified, but only 12 to 13 strains of HPV are thought to cause a neoplastic process (leading to the development of a precancer or cancer) and are classified as high-risk (hrHPV) ⁴⁷². Over 70% of women are infected with HPV during their lifetime. In a small number of those infected (8 to 10%), persistent hrHPV leads to development of precancerous lesions, which can progress to cancer over the following 10 to 30 years.

Cervical cancer is preceded by a precancerous lesion, namely Cervical Intraepithelial Neoplasia (CIN), which in most cases can be treated by surgical excision. Where implemented, call-recall cervical screening programmes have dramatically reduced incidence and mortality from cervical cancer through monitoring and treatment of CIN. Such programmes have traditionally relied on cytology from exfoliated cervical cells (ECC) (smear tests) for the primary screening test, cells were initially smeared onto a slide for examination (Papanicolaou 1941), until a new technique involving cellular suspension in a liquid medium was developed (LBC). Those with an abnormal cytology test collected by Papanicolaou smear or LBC were referred to colposcopy clinics for further evaluation. Here the cervix can be examined by a nurse or doctor Colposcopist, where under direct vision, cervical tissue biopsies are taken and examined for histological confirmation of disease status. Cytology testing involves examination of ECC under a microscope by a trained Cytologist technician, and is known to have limitations: including a low sensitivity for CIN (53 to 75%) ^{428,473,474}, high inter- and intra-observer variation and minimal future potential for robotic automation, point-of-care testing, and self-sampling. As a result, much research into alternative tests to Cytology, has been undertaken over recent years.

The association between hrHPV and Cervical Cancer led to investigation of hrHPV tests for detection for cervical lesions. Several clinical trials and now a Cochrane review have demonstrated that testing for the presence of hrHPV deoxyribonucleic acid (DNA) to be more sensitive than cytology for detection of precancerous lesions and cancer ⁴⁷⁵. Primary hrHPV DNA testing is now replacing cytology for screening in many countries, including the UK, Germany, Netherlands and USA Primary hrHPV DNA screening is anticipated to offer 60 to 70% greater protection against invasive cancer compared with cytology-based screening ⁴⁷⁶. However higher sensitivity for CIN is associated with a decreased specificity, resulting in a modest positive predictive value, in part due to the fact that the hrHPV positive prevalence in countries such as the UK is as high as 15%, reaching more than 30% in women aged 30 years or less ⁴⁷⁷. Women who screen positive are referred to colposcopy, this lower positive predictive value of primary hrHPV testing risks unnecessary examinations and over-management of women who screen hrHPV-positive. Consequently, a triage test of those found to be positive for hrHPV testing is needed to improve the specificity of the test and determine those who are truly at risk of precancerous and cancerous lesions. The best triage test for women who screen positive with a primary hrHPV-positive test is unclear and several different tests have potential. Reflex Cytology, which involves automatic cytology on hrHPV-positive samples, remains the test with the best historic safety data and is the proposed triage test used following an hrHPV-positive test in the current national guidelines in the UK (Figure 1). While in the USA, hrHPV genotyping is recommended – this test identifies specifically whether the two most common high-risk genotypes (HPV16 and HPV18) are present, which together are estimated to cause ~80% of disease. Other newer molecular tests are of increasing interest, including human and hrHPV methylation, HPV E6 and E7 mRNA testing and viral protein detection (e.g. HPV E4, E6, and E7 and P16/Ki-67 immunostaining). Such tests may offer the potential to study cellular activity and reveal underlying active disease processes, to improve accuracy in diagnosis of progressive CIN, whilst also allowing machine automation, self-sampling and point-of-care testing in future.

Human and/or hrHPV methylation tests, are anticipated to be one of the most promising triage tests for identification of hrHPV-positive women who have CIN or cancer. This systematic review aims to identify all

available diagnostic test accuracy studies of human and/or HPV methylation tests in the triage of women screening hrHPV-positive, to explore the diagnostic test accuracy.

Target condition being diagnosed

Infection by hrHPV types are necessary (although not sufficient) for the development of precancerous cervical lesions, known as cervical intraepithelial neoplasia (CIN), and cervical cancer. CIN is graded into three stages by the degree of involvement with abnormal cells in the cervical epithelium (surface layer of cervix): grade 1 involves only the lower third; grade 2 involves the lower two-thirds; and grade 3 involves the full-thickness of the epithelium ⁴⁷⁸.

CIN grades are also grouped into low grade lesions (CIN1) and high-grade lesions (CIN2-3); high-grade lesions are less likely to regress spontaneously and more likely to progress to a cancer ^{143,479,480}. In CIN all precancerous changes are limited to the epithelium, the layer lying above the barrier layer to the underlying tissue and blood vessels, called the basement membrane. Where the basement membrane is disrupted this becomes an invasive cervical cancer.

The majority of cervical cancers are of squamous cell type, involving the skin-like cells of the outer cervix (ectocervix). However, around 20% of cases lesions arise in the glandular cells of the inner cervix (endocervix). Precancerous lesions arising in the endocervix are defined as low- or high-grade cervical glandular intraepithelial lesions (CGIN) and can develop into cervical adenocarcinomas.

If identified, the majority of CIN and CGIN lesions can be effectively treated by surgical excision or ablation, to prevent progression to cervical cancer. Early stages of cervical cancer can also be effectively treated to increase survival.

Index tests to be considered

Methylation is a regulatory chemical process, which occurs at specific methylation sites in the DNA, called methylation 5-cytosine-phosphate-guanine-3 (CpG) sites. Such sites occur where cytosine and guanine DNA base-pairs lie next to each other in the genetic sequence. Methylation occurs during embryonic development, but commonly changes during the lifetime of living organisms in response to environmental stimuli. This causes the switching 'on and off' of specific genes to affect gene expression and determine downstream cellular processes.

Methylation is an early and frequent event in the development of many cancers, and is commonly observed at high levels in tumour tissues ⁴⁸¹. Technologies to examine DNA methylation have rapidly expanded over the last two decades following the sequencing of the genetic code. As such, the identification of methylation sites across the human genome has increased; millions of CpG sites are now thought to exist. Notably, viral DNA also contains methylation sites that influences gene expression.

Initial sequencing technologies included bisulphite clonal sequencing and q-MSP, pyrosequencing was then developed which allowed for a more accurate quantitative analysis of methylation at a particular site in the genetic code. More recently, next generation sequencing technologies have also been applied to estimate methylation. Pyrosequencing is currently considered the most accurate for quantitative allele quantification at a single base resolution ^{482,483}.

Methylation testing can be performed on the same sample of exfoliated cervical cells (ECC), suspended in liquid-based cytology preservative, as the original hrHPV test, reducing the need for further invasive sampling ⁴⁸⁴. Study of methylation in ECC from cervical precancer and cancer has identified multiple CpG sites in human and HPV genes where methylation levels appear to change as the process of cervical neoplasia progresses. Vaginal swabs and cervical swabs can be used to collect human and HPV DNA ⁴⁸⁵, but have a higher chance of being contaminated from cell types other than cervical epithelium, with unknown effects on site-specific methylation levels.

Diagnostic accuracy studies of human and HPV methylation as markers of high-grade cervical neoplasia have shown promising results^{486,487}, with good sensitivity and specificity^{143,488}. However, many different technologies have been used to study methylation, introducing high levels of variation in accuracy. Initial tests allowed only bivariate reporting of methylation at a CpG site, whereas more recent technologies allow a quantitative estimation from 1 to 100%. Additionally, varying cut-off thresholds have been used to declare a positive test. Further complexity is added because many different CpG sites in different genes have been studied. Methylation values may also vary by geographical environment and variation by ethnicity has not yet been adequately investigated. A wide range of sample materials have been used for methylation testing, with some studies using vaginal or cervical swabs (not considered suitable for routine cervical screening), the accuracy of sample materials other than exfoliated cervical cells remains unclear.

Clinical pathway for application of the test

This test will be evaluated as a triage test for diagnosis of high-grade CIN or Cancer in women identified as high risk (hr) HPV positive by primary cervical cancer screening. In the presence of a positive methylation test the woman would be considered at risk of high-grade disease and would need referral to colposcopy clinic for further management. In the presence of a negative methylation test the woman could be monitored by a repeat in screening (hrHPV and methylation test) at an appropriate time interval, according to the negative predictive value of the methylation test.

Alternative tests

There are two alternative tests in routine clinical use for the triage of hrHPV positive women detected at screening, which are cytology and HPV 16/18 genotyping⁴⁸⁹.

Cytology, as described above, is the traditional cervical screening test which involves smearing cervical cells on a slide for examination under the microscope. The abnormality of cellular appearance is labelled dyskaryosis and is graded dependent on pre-defined criteria as borderline, mild, moderate or severe dyskaryosis. A more recent criteria called the Bethesda system defines abnormalities as borderline, low-grade squamous intraepithelial neoplasia (LSIL) (replacing mild dyskaryosis) and high-grade squamous intraepithelial neoplasia (HSIL) (replacing moderate and severe dyskaryosis) (insert references). Glandular lesions may also be detected on cytology and is graded as low or high-grade, Table 1 provides a summary of cytological grades in comparison to histology. A cytology cut-off of HSIL or worse will be considered comparative to CIN2 or worse. Both Papanicalou smear and liquid based cytology (LBC) methods will be accepted as comparator tests.

HPV 16/18 genotyping can be performed by either DNA or mRNA testing for the presence of the HPV 16 or HPV18 virus, a wide range of commercial and non-commercial assays are available. As there is no single preferred assay then all technologies will be considered.

Rationale for the review

The best triage marker for improving detection of women with true high-grade precancerous and cancerous lesions, from women with hrHPV positive cervical screening is still unknown. This is an urgent unmet need; current screening algorithms of primary hrHPV screening with either cytology or HPV 16/18 genotyping as triage, are anticipated to greatly increase colposcopy referrals over coming years. Replacement of cytology with an improved triage marker for detection of CIN and cancer could reduce the burden of unnecessary appointments, anxiety, costs and over-treatment associated with false positive results, whilst ensuring an oncologically safe screening programme.

Methylation of human and HPV DNA have been proposed as novel molecular markers of cervical precancer and cancer with encouraging results⁴⁸⁴. But studies have explored different genes and CpG sites and applied various thresholds with inconsistent findings^{486,487}. Further work is needed to establish whether human or HPV CpG sites (alone or in combination), could be used as an accurate diagnostic triage test of hrHPV positive women.

This review aims to identify candidate CpG sites as methylation markers in both human and HPV genomes whilst summarising and exploring cut-off thresholds. Through meta-analysis we will estimate the pooled estimates for diagnostic accuracy of different candidate methylation tests, at all available thresholds and explore heterogeneity and risk of bias in the data.

Objectives

Primary Objective

The primary objective is to determine the diagnostic test accuracy of human and viral DNA methylation tests to triage women who are hrHPV positive at cervical cancer screening, for the detection of CIN grade 2 or worse.

Secondary objectives

The secondary objective is to evaluate diagnostic accuracy at different cut-off thresholds. We aim to explore the optimal cut-off thresholds for diagnostic test accuracy, by evaluating all cut-off thresholds that are provided for single (or combined) methylation CpG sites (human and/or HPV) in diagnosing CIN2+ or CIN3+ as defined by histology (or only colposcopy in normal cases) as a reference standard. Also, in the presence of any comparator test in the same study, we will analyse relative accuracy of DNA methylation to the alternative test, as provided cut-offs.

A further secondary objective is to investigate sources of heterogeneity, such as type of methylation test technology used, viral or human methylation, gene of methylation test, sample type, complete versus partial verification with the reference standard, geographical region of the study and ethnicity of the participants of the study.

Methods

Inclusion Criteria

Cross-sectional studies and longitudinal cohort studies, both prospective and retrospective studies, will be included where they assess the diagnostic test accuracy of human or HPV methylation marker (index test) in hrHPV DNA positive women, to detect high-grade pre-invasive cervical disease as confirmed by histology as the reference standard.

Randomised control trials should be included if they examine the index test(s) in the relevant population against the included reference standards, if true positives (TPs), true negatives (TNs), false positives (FPs) and false negatives (FNs) are reported, even if this means that these data are only included from one of the arms of the RCT.

We will include studies where hrHPV positivity is provided as either a) no genotyping detail, b) limited detail (if HPV16, 18, other hr types), c) extended (more detail than limited) or d) full genotyping detail (presence or absence of each HPV genotype, separately).

If available, we will separately analyse any complete diagnostic test accuracy studies providing concomitant testing of DNA methylation as compared to Cytology testing or HPV16/18 genotyping, in women with a positive hrHPV test.

Exclusion criteria

Case reports will be excluded as will studies in cell lines or tumour clones only. Studies that used only cytology as a reference standard will be excluded, and studies which do not use colposcopy directed histological biopsy for confirmation of a positive disease status (CIN1 or worse).

Population

Any population of hrHPV positive women representing a cervical screening setting or referral population will be included, without demographic restrictions, including retrospective biobanks of samples for women recruited from such populations, regardless of hrHPV genotype and sample material. The women should be close to or within an age group range similar to cervical screening (20 to 70 years).

Index tests

All DNA methylation tests will be considered i.e. human DNA methylation tests, hrHPV DNA methylation tests or tests which combine CpG sites from human and hrHPV tests into a single marker.

Regarding human DNA sites, there are millions of potential different CpG sites in the human genome, we will not limit the review to any particular genes, and will report all findings, however, we expect to require at least four studies of the same gene in order to present meta-analysis of diagnostic test accuracy results. For HPV genomes there are 8 genes, containing several hundred CpG sites, which vary according to HPV genotype. All genes will be considered but will be analysed separately according to HPV type. Assays can only be developed for a single genomic region spanning 2-300 base-pairs (bp), however many tests include 'multiple' sites by combining results of different assays into one test score. We will not restrict to either single or multiple site methylation tests but will aim to analyse separately where possible.

All human DNA and hrHPV DNA methylation technologies will be included for example we will include (but not limited to) bisulphite clonal sequencing, pyrosequencing, epiTYPER, next generation sequencing (NGS), Luminex, MS-HRM, MSP⁴⁹⁰. Both in-house laboratory assays and commercial assays will be included. As many technologies are still under investigation, all technologies and cut-off thresholds will be considered.

If any study is available with a comparison between the accuracy of cytology and/or HPV16/18 genotyping to methylation tests, then these will be treated as comparator tests and estimates of relative accuracy will be extracted and meta-analysed where possible.

Target condition

The primary target condition is high-grade cervical pre-invasive disease (CIN2 or CIN3) or Cervical Cancer, identification of which can allow treatment to prevent progression. This will be measured as CIN 2 or worse for squamous cell lesions and adenocarcinoma in situ glandular lesions by histology. For some studies a threshold of CIN3 or worse by histology may have been used. These will be included by analysed separately. The precise grades included in CIN2 or worse is therefore as follows:

- CIN2 or worse (CIN2+): CIN2, CIN3, H-CGIN, invasive squamous cell and adenocarcinoma of the cervix
- CIN3 or worse (CIN3+): CIN3, H-CGIN, invasive squamous cell and adenocarcinoma of the cervix

Reference standards

Histology, performed on a cervical tissue biopsy taken at colposcopy examination, is the preferred reference standard for diagnosis of high-grade CIN or cancer and will be included as according to colposcopy guidelines in the most recent NHS Cervical Screening Programme (NHSCSP) guidelines⁴⁷⁹. Where the colposcopic examination is abnormal, we will assume that the histological material taken as a reference standard is fully representative of the disease status (although full excision of the cervix and subsequent histological examination of the cervix would be necessary for full certainty of disease status, this causes significant morbidity and would not be ethically justifiable in precancer, if not clinically indicated). Of note, the NHSCSP 2020⁴⁷⁹ guidelines state that a tissue biopsy is not clinically indicated in the presence of a normal colposcopy. Therefore, for ethical considerations, we will not require a biopsy for the normal histological grading of disease. However, the confirmation of normal status by colposcopy alone, or colposcopy plus biopsy, will be considered in the risk of

bias assessment. If there are sufficient data then we will examine studies separately, that did or did not perform colposcopic biopsy for all patients. Verification bias will also be explored within the assessment of study quality.

Search methods for identification of studies

The literature search will start from January 1990 when the first methylation assays were developed and will include references published up to present day.

Separate search strategies will be developed for MEDLINE Ovid and Embase Ovid, according to the guidance provided in Chapter 7 of the Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy (de Vet 2008). Searches will be conducted without language or publication status restrictions. Draft Medline strategy can be found in Appendix 1. For databases other than MEDLINE, the search strategy will be adapted accordingly. The following trial registries will be searched for ongoing studies: US National Institutes of Health Ongoing Trials Register ClinicalTrials.gov; World Health Organization International Clinical Trials Registry Platform.

We will also search the MetaRegister of Controlled Trials (mRCT), National Cancer Institute (NCI) Physicists Data Query (PDQ), ClinicalTrials.gov and NCI Clinical Trials will be searched for ongoing studies. The main investigators of ongoing trials will be contacted for further information. Forward citation searching will be performed in Scopus/Web of Science. To identify studies which might have been missed during the electronic search, the citation lists of included studies will be hand-searched and experts in the field, including directors of UK cancer and colposcopy registries, contacted to identify further reports of studies. We will further check the proceedings of conferences via the Conference Proceeding Citation Index-science (CPCI-S) and specifically the following relevant conferences:

- Annual Meeting of the British Society of Colposcopy and Cervical Pathology.
- Annual Meeting of the International Federation of Cervical Pathology and Colposcopy.
- European Research Organisation on Genital Infection and Neoplasia (EUROGIN).
- European Federation of Colposcopy Congresses.
- Annual Meeting of the American Society of Colposcopy and Cervical Pathology.

Both published and unpublished studies will be included, if they meet the inclusion criteria for the review.

Data collection

Two review authors (SL and IK) will download all titles and abstracts retrieved by electronic searching to the reference management database EndNote (ref). We will remove duplicates and the remaining references will be examined independently by two authors. We will add titles and abstracts retrieved from other sources to Endnote. We will exclude those studies which clearly do not meet the inclusion criteria and will obtain copies of the full text of potentially relevant references. Two review authors (SL and IK) will independently assess the eligibility of retrieved papers. We will then compare the results and resolve any disagreements by discussion. Disagreements will be resolved by consensus, with discussion with a third review author (MK) if necessary. We will document reasons for exclusion.

Data extraction and management

Two review authors (SL and IK) will independently perform data extraction using a data extraction form. Disagreements will be resolved by consensus, with discussion with a third review author (MK) if necessary. We will extract data on the following items:

- Study design, population and setting: author, year, study design, any randomisation, study population, geography, ethnicity, total number of participants, hrHPV status (positive or negative), hrHPV assay used to define positivity, hrHPV genotype of HPV positive participants;
- Index test: t technology, CpG site(s) and location, gene, if HPV methylation test then hrHPV genotype, cut-off threshold used;

- Sample material: e.g. vaginal swab, cervical swab, ECC;
- Results: true positives, false positives, true negatives, false negatives will be extracted for each test and outcome (including for comparator test where available);
- Comparator: any comparator test including details of the technology;
- Reference standard: histological grade, cytological grade;
- Outcome measures e.g. CIN2+, CIN3+

We will extract data for different HPV types separately and for both individual CpG sites and combinations of CpG sites where available. Where data are missing (i.e. individual CpG sites data, or the absolute number of true- or false- positive or negative), we will contact the authors. and collate additional data for studies with missing data. In order to perform the analysis, the data for true positive, false positive, true negative and false negatives are all needed - where this data is still missing, we will exclude the study from the analysis. Where data for individual CpG sites is missing, we will perform the analysis at the gene level, or using combinations of CpG sites and present this appropriately.

Assessment of methodological quality

Two authors will (SB and KL) assess the risk of bias for selected studies using the QUADAS-2 tool independently^{491,492}. We will assign a judgement of low, moderate or high risk of bias to each of the domains (A) patient selection; B) index test; C) reference test; D) flow and timing, according to the criteria outlined in the Cochrane Handbook⁴⁹³. We will assess each domain using the signalling questions and answering yes (Y), No (N) or Unclear (U). We will tailor the signalling questions to the review as summarised in Appendix 2. An overall judgement will be formed in terms of risk of bias by recording information used to support the risk of bias judgement, the signalling questions and the judgement of high, low or unclear. For applicability we will record information on the applicability of each study, and rate as low, high or unclear (no score is applied for the flow and timing domain under applicability as according to QUADAS-2 guidance). In the case of RCTs, we will apply the Cochrane tool for assessment of the risk of bias in randomised trials and will verify whether used procedures are applicable in a real world setting.

Statistical analysis and data synthesis

In order to define methylation positivity, we will aim to explore and summarise different cut-off points of methylation used in the literature. When several studies present duplicate data from the same cohort, the study with the largest sample size will be selected for inclusion in the analysis.

For absolute diagnostic accuracy, we will use the true positive (TP), true negative (TN), false negative (FN) and false positive (FP), as defined by the individual studies in terms of presence of methylation positivity in the index test with comparison to presence of high-grade CIN or cancer (CIN2/HSIL+) in the reference standard. Where data for the absolute sensitivity and specificity for the detection of CIN3/HSIL+ is provided, we will calculate this separately.

It is important to note that different HPV types and genes require different test assays and these cannot be included in the same meta-analyses. We will therefore aim to pool accuracy from tests that identify similar targets, which we anticipate will involve several different analyses:

a) Analysis of tests using the same HPV type and CpG sites (human or viral); b) Analysis of tests using the same HPV types and genes (individual CpG sites are not available); c) Analysis of tests using multiple CpG sites across different genes and viral or human species (where the exact same combination of multiple CpG sites has been performed in different populations).

Different technologies will be combined in the above analyses, if the same CpG sites or same genes have been included.

We will first restrict to studies reporting a common threshold and will use the bivariate model ⁴⁹⁴ to estimate a summary sensitivity and specificity. Each study will contribute to the analysis once using TP, FN, TN, and FP. If there are sufficient data, we will additionally explore how different thresholds impact on the estimation of sensitivity and specificity using separate bivariate models. If there are no sufficient data to perform separate bivariate models, we will apply the HSROC model ⁴⁹⁵ to estimate a summary curve from studies that have used different thresholds. In case of multiple thresholds within each study, with different thresholds between studies, we will perform a linear mixed effects model accounting for heterogeneity and correlation between groups and using the TP, TN, FP, and FN values from all thresholds of each study. This model will estimate the optimal threshold, that is the point where the test is likely to perform best using the maximum value of the weighted sum of sensitivity and specificity ⁴⁹⁶. To explore heterogeneity, we will visually inspect forest plots and SROC plots, as well as by adding covariate terms to the HSROC/bivariate model to assess the effect of each study characteristic on the observed heterogeneity in the accuracy and threshold parameters (see also the Investigation of heterogeneity section). Analyses will be performed using the *mada* ⁴⁹⁷ and *diagmeta* ⁴⁹⁸ R packages.

In the case of any studies presenting concomitant data for methylation tests compared to alternative tests (cytology or HPV16/18 genotyping) we will examine relative accuracy. In METADAS, a SAS macro for meta-analysis of diagnostic test accuracy studies, the pooled relative sensitivity and specificity of the index tests versus comparator tests will be assessed by including the test as a covariate in the bivariate model ^{493,499}. From the point estimates, ratios of sensitivity and specificity can be computed.

Although we will not assess publication bias as according to latest Cochrane DTA guidance, we will assess whether only negative results are identified or whether only small studies provide supporting evidence for the test.

We will use empirical evidence to estimate the prevalence of high-grade CIN or cancer in the hrHPV positive population and report the expected TP, TN, FP and FN per 1000 women.

In case of multiple reports from the same study we will select the one with largest number of subjects included, unless the risk of bias is substantially higher than in the smaller report

Investigations of heterogeneity

We will assess the between-study heterogeneity through visual inspection of forest plots (variability in sensitivity and specificity). We will also sort paired forest plots by sensitivity to explore if a reverse trend in estimates of specificities is detected. If there is variation in threshold, we will also visually inspect whether the study-specific effects lie close to the summary ROC curve. The higher the scatter of the study-specific effect sizes and the larger the prediction ellipse, the higher the between-study heterogeneity. If variability can be explained by sample size, threshold effects, test variations or study characteristics, then more confidence can be placed in the meta-analysis results. If there are more than 10 studies available for a test, we will explore heterogeneity by adding covariate terms using sub-group meta-analysis or meta-regression if sufficient and equally categorized covariate information is available for studies. We intend to assess the influence of the following covariates: the geographical location where the study was conducted (continent; high vs low and middle income country), age groups, the ethnicity of the study population, the reference standard used, the type of index test used (viral, human or mixed methylation; targeted genes).

Sensitivity analyses

We plan to complete sensitivity analyses by restricting to studies free of concern for the QUADAS-2 domains based on the signalling questions we have identified as the most important. Therefore, sensitivity analyses will be performed including only those studies answering yes for each of the four domains (a-d), where:

- a) appropriate patient selection where selection was consecutive or random and reflects a hrHPV screen positive population (QUADAS-2 item 1)
- b) where the index test used an appropriate methylation test and sample material (QUADAS-2 items 6 and 7)

c) Appropriate reference standard was used (QUADAS-2 item 8)

D) Flow and timing was appropriate (QUADAS-2 items 10 and 12)

Assessment of reporting bias

We will assess publication bias using visual inspection of funnel plots (i.e. scatter plots for the logarithm of the diagnostic odds ratio against the reciprocal of the square root of the effective sample size [$ESS = (4n_1n_2)/(n_1+n_2)$, with n_1 and n_2 representing the number of diseased and non-diseased, respectively]) on analyses with greater than 10 studies and the Deeks asymmetry test⁵⁰⁰. The regression line of the Deeks funnel plot will be monitored, and if the included angle of the diagnostic odds ratio axis is close to 90 degrees, it will suggest low or absence of publication bias.