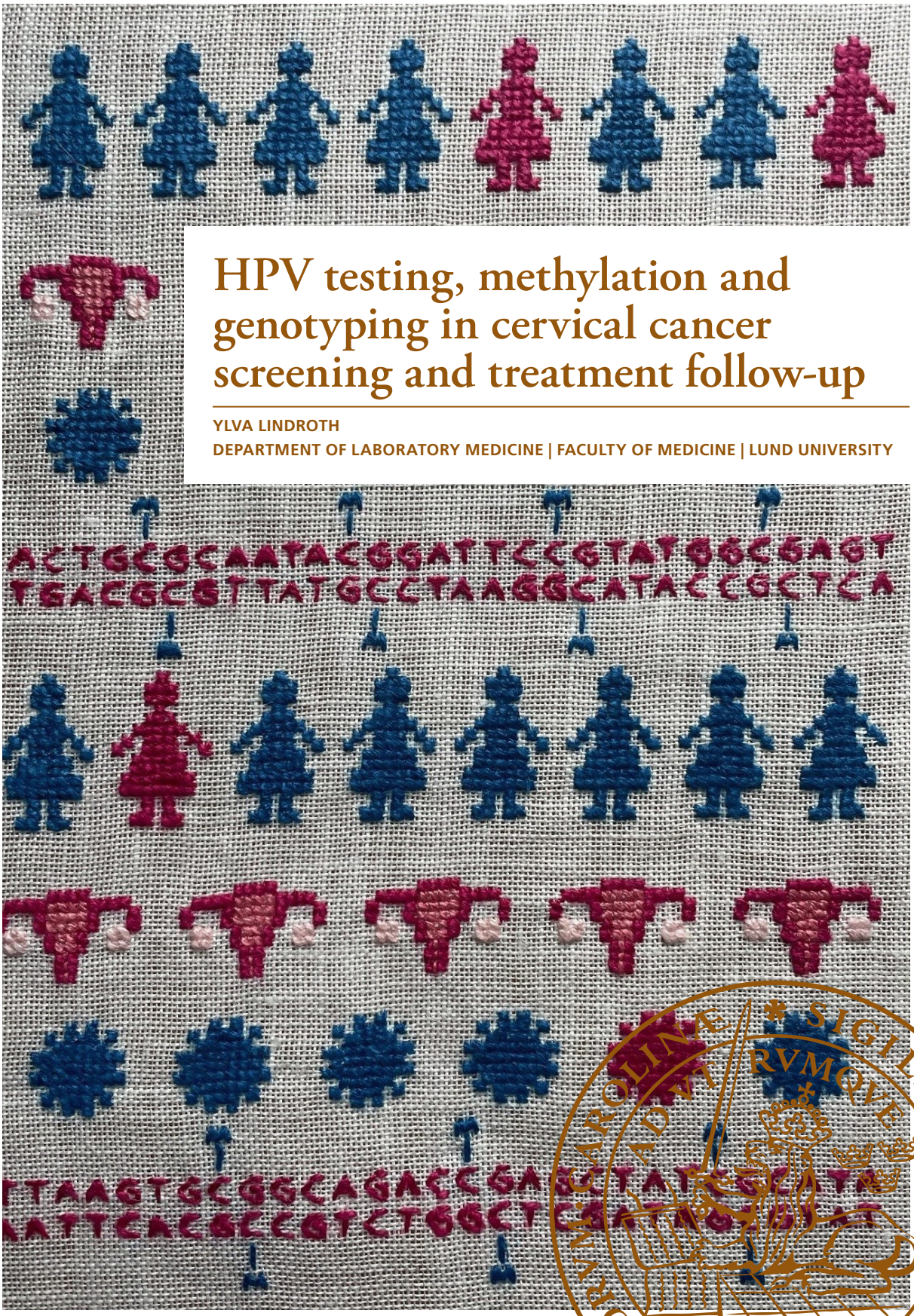
HPV testing, methylation and genotyping in cervical cancer screening and treatment follow-up

Lindroth, Ylva

2024

Document Version:
Publisher's PDF, also known as Version of record



HPV testing, methylation and genotyping in cervical cancer screening and treatment follow-up

YLVA LINDROTH
DEPARTMENT OF LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

YLVA LINDROTH is a consultant in clinical virology and bacteriology at Clinical Microbiology in Lund. The laboratory is a part of Region Skåne and provides microbiology diagnostics for the 1.4 million people living in Skåne, Sweden. Her daily work includes interpretation of microbiological and serological findings, evaluation of new diagnostics and giving advice to laboratory technicians and clinicians. She has a special interest in diagnostics for sexually transmitted diseases, including HPV.

During her doctoral studies, she has studied how different HPV types are affected by treatment of cervical dysplasia, the effect of primary HPV testing in the cervical cancer screening program in Skåne, and the possibility to use methylation analysis to improve the cervical cancer screening.

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Laboratory medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2024:149
ISBN 978-91-8021-646-3
ISSN 1652-8220

HPV testing, methylation and genotyping in cervical cancer screening and treatment follow-up

HPV testing, methylation and genotyping in cervical cancer screening and treatment follow-up

Ylva Lindroth

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 6th of December 2024 at 09.00 in Segerfalksalen, BMC, Sölvegatan 17, Lund

Faculty opponent
Ulf Gyllensten

Department of Immunology, Genetics and Pathology, Uppsala University

Organization: LUND UNIVERSITY

Document name: Doctoral dissertation **Date of issue** 2024-12-06

Author(s): Ylva Lindroth **Sponsoring organization:**

Title and subtitle: HPV testing, methylation and genotyping in cervical cancer screening and treatment follow-up

Abstract:

HPV testing has a higher sensitivity than cytology for identification of women at risk of cancer. Primary HPV testing was introduced in the organized screening program in Skåne, Sweden in 2017 for women 30 years and older. High-risk HPV E6/E7 mRNA was detected in 7.0% of these women. Primary HPV-screening identified a similar proportion of abnormal cytology (ASCUS+) as cytology-based screening (3.70% vs 3.52%). All co-tested women 40-42 years old, with high-grade cytology were HPV positive, indicating that primary HPV testing did not miss cases of cervical dysplasia. The introduction of primary HPV screening resulted in a 54% increased follow-up rate.

After treatment of high-grade cervical lesions by conization, persistent infection with at least one high-risk HPV type was seen in 16% of the women. Low-risk HPV infections more often persisted after treatment. Testing for high-risk HPV showed high sensitivity (92%) for detection of residual high-grade cytology.

Testing for DNA methylation is a promising alternative to cytology for triage of HPV positive women in the screening. Analysis of DNA methylation of the human genes *FAM19A4* and *miR124-2* in cytology samples collected up to eight years prior to histologically confirmed severe dysplasia and cancer showed a higher sensitivity than cytology. Screening samples collected within three years from histologically diagnosed cancer were positive for methylation of *FAM19A4/miR124-2* in 12/14 (86%) cases, while 13/17 (76%) showed abnormal cytology. However, the methylation test was less sensitive than cytology for detection of precancerous lesions (HSIL and AIS). Approximately one third of samples with normal cytology prior to cancer or severe dysplasia were positive in the methylation assay. Adding methylation analysis to HPV positive samples with normal cytology would thus increase the sensitivity for detection of cancer in the screening program.

Key words: HPV, cervical cancer screening, methylation, HPV genotypes

Lund University, Faculty of Medicine Doctoral Dissertation Series 2024:149

Language English **Number of pages**: 91
ISSN and key title: 1652-8220
ISBN: 978-91-8021-646-3

HPV testing, methylation and genotyping in cervical cancer screening and treatment follow-up

Ylva Lindroth

LUND
UNIVERSITY

Cover illustration by the author (linen embroidery)

Faculty of Medicine
Department of Laboratory medicine, Lund University

ISBN 978-91-8021-646-3
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2024

To my family

Table of Contents

Abstract

HPV testing has a higher sensitivity than cytology for identification of women at risk of cancer. Primary HPV testing was introduced in the organized screening program in Skåne, Sweden in 2017 for women 30 years and older. High-risk HPV E6/E7 mRNA was detected in 7.0% of these women. Primary HPV-screening identified a similar proportion of abnormal cytology (ASCUS+) as cytology-based screening (3.70% vs 3.52%). All co-tested women 40-42 years old, with high-grade cytology were HPV positive, indicating that primary HPV testing did not miss cases of cervical dysplasia. The introduction of primary HPV screening resulted in a 54% increased follow-up rate.

After treatment of high-grade cervical lesions by conization, persistent infection with at least one high-risk HPV type was seen in 16% of the women. Low-risk HPV infections more often persisted after treatment. Testing for high-risk HPV showed high sensitivity (92%) for detection of residual high-grade cytology.

Testing for DNA methylation is a promising alternative to cytology for triage of HPV positive women in the screening. Analysis of DNA methylation of the human genes FAM19A4 and miR124-2 in cytology samples collected up to eight years prior to histologically confirmed severe dysplasia and cancer showed a higher sensitivity than cytology. Screening samples collected within three years from histologically diagnosed cancer were positive for methylation of FAM19A4/miR124-2 in 12/14 (86%) cases, while 13/17 (76%) showed abnormal cytology. However, the methylation test was less sensitive than cytology for detection of precancerous lesions (HSIL and AIS). Approximately one third of samples with normal cytology prior to cancer or severe dysplasia were positive in the methylation assay. Adding methylation analysis to HPV positive samples with normal cytology would thus increase the sensitivity for detection of cancer in the screening program.

Populärvetenskaplig sammanfattning på svenska

Livmoderhalscancer är en av de vanligare cancerformerna i Sverige och varje år insjuknar cirka 500 kvinnor varav ungefär hälften är under femtio år. I hela världen drabbas över en halv miljon kvinnor av livmoderhalscancer årligen. Sjukdomen utvecklas långsamt och tack vare screening med så kallat cellprov som har funnits i Sverige sedan slutet av 1960-talet kan man ofta hitta och behandla förstadier som annars skulle ha utvecklats till livmoderhalscancer. Eftersom nästan all livmoderhalscancer orsakas av humant papillomvirus (HPV) har cellprovet ersatts av HPV-test. Om man inte är infekterad med HPV har man mycket låg risk att få livmoderhalscancer. HPV-testning är känsligare än cellprov för att upptäcka kvinnor som riskerar att utveckla livmoderhalscancer. I Skåne bytte man från cellprov till HPV-testning i screeningen i januari 2017, för kvinnor som var 30 år och äldre. Det innebär att man tar ett cellprov, men att man bara studerar cellerna i mikroskop om man detekterar HPV i provet. I den första studien har vi visat att lika många kvinnor med allvarliga cellförändringar upptäcktes efter införandet av HPV-test i screeningen. 7% av alla kvinnor över 30 år var infekterade med HPV.

Utan HPV utvecklas inte livmoderhalscancer. Men det är inte samma sak som att HPV nödvändigtvis ger livmoderhalscancer. Tvärtom är det bara en liten del av alla HPV-positiva som får allvarliga cellförändringar. För att inte alla HPV-positiva ska behöva utredas vidare använder man ytterligare ett test. Hittills har det varit cytologi, alltså att man tittar på cellprovet i mikroskop. De kvinnor som både är HPV-positiva och har avvikande celler följs upp på kvinnokliniken medan de som har normala celler får avvakta med uppföljning och i stället ta ett nytt HPV-test senare. Ofta har HPV-infektionen läkt ut till nästa prov och då behövs ingen uppföljning. Om infektionen finns kvar ska kvinnan följas upp med undersökning på kvinnokliniken där man studerar livmodertappen i uppförstoring och eventuellt tar vävnadsprover. Cytologi är en ganska bra metod, men kräver lång upplärning och kontinuerligt arbete för att upprätthålla god kvalitet. En alternativ metod som föreslagits för att avgöra vilka HPV-positiva kvinnor som behöver följas upp är testning av DNA-metylering. Vår arvsmassa utgörs av DNA. Fel kan uppstå när DNA:t kopieras inför att cellerna ska delas. Om dessa inte upptäcks och rättas till blir det ett skrivfel i DNA-sekvensen, en mutation. Men DNA :t kan även förändras på andra sätt som är mer flexibla. DNA-metylering är ett exempel på en så kallad epigenetisk förändring där metylgrupper läggs till på vissa DNA-baser. Det gör DNA-molekylen på dessa ställen mer fettlöslig och den drar ihop sig och blir mer svårtillgänglig för de delar av cellmaskineriet som skriver av DNA:t så att

proteiner kan bildas. Om proteiner som skyddar mot cancer inte uttrycks ökar risken för cancer. Genom tidigare studier vet man att de två generna *FAM19A4* och *miR124-2* oftast är metylerade vid livmoderhalscancer. I studie II undersökte vi om det gick att testa cellprov som togs upp till åtta år innan allvarliga cellförändringar och cancer, och redan då se att cellproven var metylerade. Frysta cellprov från kvinnor som senare fick cellförändringar eller cancer fanns sparade i en biobank. Man hade sett vid undersökning av vävnadsprov från dessa kvinnor att de verkligen hade allvarliga cellförändringar eller cancer. Vävnadsprov kan ge säkrare diagnos än cytologi. Alla cellprov som var tagna på kvinnor som fick cancer var inte metylerade. Testet kunde alltså inte med perfekt säkerhet förutsäga alla fall av cancer. Men det kunde å andra sidan inte cytologi heller. Metylering kunde påvisas i 33 av 77 (45%) av prov där cellerna såg normala ut. Andra prov var negativa för metylering men hade en cellbild som var onormal. När det gäller adenocarcinom, en cancertyp som utgår från körtelceller, kunde metylering ofta upptäckas i cellprov som var tagna långt tidigare, trots att cellbilden inte hade avslöjat något onormalt.

Eftersom proven i studie II var ganska gamla och i vissa fall tagna lång tid innan cancer eller allvarliga cellförändringar utvecklades, ville vi även studera metylering i prov som togs under första året med HPV-screening, 2017. De kvinnor som då var HPV-positiva har nu haft några år på sig att utveckla cellförändringar och cancer. Av ca 4 300 kvinnor som var HPV-positiva 2017 hade knappt hälften onormala celler och skulle följas upp med gynekologisk undersökning där vävnadsprov eventuellt skulle tas. Bland dem hittades allvarliga cellförändringar i vävnadsprov hos knappt 600 och cancer hos 20 kvinnor. Bland dem som hade normala celler hade ett sjuttiotal allvarliga cellförändringar enligt vävnadsprov och fem hade cancer. De flesta allvarliga cellförändringar upptäcktes alltså tack vare undersökning av cellprovet i mikroskop. Det var många HPV-positiva kvinnor som hade onormal cytologi, men som ändå inte utvecklade allvarliga cellförändringar eller cancer. Nästan hälften av de HPV-positiva kvinnorna behövde följas upp på grund av onormal cytologi. (De flesta i onödan, med facit i hand). Med metyleringsanalys skulle förmodligen färre kvinnor behöva följas upp. Ungefär 10% av HPV-positiva kvinnor med normal cytologi, som läkte ut HPV-infektionen, testade positivt i metyleringstestet. Metyleringstestet var ungefär lika bra som cytologi för att förutsäga vilka kvinnor som hade cancer eller skulle utveckla cancer innan nästa cellprov. Men när det gäller att upptäcka allvarliga cellförändringar som ännu inte utvecklats till cancer var cytologi bättre. Det skulle därför inte vara rimligt att byta ut cytologi mot analys av metylering. Eftersom en del prov var positiva i metyleringstestet trots att cellerna såg normala ut, skulle man kunna

lägga till metyleringstest för normala cellprov och på så sätt upptäcka fler kvinnor som håller på att utveckla allvarliga cellförändringar eller cancer. Tyvärr skulle det öka kostnaderna för screeningen och ännu fler kvinnor skulle behöva följas upp hos gynekolog.

När allvarliga cellförändringar är ett faktum, behöver de behandlas genom att en bit av livmodertappen skärs bort, så kallad konisering. Det finns inget effektivt sätt att bli av med HPV-infektionen utan att ta bort den infekterade vävnaden. I studie IV beskrivs hur förekomsten av olika HPV-typer påverkades av konisering. Det finns många olika typer av HPV, där högrisktyper ger ökad risk för cancer till skillnad från lågrisktyper. Målet med behandlingen är att HPV-infektionen ska läka ut och att cellförändringen ska försvinna, vilket lyckades i de flesta fall. Men efter behandlingen hade 16% kvar samma högrisk-HPV som innan behandlingen och 17% hade fortfarande onormalt cellprov. Lågrisk-typer påverkades inte av behandlingen, vilket kan bero på att infektionen ofta sitter i vävnad som inte tas bort vid behandlingen, eller på att lågrisktyper i allmänhet läker ut efter kortare tid och ersätts av nya infektioner. Hos kvinnor där högrisk-HPV inte kunde påvisas efter behandling var risken för kvarvarande cellförändringar mycket låg.

Abbreviations

HPV	Human papillomavirus
HR HPV	High-risk HPV
LR HPV	Low-risk HPV
CIN	Cervical Intraepithelial Neoplasia
CIN1	Cervical Intraepithelial Neoplasia grade 1
CIN2	Cervical Intraepithelial Neoplasia grade 2
CIN2+	Cervical Intraepithelial Neoplasia grade 2 or worse
CIN3	Cervical Intraepithelial Neoplasia grade 3
CIN3+	Cervical Intraepithelial Neoplasia grade 3 or worse
ASCUS	Atypical squamous cells of undetermined significance
ASC-H	Atypical squamous cells, cannot exclude high-grade lesion
LSIL	Low-grade squamous intraepithelial lesion. Corresponds to CIN1.
HSIL	High-grade squamous intraepithelial lesion. Includes CIN2 and CIN3.
AIS	Adenocarcinoma in situ
ADC	Adenocarcinoma
SCC	Squamous cell carcinoma
ADC	Adenocarcinoma
AGC	Atypical glandular cells
NIML	Negative for intraepithelial lesion or malignancy
LBC	Liquid Based Cytology
NKCx	Swedish National Cervical Screening Registry

Overall aims

The overall aim of the thesis was to validate and improve the population-based cervix cancer screening program.

The aim of

Study I: To evaluate the first year with primary HPV testing for women 30 years and older in the organized cervical screening program.

Study II: To investigate if methylation of *FAM19A4/miR124-2* could be detected in cytology samples collected before development of severe dysplasia or cancer.

Study III: To further evaluate the sensitivity of the methylation test for detection of cancer or severe dysplasia within one screening round, with the intention to implement this test in the screening program.

Study IV: To study the dynamics of different HPV types and to evaluate HPV testing as a test of cure after conization treatment.

List of papers

The thesis is based on four papers, referred to by the Roman numerals below**.**

Paper I **Lindroth Y**, Borgfeldt C, Thorn G, Bodelsson G, Forslund O. Population based primary HPV mRNA cervical screening compared with cytology screening. Preventive Medicine 2019 Jul;124:61-66.

Paper II **Lindroth Y**, Pedersen L, Alssamaray J, Berglund T, Sundqvist A, Borgfeldt C, Forslund O. Cervix cytology samples revealed increased methylation of the human markers *FAM19A4/miR124-2* up to 8 years before adenocarcinoma. Acta obstetricia et gynecologica Scandinavica. 2024;103(2):378-86.

Paper III **Lindroth Y**, Borgfeldt C, Forslund O. *FAM19A4/miR124-2* methylation analysis in a real-life screening-cohort. Manuscript. 2024.

Paper IV **Lindroth Y,** Bjelkenkrantz K, Forslund O. Spectrum of HPV types before and after treatment of cervical intraepithelial neoplasia grade 2 and 3. J Clin Virol. 2017;97:38-43

Papers not included in the thesis

Lindroth Y, Hansson L, Forslund O. An automated commercial open access assay for detection of Mycoplasma genitalium macrolide resistance. APMIS. 2024 (in press).

Albinsson B, Hoffman T, Kolstad L, Bergström T, Bogdanovic G, Heydecke A, Hägg M, Kjerstadius T, Lindroth Y, Petersson A, Stenberg M, Vene S, Ellström P, Rönnberg B, Lundkvist Å. Seroprevalence of tick-borne encephalitis virus and vaccination coverage of tick-borne encephalitis, Sweden, 2018 to 2019. Euro Surveill. 2024;29:2300221.

Lexner J, Lindroth Y, Sjöberg K. The risk for celiac disease after Covid-19 infection. BMC Gastroenterol. 2023;23:174.

Hadad R, Golparian D, Velicko I, Ohlsson AK, Lindroth Y, Ericson EL, Fredlund H, Engstrand L, Unemo M. First National Genomic Epidemiological Study of Neisseria gonorrhoeae Strains Spreading Across Sweden in 2016. Front Microbiol. 2022;12:820998.

Strand R, Thelaus L, Fernström N, Sunnerhagen T, Lindroth Y, Linder A, Rasmussen M. Rapid diagnostic testing for SARS-CoV-2: Validation and comparison of three point-of-care antibody tests. J Med Virol. 2021;93:4592-4596.

Hober S, Hellström C, Olofsson J, Andersson E, Bergström S, Jernbom Falk A, Bayati S, Mravinacova S, Sjöberg R, Yousef J, Skoglund L, Kanje S, Berling A, Svensson AS, Jensen G, Enstedt H, Afshari D, Xu LL, Zwahlen M, von Feilitzen K, Hanke L, Murrell B, McInerney G, Karlsson Hedestam GB, Lendel C, Roth RG, Skoog I, Svenungsson E, Olsson T, Fogdell-Hahn A,

Lindroth Y, Lundgren M, Maleki KT, Lagerqvist N, Klingström J, Da Silva Rodrigues R, Muschiol S, Bogdanovic G, Arroyo Mühr LS, Eklund C, Lagheden C, Dillner J, Sivertsson Å, Havervall S, Thålin C, Tegel H, Pin E, Månberg A, Hedhammar M, Nilsson P. Systematic evaluation of SARS-CoV-2 antigens enables a highly specific and sensitive multiplex serological COVID-19 assay. Clin Transl Immunology. 2021;10:e1312.

Unemo M, Hansen M, Hadad R, Lindroth Y, Fredlund H, Puolakkainen M, Sundqvist M. Finnish new variant of Chlamydia trachomatis escaping detection in the Aptima Combo 2 assay also present in Örebro County, Sweden, May 2019. Euro Surveill. 2019;24:1900370.

Lantto J, Lindroth Y, Ohlin M. Non-germ-line encoded residues are critical for effective antibody recognition of a poorly immunogenic neutralization epitope on glycoprotein B of human cytomegalovirus. Eur J Immunol. 2002;32:1659-1669.

Introduction

The human papillomavirus

The human papillomavirus (HPV) is non-enveloped and has a genome consisting of an 8 kb double stranded, circular DNA molecule. The capsid is 50-60 nm in diameter, has an icosahedral structure, and is built up by 72 capsomeres, each made up of five L1 molecules, in total 360 copies of the L1 protein, and a variable number of L2 molecules. The L1 and L2 proteins are coded by the late genes L1 and L2. In addition to the late genes, the HPV genome contains the early genes E1, E2, E4, E5, E6, E7 and a regulatory element named the long control region (LCR). The virus has two promotors, the early promotor p97 located in the LCR, and the late promotor p670 which is dependent on differentiation of the cell and located within the E7 coding region.

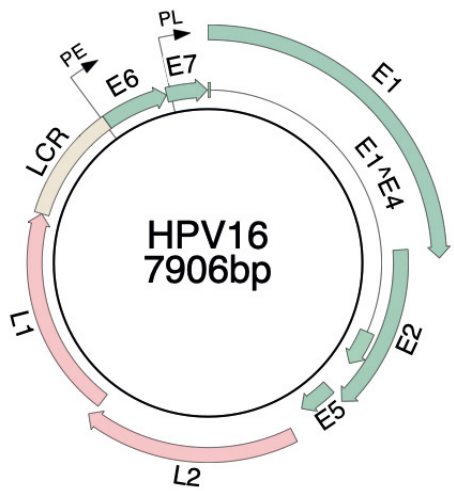


Figure 1. The HPV16 genome.

Abbreviations: Long control region (LCR), early promotor (PE), late promotor (PL), early genes (E1-E7), late genes (L1-L2). Genes are defined according to the HPV reference genome (NCBI accession number NC_001526.4).

During infection, the L1 protein binds to heparin proteoglycans in the basal membrane, possibly through a microabrasion in the epithelium. Conformational changes of the virus particle cause exposure of the L2 protein on the surface. Following cleavage of L2 by furin (a protease which cleaves precursor proteins of parathyroid hormone, the von Willebrand factor and numerous other human proteins) the virus particle is internalized in the epithelial basal stem cell (1, 2). The cellular receptor in not fully characterized. After transportation to the cell nucleus, the E1 protein, a helicase, unwinds the viral DNA and binds to the origin of replication within the LCR. The binding is enhanced by the E2 protein, and DNA replication can begin (3). Initially, the early promotor is used for RNA transcription. This promotor allows expression of the early proteins E6 and E7. Expression of these early proteins causes cell cycle re-entry in cells that would normally exit the cell cycle and undergo termination differentiation. Through this mechanism, the virus can use the replication-machinery of the host cell and production of viral DNA can continue (4). When the virus reaches the upper layers of the epithelium, the late promotor is activated and allows for expression of the proteins L1 and L2. The process is facilitated by expression of higher levels of E2 that down-regulate the early promotor (5). The L1 and L2 proteins make up the capsid of new virus particles, which are released by shedding of epithelial cells. The virus can evade the immune system by not expressing the immunogenic capsid proteins until late during the infection. The L1 protein can self-assemble to icosahedral particles. Indeed, empty shells of recombinant L1, so called virus like particles (VLP) are used in the HPV vaccine.

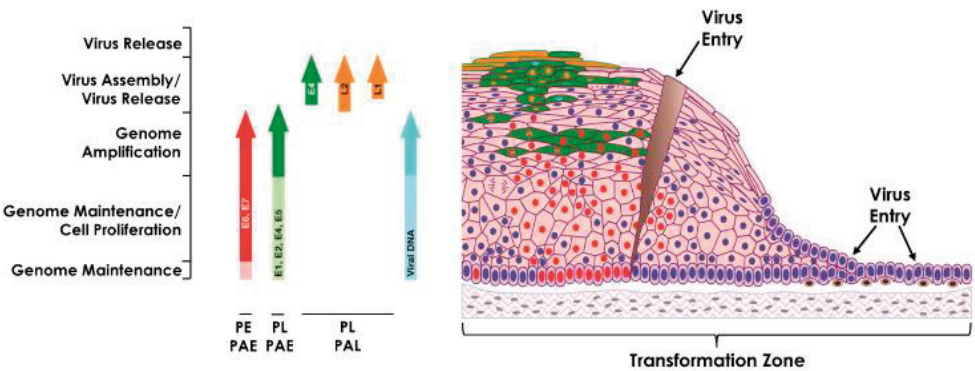


Figure 2. Life cycle of high-risk HPVs in cervical epithelium.

Reprinted from Vaccine, vol. 30 Suppl 5, Doorbar J et al., The biology and life-cycle of human papillomaviruses, F55-F70, Copyright (2012), with permission from Elsevier.

Sometimes the HPV infection persists in the lower epithelial layers, with expression of E6 and E7 but without production of viral particles. Prolonged expression of these proteins can cause DNA damage by forcing the cell to stay in the cell cycle. E7 interacts with members of the retinoblastoma protein (pRb) family (6). Through binding of E7 to pRb, the transcription factor E2F is released, and DNA synthesis and proliferation of cells is upregulated (7, 8) (Figure 3). Normally, this elevated DNA synthesis would lead to apoptosis of the cell. But E6 binds to and causes degradation of p53, with reduced apoptosis as the effect (9, 10). The long-term consequence is an increased risk for cancer development. HPV is the known to cause cervical cancer, but also other types of anogenital cancers as well as oropharyngeal cancer (11). In many cases of cervical cancer, the viral DNA is integrated in the chromosomal DNA (12). When the viral DNA is integrated, cleavage of the circular DNA can cause disruption of the E2 gene (13). The early promotor is thought to be constitutively active during most of the viral life cycle but is downregulated by E2 (14). When E2 is no longer expressed, the downregulation of the early promotor is ceased and expression of E6 and E7 increases (15). But integration is not necessary for oncogenicity - also episomal HPV DNA can cause cancer (16).

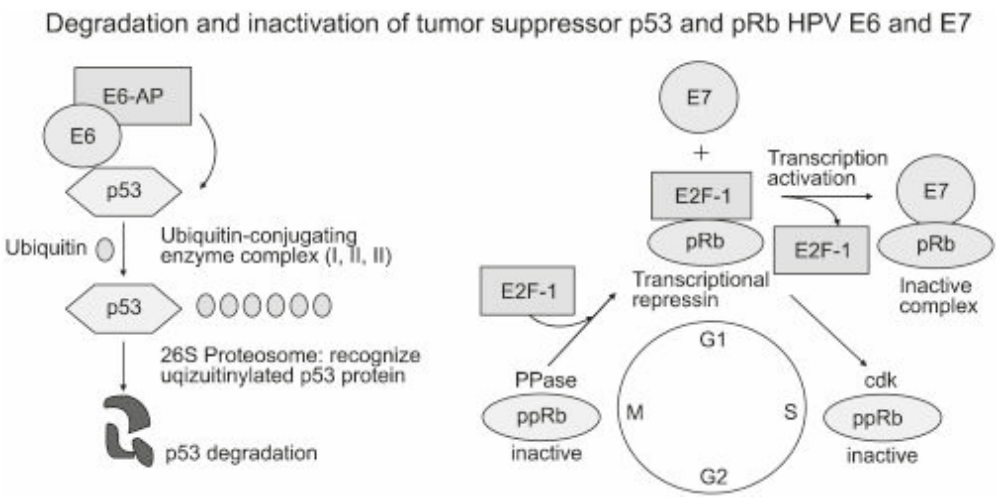


Figure 3. HPV E6 and E7 oncogenes.

The tumor supperssor protein p53 is marked for degradation through ubiquitination facilited by E6 and E6-AP (Ubiquitin ligase E6-associated protein). Binding of E7 to pRb releases the transcription factor E2F-1 which promotes re-entry to the cell cycle. (Source: Yim EK, Park JS. The Role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. Cancer Res Treat. 2005;37:319-324.(17) ©2005 Korean Cancer Association)

HPV types

The human papilloma virus is ubiquitous, and the majority of all humans carry HPV on their skin surface without any symptoms (18, 19). Actually, many other vertebrates also carry papillomaviruses (20). Hundreds of HPV types have been identified. They are divided into five different genera alfa, beta, gamma, mu and nu, based on differences in the DNA sequence of the L1 gene (21). Different genera share less than 60% nucleotide sequence identity in the L1 (22). A HPV type is defined as a HPV with at least 10% dissimilar L1 sequence from that of other HPV types (23). The alfa genus includes both cutaneous types and mucosal types. Low-risk mucosal alfa genus types, such as HPV6 and HPV11, cause genital warts (condyloma) while high-risk types, especially HPV16, are known to cause cancer (24-26). Skin warts on hands and feet are often caused by HPV2 from the alpha genus or HPV1 from the mu genus. The 2009 Nobel prize laureate Harald zur Hausen and coworkers cloned HPV16 in 1983 and noted that DNA from this HPV typed was present in about 50% of cervical cancer biopsies (27). The following year, 1984, HPV18 was identified by the same group (28). HPV16 has a particularly high oncogenic capacity (29, 30). HPV16 is the most frequent cause of cervical squamous cell carcinoma, while HPV18 is more often associated with adenocarcinoma (31). HPV16 and HPV18 together cause 70% of cervical cancer (32, 33). In 2012, the International Agency for research on Cancer (IARC) published a report on biological agents which present carcinogenic risks to humans (34). Twelve HPV types were classified as having a clear carcinogenic potential, while others were classified as probably carcinogenic or possibly carcinogenic (Table 1). During the last years, focus on different oncogenicity for different high-risk types has been emphasized (35, 36). The reason is that both the prevalence and the oncogenic potential varies hugely between different high-risk types, which has implications when primary HPV testing is used in cervical cancer screening. Young women infected with a low-oncogenic HPV type may not need follow up, since the risk for cancer is very low. The new classification, defined in the IARC Handbook for cervical cancer screening (35), is presented in Table 2. This classification is also used in the 2022 Swedish national guidelines for cervix cancer prevention (37).

Table 1. Classification of HPV types according to IARC in 2012 (34).

CLASSIFICATION	HPV TYPES
Carcinogenic types	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Probably carcinogenic types	68
Possibly carcinogenic types	26, 53, 66, 67, 70, 73, 82

Table 2. Classification of high-risk HPV types according to the 2022 IARC Handbook of Cervical cancer screening (35)**.** This classification is used in the current Swedish national guidelines (37).

CLASSIFICATION	HPV TYPES
High-oncogenic	16, 18, 45
Mid-oncogenic	31, 33, 52, 58
Low-oncogenic	35, 39, 51, 56, 59, 68

Natural course of HPV infections

HPV infections are common in young women and are usually cleared within a few months or years without causing cervical cancer (33). A longitudinal study of teenage girls in Indianapolis (USA) showed a median duration of persistence of a specific HPV type of 168 days (38). Nobbenhuis et al. showed that the 46% of women with normal cytology clear the HPV-infection within one year, and after 36 months approximately two thirds had cleared the infection (39). Low-risk types seem to clear faster than high-risk types (33, 40-42). Persistence of the same type is associated with a higher risk for cervical cancer (43). What determines if an HPV infection will persist or regress, is not yet fully understood. The virus can escape the immune system in many ways, also in an immune competent host. The HPV virus can stay unnoticed by the immune system since its entire life cycle is within the epithelial cell. The activity of antigen presenting cells is low after the HPV infection and so is the release of cytokines (44). Anti-viral effects of interferons are disrupted by HPV E6 and E7 genes (45). Sustained inflammatory response with infiltration of macrophages and neutrophils is associated with persistence of infection. Repression of the expression of TLRs by HPV is another feature related to persistence, while increased levels of TLR2, TLR7 and TLR8 have been

observed prior to regression of cervical lesions (CIN2) caused by HPV (46). Cell-mediated immunity, with CD4 responses to E6 and E7, plays an important role for clearance of HPV infection, whereas only about half of women infected with HPV16 develop antibodies against HPV16 L1. Thus, antibodies are not necessary for clearance of HPV infections, and protection against reinfection with the same HPV type is often not obtained. Antibodies, however, are crucial for prevention of HPV infection after vaccination (45).

Microbiome studies have shown correlation between progression of cervical dysplasia and high vaginal microbial diversity and high abundancy of bacteria associated with bacterial dysbiosis, such as *Gardnerella vaginalis* (47). Lactobacillus dominance, especially of *L. crispatus*, is associated with regress of cervical intraepithelial dysplasia (48-50). However, it remains unclear whether the dysbiosis is caused by a persistent HPV infection and development of dysplasia, or vice versa.

Cervical cancer

Cervical cancer has an incidence of 500 cases per year in Sweden. For comparison, 8 600 women were diagnosed with breast cancer and 2 600 women were diagnosed with colon cancer in 2021 (51). But while the median age of women diagnosed with breast cancer and colon cancer was 66 years and 75 years, respectively, the median age for women diagnosed with cervical cancer was only 51, and one third was under forty years of age. Cervical cancer is a disease which often affects young women and deprive them of healthy years of life. In Sweden, approximately 150 deaths per year are caused by cervical cancer.

Since cervical cancer develops slowly and precursors can be treated, it is suitable for screening. After the introduction of cervical screening in the late 1960s the incidence has decreased dramatically, from 1000 to 500 cases per year today (52). The decline ceased in the mid-2010s, when an increase from about 450 cases to about 550 per year was seen. Cervical cancer had increased among women who had participated in the screening (53). Rereview of cytology slides collected before cancer revealed an increase in false negative smears (54). Fortunately, the incidence of invasive cervical cancer in Sweden has decreased since 2019, although an increase is seen in some regions (55).

Globally, the estimation was 570 000 new cases in 2018. The incidence of cervical cancer is higher in low- and middle-income countries, and 90% of the annual more than 300 000 deaths worldwide occur in these countries (56).

The vast majority, 99.7%, of cervical cancers are caused by HPV (57). Only in a few cases of cervical cancer, HPV has not been detected despite modern detection methods (58). Risk factors for cervical cancer are early sex debut, multiple sex partners, use of oral contraceptives, high parity, smoking, HIV infection, immunodeficiency, and last but not least: not attending the screening program (59-64).

The most common histological type is squamous cell carcinoma (SCC) which accounts for about two thirds of the cases followed by adenocarcinoma (ADC) which accounts for most of the remaining cases. Whereas SCC has decreased since the introduction of screening, ADC is still increasing (65). Both histotypes are strongly correlated to HPV infection. HPV16 is the most common cause of SCC while HPV18 is more often present in ADC (66).

Cervical cancer usually arises in the squamocolumnar junction where the squamous epithelium of the ectocervix meets the glandular epithelium of the endocervix. During childhood and after menopause, the squamocolumnar junction is located inside the cervical canal. When oestrogen level rise during puberty, the glandular epithelium grows out on the endocervix. After exposure to the acidic environment of the vagina, the squamous epithelium grows back, to some extent. The reason why the squamocolumnar junction is vulnerable to cancer development, is thought to be that the HPV can come through and infect the basal membrane, or that metaplastic cells present at this site are more susceptible to HPV-infection, particularly at puberty (23). This could explain why early sex debut is associated with increased risk for cervical cancer. When collecting a cytology sample, it is important to include cells from the squamocolumnar junction, which can be difficult when sampling post-menopausal women.

Early stages of cervical cancer, without spread outside the cervix or the upper third of the vagina are usually treated by hysterectomy. For small tumours, fertility sparing surgery, trachelectomy, or conization can be a possibility. More advanced cases are treated by local radiation (brachy therapy) or external radiation in combination with chemotherapy. In a study by Hellsten et al., 247 women with cervical cancer were identified in Skåne between 2017 and 2020, of which 11% received fertility sparing surgery, 30% hysterectomy, 44% radiotherapy and/or chemotherapy and 11% a combination of hysterectomy and radiotherapy and/or chemotherapy (67).

The overall five-year survival for cervical cancer in Sweden in 2014 was approximately 70% (65), but the prognosis differs between different stages of the disease. The overall survival has improved since 1960 but the survival for older women has not improved (65).

Prevention of cervical cancer includes detection of cancer precursors through screening, treatment of precursors and HPV vaccination. Precursors of cervical cancer as well as small, localized tumours are treated by conization, which is excision of the lesion in an approximate cone shape, including the outmost part of the cervical canal. Normally an electric loop is applied. Treatment of high grade squamous intraepithelial lesions radically lowers the risk for cancer (68). On the other hand, dysplasia can regress, especially in young women (69, 70). Low grade squamous intraepithelial lesions have a regression rate of 90% in young women (71, 72) and is not treated, but also high degree lesions can regress. Studies have shown that 70% of CIN2 in young women regress within three years without treatment (73, 74). An unwanted side effect of the treatment is increased risk for preterm delivery in future pregnancies (75), which advocates against overtreatment of young women.

The World Health Organization (WHO) presented a strategy for elimination of cervical cancer in 2020 (56). In this context, elimination means an incidence of less than four per 100 000 women per year. For Sweden, with a population of 5 million women, this corresponds to less than 200 new cases per year, which is a decrease of more than 50% from the current situation. For some low- and middle-income countries the incidence in 2018 was more than 26 cases per 100 000 women. For the WHO strategy to be successful, by 2030 the 90-70-90 targets must be met, meaning 90% of girls fully vaccinated by the age of fifteen, 70% of women tested by a high-performance test by the age of 35 and again by 45 years of age, 90% of women with precancerous lesions and 90% of women with cervical cancer will receive treatment.

In Sweden HPV vaccination commenced in 2012, for girls 11-12 years of age, with catch up vaccination for girls 13-18 years of age (37). The first vaccine used was the four-valent Gardasil 4 which protects against the most common high-risk types HPV16 and HPV18 and the low-risk types HPV6 and HPV11. The vaccine gives protection from the 70% of HPV-related cancer caused by HPV16 and HPV18, and from condyloma caused by HPV6 and HPV11. The vaccine consists of recombinant L1 protein which makes up virus like particles (empty capsids) (76). Since 2019, the nine-valent vaccine Gardasil 9 has been used in the vaccination program. This vaccine gives protection against HPV6, 11, 16, 18, 31, 33, 45, 52 and 58, genotypes which cause 90% of cervical cancer. Since 2020 it has been offered also to boys.

Cervical cancer screening

Cervical cancer screening was introduced in Sweden in the 1966 and was implemented throughout the country in 1977 (37). The overall goal of the organized cervical cancer screening is to prevent cervical cancer through detection and treatment of precancerous lesions. The screening starts at age 23 with a three-year interval for women up to 50 years of age and a five-year interval for women over 50. During the first forty years, cytology was the screening method. A sample collected from the cervix, including the squamocolumnar junction mentioned above, was directly applied to a microscopy slide and dyed with hematoxylin and eosin. The method, called Pap smear, was invented in the 1940s by Papanocolau (77). Cells affected by HPV get a different appearance in the microscope. Although the relatively low sensitivity of cytology (60-70%) the screening program has been successful since the slow development of cancer allows for repeated tests to detect each case (78). Cytology results are graded according to the Bethesda system (79) in the following cytological categories: negative for intraepithelial malignancy (NILM); atypical squamous cells of undetermined significance (ASCUS); atypical squamous cells, cannot exclude high grade lesion (ASC-H); low-grade squamous intraepithelial lesion (LSIL); high-grade squamous intraepithelial lesion (HSIL); squamous cell carcinoma (SCC); atypical glandular cells (AGC); adenocarcinoma in situ (AIS) or adenocarcinoma (ADC). The former system used three categories of cervical intraepithelial dysplasia: CIN1, which in the Bethesda system corresponds to LSIL, and CIN2 and CIN3 which both are included in the HSIL classification.

Introduction of liquid-based cytology (LBC) around 2010 allowed for HPV testing of the same sample (reflex testing). Before introduction of primary HPV screening, HPV testing of women 35 years and older, with the less severe cytology diagnoses ASCUS and LSIL, was used as a triage, where HPV negative women were followed up with repeated cytology instead of immediate colposcopy.

Since HPV testing has shown a higher sensitivity than cytology for detection of cervical cancer (80-82) a natural consequence was to implement primary HPV screening. Recommended by the National Board of Social Affairs and Health in 2015, primary HPV screening was introduced in the Swedish national guidelines in 2017 (52). Primary HPV screening was implemented in Skåne in 2017, for women 30 years of age and older. The cytology was only assessed if the HPV test was positive (Figure 4). Women with positive HPV test and cytology of ASCUS or worse (ASCUS+) were referred for colposcopy

within three months. HPV positive women with normal cytology were retested after three years. If persistency of HPV was seen in the follow up test after, the woman was referred for colposcopy regardless of cytology result. If the HPV test was negative, the woman returned to the screening program, with an invitation every three years for women under 50 years of age and every five years for women over 50 years. Primary cytology screening continued for women under 30 years. Since younger women more often are HPV-infected and usually clear the infection without development of high-grade lesions, primary HPV testing was not implemented for screening of young women, as it would give rise to unnecessary colposcopies and unnecessary anxiety. For screening samples with abnormal cytology, reflex HPV testing was performed on samples from women under 30 years.

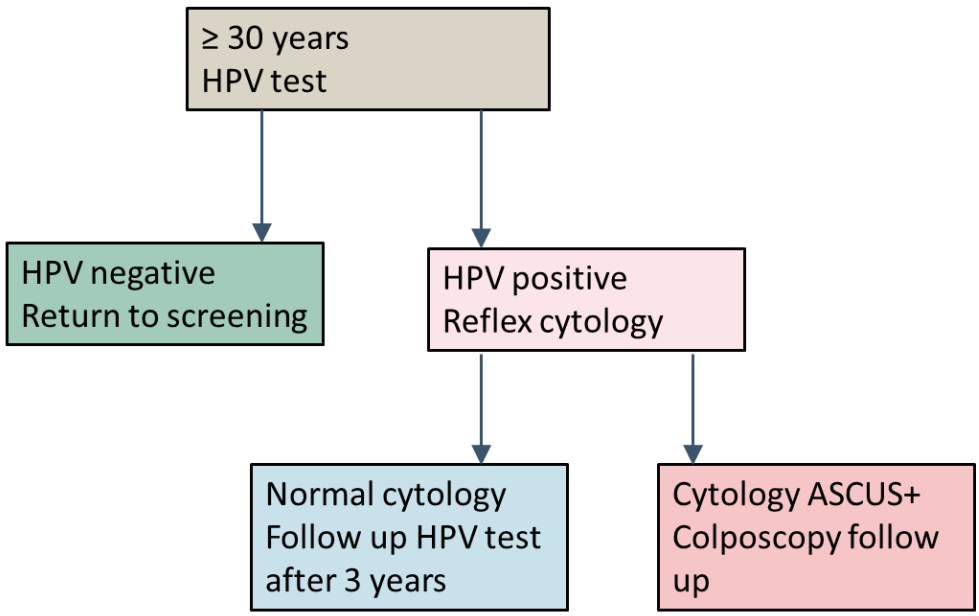


Figure 4. Overview of the primary HPV screening introduced in 2017.

An often observed drawback of primary HPV screening is an increase in the number of women who need follow up (83-86). The reason is low specificity of HPV testing, in the sense that most women will clear the infection without development of cancer or precancerous lesions. Even though a complementary test, usually cytology, is used to triage HPV positive women, the number that

need follow up tend to increase. Alternative triage strategies could be retesting of HPV positive women (87, 88), HPV genotyping (36) or analysis of DNA methylation of either viral DNA, human genes or a combination thereof (89).

During the covid pandemic, the demand for self-collected samples increased, since all unnecessary social contacts, including health care contacts, should be avoided. In Skåne, kits for self-collected samples had previously been sent out to women who had not participated in the screening for over seven years. Self-collected vaginal samples give a similar sensitivity for detection of HSIL, AIS and cancer as clinician-taken cervical HPV-samples (90). Since September 1, 2021, self-collected vaginal samples for HPV testing has been the principal method for cervical screening in Skåne, including women of all ages, starting from 23. The recommendation from the National Board of Social Affairs and Health in 2022 is that HPV test should be used for cervical cancer screening of women from 23 years of age, and that self-sampling can be offered as an alternative to sample collected by a health care professional (37). So far, self-sampled HPV positive women need to go to a midwife for a second sample from the cervix with possibility of both HPV test and reflex cytology. In the future it might be possible to decide directly from the self-collected sample, by HPV genotyping or methylation analysis, if the woman needs follow up.

Methods

HPV testing

HPV tests used for cervical cancer screening

In 2009 Meijer et al. proposed requirements for HPV DNA tests used for primary cervical screening of women of at least 30 years of age (91). The FDA (US Food and Drug Administration) approved Hybrid Capture 2 (hc2, Qiagen) or the GP5+/6+ PCR-enzyme assay was used as a reference method in the Meijer criteria. These two assays had been evaluated with a follow-up of eight years or more. The hc2 had shown a clinical sensitivity of 95-98% for ≥CIN2 and a specificity 91-94%. A candidate test should have a clinical sensitivity of at least 90% and a specificity of no less than 98% of the hc2. When these cut offs were set, special consideration was taken to the redundant follow-up that a test with low specificity would result in. During the years, several assays have been validated according to the Meijer protocol, of which the majority are systems for detection of HPV DNA.

The Aptima HPV assay (Hologic, San Diego, CA) detects HPV E6/E7 mRNA from the 14 high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 82 along with the potential high-risk types 26, 66, 67 and the low-risk type 70. The mRNA is captured on magnetic beads through hybridization to complementary DNA probes and amplified through transcription mediated amplification (TMA). TMA is an isothermal amplification method, where RNA is transcribed to DNA by reverse polymerase. The resulting DNA, which includes a promotor sequence for T7 RNA polymerase, is transcribed by the RNA polymerase and an abundance of RNA copies are created. The RNA amplicons are detected through hybridization to fluorescein labelled DNA. Non-hybridized probes are degraded, whereas probes bound to RNA amplicons emit light. The emitted light is registered by the highly automated instrument.

This assay, which has been used in Skåne for cervical screening of women 30 years and older since January 2017, was validated in 2013 and fulfilled the requirements (92), but the utility in screening at five-year or longer intervals was not shown (93). Forslund et al. evaluated the Aptima HPV assay for cervical cytology samples taken up to seven years before CIN3 or cancer, and found a similar longitudinal sensitivity as the HPV-DNA test Cobas 4800 (Roche) which had previously been shown to perform according to the Meijer criteria (91), The sensitivity was 76.3% (95% CI: 65.8%-84.3%) for the Aptima assay and 82.5% (95% CI: 72.6%-89.4%) for the Cobas assay. Since HPV E6/E7 mRNA is detected in the Aptima assay, and E6/E7 expression is a key feature of neoplastic progression, it is reasonable to assume that the assay efficiently identifies the relevant HPV-infections. A higher specificity for the Aptima assay than for hc2 was seen in a review by Haedicke and Iftner in 2016 (85). A meta-analysis from 2022 (94) confirms that mRNA testing with Aptima has a similar sensitivity for CIN2+ and CIN3+ and a slightly higher specificity than DNA-based assays. However, due to scarce data for longer intervals than five years, the authors only recommend use of mRNA testing for screening intervals up to five years. The Swedish national guidelines recommend a five year screening interval for women aged 23-49 and a seven years screening interval for women 50-64 years old (37). In Skåne, the screening intervals have been kept at three years for the younger women and five years for the older women.

In study I, primary HPV screening by use of Hologic's system for HPV mRNA detection is an evaluated. The women in study III have participated in the cervix cancer screening program and tested positive by this assay.

HPV genotyping

In recent years, genotyping has gained increased attention. Large epidemiological studies have increased the knowledge of how the HPV types differ in prevalence and oncogenicity (36). Commercial systems for high-throughput detection of HPV with so called extended genotyping have evolved. Instead of just reporting the presence of any high-risk type in the sample, the systems give information on the type or the oncogenicity group (Table 2) that is detected.

As mentioned above, the Aptima HPV assay currently used in Skåne reports 14 high-risk types and four other types. Positive samples are further tested with Aptima GT, a genotyping assay which detects the high-oncogenic types HPV16, and 18/45.

MGP-PCR

For wide use in the screening program, automated systems for genotyping are required. For genotyping of selected samples or for research use, other methods can be used, for example a bead-based multiplex PCR. The samples in study IV had been analysed by the modified general primer PCR system developed by Söderlund-Strand et al. (95). The system was a modification of the classical HPV general primer system GP5+/6+ (96), with primers that could be used to amplify the L1 region of many different HPV types. The resulting amplicons were detected through hybridization to short oligonucleotide probes linked to fluorescence-labelled polystyrene beads. Every probe, specific for a certain HPV type, was linked to a bead with a specific fluorescence. Beads with hybridized amplicons were identified, and counted, by a type of flow cytometer, the Bioplex 200 Luminex system. The assay could identify twelve different high-risk types: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59, as well as the former probable or possible high-risk types 26, 53, 66, 67, 68, 69, 73, 82 and the low-risk types 6, 11, 30, 40, 42, 43, 54, 61, 62, 70, 74, 81, 83, 85, 86, 87, 90, 91 and 114. In total 39 different HPV types could thus be detected.

Methylation analysis

Epigenetics is the study of DNA alterations which are generally not inherited and where the nucleotide sequence is not affected. During development of the human body, genes can be activated or silenced due to epigenetic changes. Methylation is an important epigenetic mechanism where a methyl group (CH_3) is added to a protein or DNA. DNA-methylation generally means

addition of a methyl group to the 5-position of the aromatic ring of a cytosine (C) next to a guanine (G), a so called CpG motif, often but not necessarily in a CpG island, a section of DNA with a high concentration of CpG sites. The methyl group increases the hydrophobicity of the DNA, which can become more condensed, so that genes or promoter regions may be switched off, or at least be less accessible for transcription. Methylation of tumor suppressor gene promoters increases the risk for cancer. Aberrations in the DNA methylation pattern have an important role in cancer and other types of diseases (97). Interestingly, smoking changes the methylation pattern, and increased methylation of certain sites can predict lung cancer in buccal samples collected more than 20 years before the diagnosis (98). Methylation is closely linked to aging and studies are performed to find methylation biomarkers for biologic age (99). In summary, DNA methylation profiling has been described as an emerging tool in pathology, which as an addition to histomorphology can increase accuracy of diagnosis (100).

Methylation of various human genes, HPV genes and combinations of human genes and HPV genes have been investigated for detection of cervical cancer and its precursors. Human genes shown to have elevated methylation in cervical cancer and HSIL (CIN2 and CIN3) are among others *CADM1*, *MAL* (101), *EPB41L3* (102), *FAM19A4* (103, 104), *miR124* (105), *PAX1* and *SOX1* (106).

A common method for DNA methylation testing is by quantitative methylation-specific PCR. The Ct value (cycle threshold) of the methylated gene is compared to the Ct value of a housekeeping gene, for example β-actin, and if the difference is less than a set cut off, the result is considered positive.

The S5 classifier is a detection system based on methylation of the late regions of HPV16, HPV18, HPV31 and HPV33 and the promoter region of the human gene EPB41L3 (102). The S5 classifier uses pyrosequencing.

Prior to PCR or sequencing, the DNA must undergo bisulfite conversion. By this approach, unmethylated cytosines are converted to uracil whereas methylated cytosines are conserved (Figure 5). PCR probes can be designed for detection of DNA segments with conserved cytosines, which indicate that these nucleotides were methylated in the gene of study.

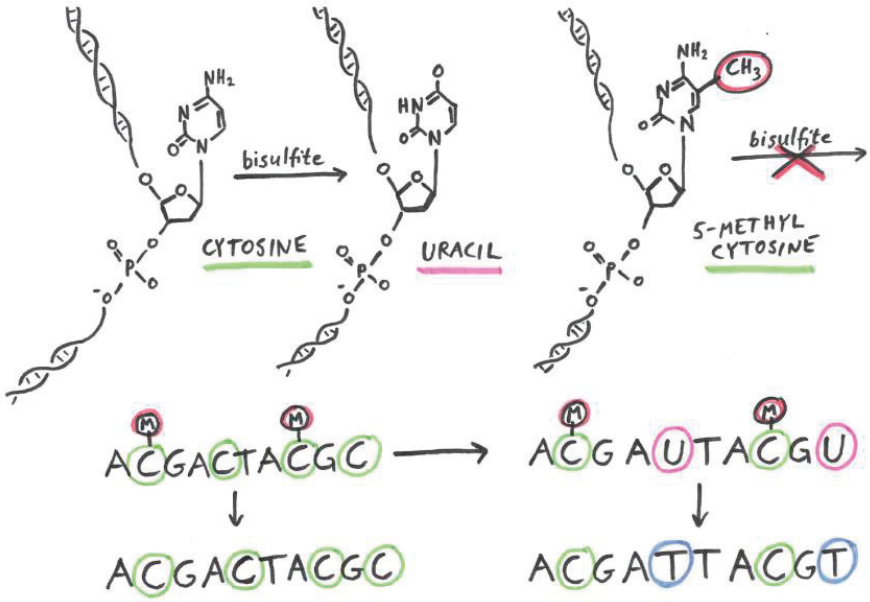


Figure 5. Principles of bisulfate conversion
Bisulfite treatment will convert not methylated cytosine residues to uracil, which during PCR will be replaced by thymidine. Methylated cytosine residues will not be affected by the bisulfate treatment but will remain cytosines. Sequencing or methylation specific PCR will elucidate whether the cytosines were methylated or not.

FAM19A4/miR124-2 methylation

Methylation of the promotor region of the gene *hsa-miR124-2* is seen in different types of cancer, including colon cancer, breast cancer, leukemia and cervical cancer, and causes decreased expression of this microRNA (107). MicroRNAs are 20-24 nucleotide long single stranded RNAs known to interact with mRNAs and regulate gene expression post-transcriptionally. The exact role of miR124 in cervical cancer is not fully elucidated, but decreased levels of miR124 is associated with increased levels of *IGFBP7* mRNA (107). *IGFBP7* belongs to a gene family associated with gynaecological malignancy.

FAM19A4 (also known as TAFA4) is a chemokine-like protein involved in the regulation of immune responses in the central nervous system and regeneration after brain injury (108). Genome wide search for methylation markers revealed increased methylation of *FAM19A4* in human keratinocytes transduced with HPV16E6E7, as well as in cervical carcinomas (109). Further

evaluation has confirmed that methylation of *FAM19A4* is a promising marker for cervical cancer and advanced cervical lesions (103, 110).

In study II and study III, *FAM19A4/miR124-2* methylation in liquid-based cytology samples was evaluated for use in the cervical cancer screening program. The assay is available as a commercial kit, the QIAsure test by QIAGEN. It has shown high sensitivity for detection of cervical cancer (111). Unlike the S5 classifier, the QIAsure test does not require pyrosequencing, and could therefore be more readily implemented in routine clinical use. After bisulfite treatment and the subsequent purification step, quantitative PCR is performed on a Rotorgene instrument. ACTB (actin β) is used for quality control of the specimen and the bisulfite conversion. If the Ct for ACTB exceeds 26.4, the analysis is invalid. If the Ct value for the methylated FAM19A4 is less than about 10 cycles higher than the Ct value for ACTB (exact cut off is not communicated by the manufacturer) the samples is positive for FAM19A4 methylation. For miR124-2, a Ct value lower than approximately 6 cycles higher than the Ct value of ACTB is required for a positive result. If FAM19A4 or miR124-2 are amplified with a higher Ct value, indicating low amounts of methylated DNA, the result is negative. If methylated FAM19A4 or miR124-2 are not amplified in the PCR, but the ACTB is amplified with a Ct value ≤26.4, the sample is negative for methylation.

Study designs

Study I

The study is an evaluation of the population-based cervical cancer screening in Skåne after the introduction of primary HPV testing in January 2017. The number of identified cases of pathologic cytology and the number of women who needed follow up during a twelve-month period before introduction of primary HPV-screening (January 21, 2016 - January 22, 2017) and after the introduction of primary HPV-screening (January 23, 2017- January 21, 2018) was compared. The HPV test used was the Aptima assay mentioned above, which detects HPV E6/E7 mRNA from the 14 high-risk HPV types. Samples were collected (by midwives) in Thinprep (Hologic) liquid-based cytology medium, which made reflex cytology after HPV testing possible. The primary HPV-screening only included women 30 years and older, while women between 23 and 29 were screened by cytology. The women participating in the screening could be up to 70 years, since yearly re-invitations were sent to them who had not taken part in screening at age 64 or older.

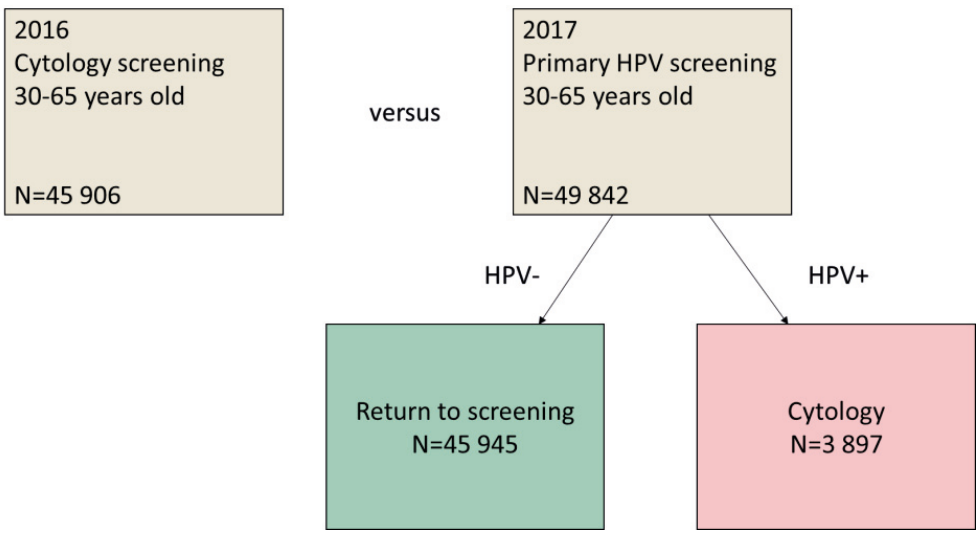


Figure 6. Design of Study I
Overview of the groups of screened women compared in the audit.

A head-to-head comparison of cytology screening and primary HPV screening was performed for a control group consisting of women 40-42 years old, n= 4433 (Figure 7). The cytology samples from women in this age group were assessed regardless of the HPV-result. For the rest of the samples, the cytology was only assessed if the HPV test was positive. Women with a negative HPV test returned to the screening program, which meant a new invitation to screening three years later for women under 50 and five years later for women over 50 years of age. Women who were HPV positive with cytology ASCUS+ should be followed up by colposcopy. Before 2017, women with cytology ASCUS or LSIL were referred for colposcopy only if HPV positive, otherwise they were reinvited for a new test one year later.

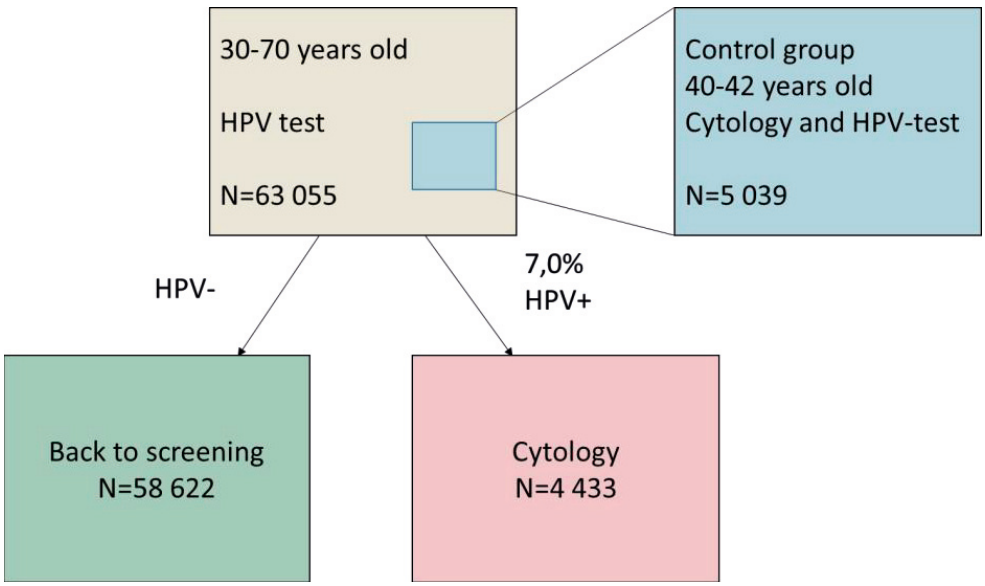


Figure 7. Design of Study I
Women 40-42 years old were co-tested with cytology and HPV test.

All samples were analyzed at Clinical Pathology, Lund, where also the cytology was assessed by cytology technicians and graded according to the Bethesda system. Data on HPV- and cytology-results were collected from the pathology laboratory information system.

Study II

We had access to a collection of 1 225 LBC (liquid-based cytology) samples stored at -80°C, from women who participated in the cervical cancer screening program from May 2007 to January 2012 and who later developed CIN3+ diagnosed by histology. A selection of 113 of these samples were analyzed for methylation of the human genes *FAM19A4* and *miR124-2* with the QIASure® kit (Qiagen) described above. All available samples from women with invasive cancer and AIS (adenocarcinoma in situ) were selected, along with a fraction of samples from women who developed CIN3 (Figure 8). The women had an average age of 38.0 years (range 19-80 years) at the time of the histology diagnosis (sampling time). The time between the histology diagnosis and the previous cytology sample was in average 2.8 years (range 4-95 months).

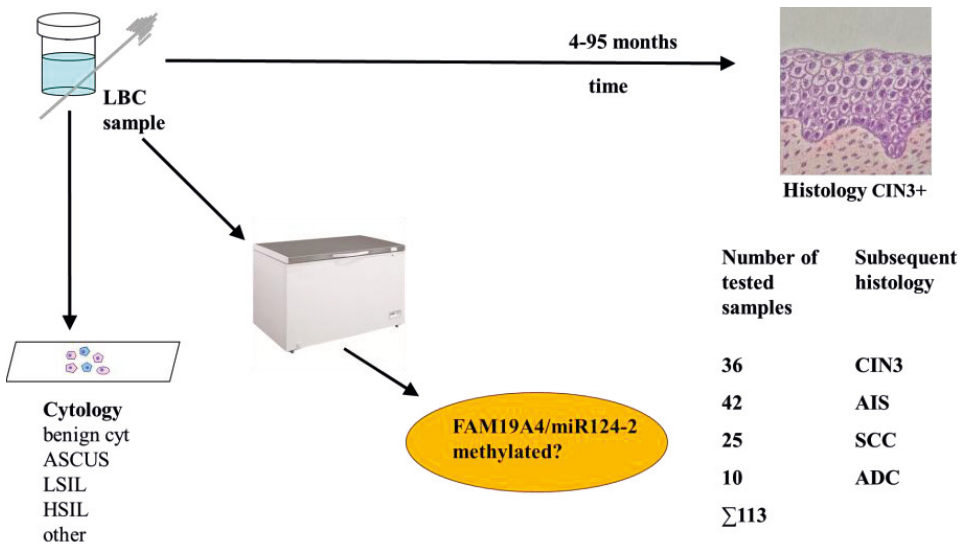


Figure 8. Design of Study II
113 LBC samples collected 4-95 months before histology CIN3+ were selected for methylation analysis.

Samples from women who took part in the screening program 2007-2012 with normal cytology (n=31) were analysed to estimate the specificity of the methylation test. These women, with an average age 41.4 years (range 20-66) had normal cytology and/or a negative HPV result in the next round of screening 3-5 years later, and no abnormal histology result registered in the pathology data base.

Study III

During 2017, the first year of primary HPV-screening in Skåne, 4 227 women 30 years and older were positive for at least one of the HPV types included in the Aptima HPV mRNA assay. These women took part in the population-based screening and did not have any known history of dysplasia. Data was collected from the pathology laboratory information system, and a review was made including reflex cytology results of the samples from 2017 and future histology diagnoses of HSIL, AIS, SCC and ADC for these women. The result of follow up HPV testing in 2020, for HPV positive women with normal cytology in 2017 was retrieved from the pathology database.

All available cytology samples from 2017 from women who developed AIS (n=34), SCC (n=13) or ADC (n=8) before 2023 were analyzed for methylation of *FAM19A4* and *miR124-2* with the QIASure® assay (Qiagen) described above, together with a selection of samples from women who developed HSIL (n=89) before the end of 2020.

Methylation analysis was also performed for samples from HPV positive women with normal cytology (n=58) and ASCUS (n=65) in 2017 and a negative HPV test by 2020, to estimate the specificity of the assay.

Study IV

From January 2010 through October 2012, 2 031 women in the Malmö area were treated for cervical dysplasia by loop electrosurgical excision procedure (LEEP) with histology diagnosis CIN2 or CIN3. 446 women (22%) who had been HPV tested before and after the treatment with were identified in the pathology registry. The HPV test had previously been performed with the MGP PCR Luminex assay described above, identifying 39 different HPV types, including 12 high-risk types (Table 3). The samples were collected in average 4 months before treatment (range 0-32 months) and 7 months after treatment (range 0-26 months). The average age of the women was 34.8 years (range 18-59 years) at the time of the LEEP cone.

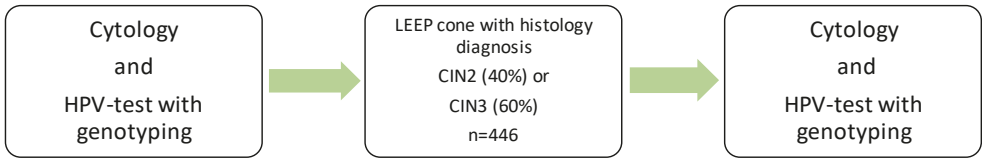


Figure 9. Design of Study IV
446 women with cytology samples analyzed with the MGP PCR Luminex assay both before and after treatment of CIN2+ were identified in the pathology data base. The samples were collected in average 4 months before treatment (range 0-32 months) and 7 months after treatment (range 0-26 months).

Table 3. HPV types identified by the MGP PCR Luminex assay. The assay could identify 12 different high-risk HPV types (HR), 8 different probable or possible high-risk types (PHR) and 19 different low-risk types (LR). Classification of HPV types according to IARC in 2012 (34).

CLASSIFICATION	HPV TYPES
High-risk (HR)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Probable/possible high-risk (PHR)	26, 53, 66, 67, 68, 69, 73, 82
Low-risk (LR)	6, 11, 30, 40, 42, 43, 54, 61, 62, 70, 74, 81, 83, 85, 86, 87, 90, 91, 114

Statistics

Chi-square test with Yates's correction was used for analysis of differences between the rates of different cytology diagnoses before and after the introduction of primary HPV screening (paper I). GraphPad Software was used (www. Graphpad.com).

Pearson's Chi-square test for trend was used for differences in HPV prevalence between age groups in paper I and differences in methylation rates between histology diagnoses in paper II. We used the Epitool epidemiological calculators (epitools.ausvet.com.au).

Fisher's exact test (two-tailed) was used in paper IV for comparison of the number of HPV infections before and after treatment, for comparison of the sensitivity of methylation analysis and cytology for identification of future high-grade dysplasia and cancer in paper II, and for comparison of methylation rate in samples with ASCUS and normal cytology prior to clearance of HPV in paper III. GraphPad Software was applied.

To analyze whether the prevalence of HPV types had changed significantly after treatment of high-grade dysplasia (paper IV) Mc Nemar's test was used (MedCalc for Windows, MedCalc Software).

Cohen's kappa was calculated in paper II and III for assessment of the agreement between methylation and abnormal cytology, by use of GraphPad Software.

The Epitool website was used for calculation of confidence intervals for proportions (paper II-III). The Clopper-Pearson exact method was applied.

G*Power was used for power calculation (112).

Ethical considerations

The studies included in this thesis used previously collected liquid cytology samples (study II-III) and previously collected data on the studied women (study I-IV). No sampling or other interventions were added to the routine procedure. When patients have samples taken, they agree to have their sample stored in a biobank for future use in diagnostics, healthcare, quality control and research, unless the opposite is stated. The person who takes the sample has the duty to inform the patient on the purposes for which the sample may be used, according to the Swedish Biobank Act. The patients should also be informed that she has the right to limit the use of her sample. The patient can always ask to have her sample removed without explaining why. Study II was approved by the Swedish ethics review authority, reference number Dnr 2019-01464, as well as study III, Dnr 2023-05041-01. Study III required an advertisement in a newspaper to inform women that the study was planned, and who they could contact if they had questions or objected to the use of their sample in the study. Study I and IV were approved by the Ethical review board in Lund, Dnr 2013/390.

A permit from the consultation group for quality registers and healthcare databases (KVB) to extract data from the Regon Skåne databases, in this case the pathology laboratory information system, was required for study III, as well as a permit to use samples from the biobank. The samples were tested anonymously.

Results

Study I

63 055 women 30-70 year of age participated in the 2017 screening, of which 49 842 were aged 30-65. In 2016, 45 906 women aged 30-65 participated in the screening (Figure 6 and 7). The overall HPV mRNA prevalence in 2017 was 7.0% (4433/63055) for women aged 30-70. The HPV-prevalence decreased by age, from 11.2% for 30–49-year-old women to 3.95% for the oldest groups aged 66-70 (Figure 10). The rate of women with cytology ASCUS+ also decreased with increasing age and was similar before and after the introduction of primary HPV-screening for each age group (Figure 10). The total proportion of women aged 30-70 years with cytology ASCUS+ was 3.13% in 2017. The proportion of abnormal cytology among HPV positive women also decreased with increasing age, with proportions of 51% for women aged 30-49, 34% for women aged 50-65 and 26% for women aged 66-70.

The proportion of women aged 30-65 with cytology ASCUS+ was 3.52% (95% CI: 3.36%-3.70) in 2016 and 3.70% (95% CI: 3.53-3.86%) in 2017 (Figure 11). In absolute numbers, 1 618 of 45 906 women had ASCUS+ in 2016, compared to 1 842 of 49 842 in 2017. The number of women with cytology ASCUS+ did not differ significantly between 2016 and 2017 (p=0.16).

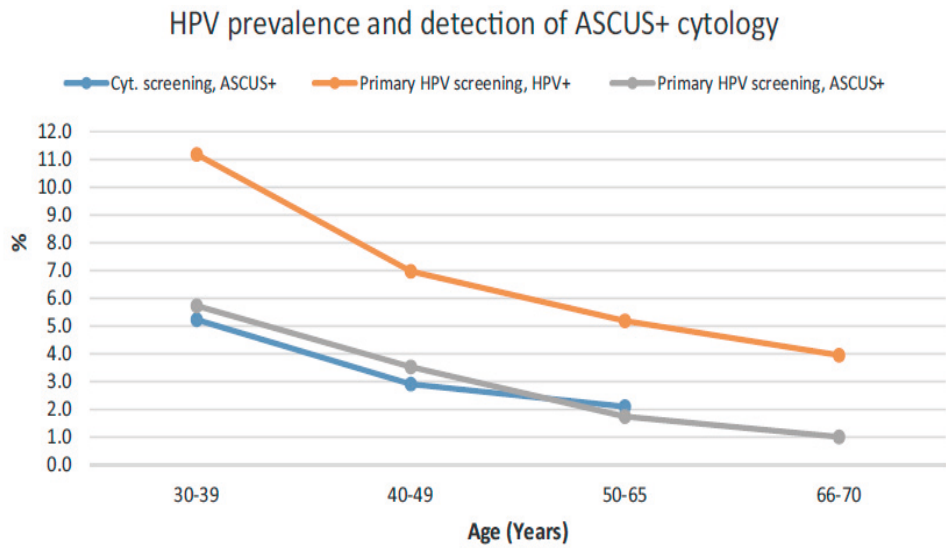


Figure 10. Prevalence of HPV and cytology ASCUS+ for different age groups
The overall HPV prevalence in 2017 ranged from 4.0-11.2% with the highest prevalence for the youngest women. The proportion of women with ASCUS+ cytology was similar between 2016 (shown in blue) and 2017 (shown in gray). Women 66-70 years did not take part in the screening in 2016. Figure from Paper I.

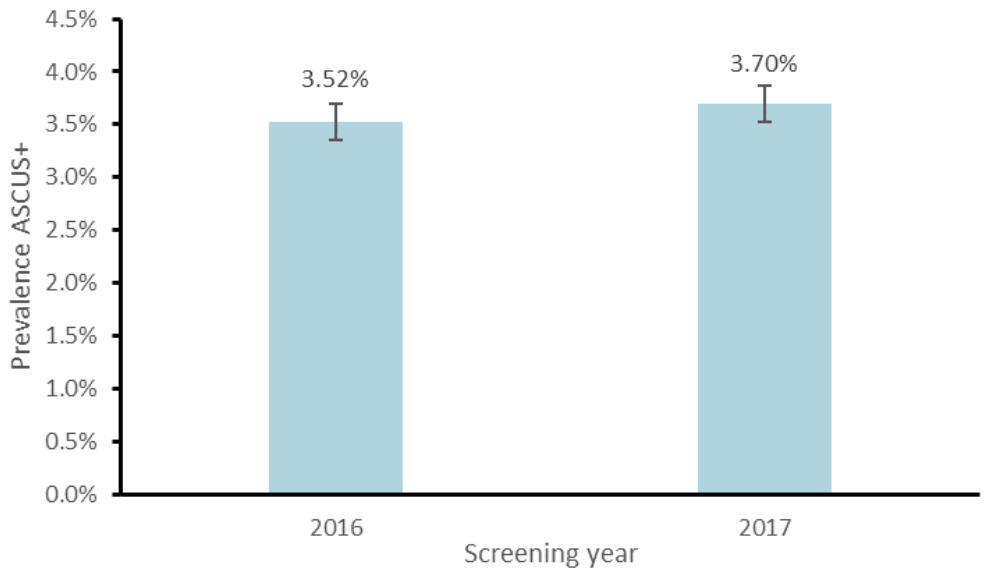


Figure 11. Prevalence of cytology ASCUS+ among women 30-65 years of age.
The prevalence of cytology ASCUS+ was the same after introduction of primary HPV screening. 1618 of 45 906 women had ASCUS+ in 2016 and 1842 of 49 824 in 2017. 95% CI is shown in the histogram.

Stratified by cytology diagnosis, a significant difference was seen only for cytology ASC-H (Atypical squamous cells, cannot exclude high-grade lesion), where the proportion increased from 0.13% to 0.23% of screened women ($p<0.0001$). For the cytology diagnoses ASCUS, LSIL, HSIL, AGC (atypical glandular cells) and cancer, no significant difference was seen (Figure 12).

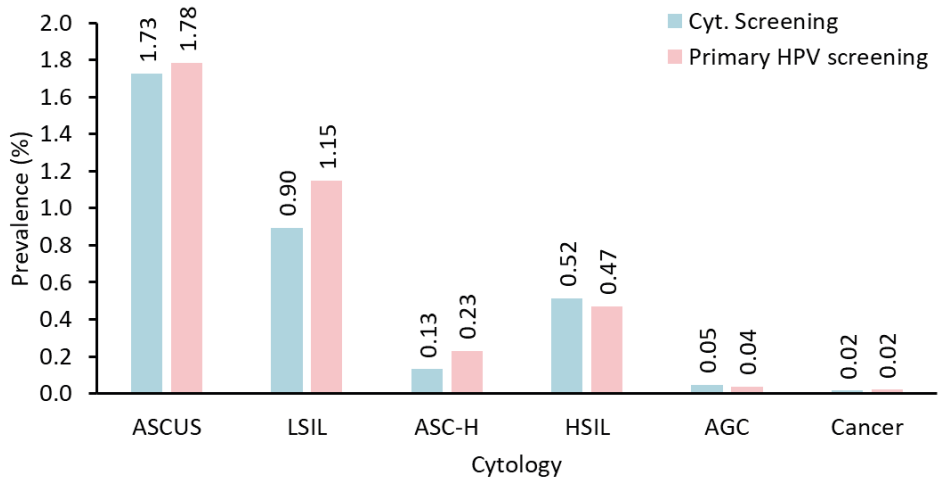


Figure 12. Prevalence of cytology diagnoses in women screened with cytology based screening in 2016 and primary HPV screening in 2017.

Women 40-42 years old were co-tested with cytology and HPV test. In this age group, the HPV prevalence was 7.2% (364/5039) As shown in Figure 13, all women with HSIL, and ASC-H were HPV positive. Four out of five women with atypical glandular cells, 90% (56/62) of women with LSIL and 58% (79/136) of women with ASCUS were HPV positive. Of women with normal cytology, 3.9% (188/4787) were HPV positive.

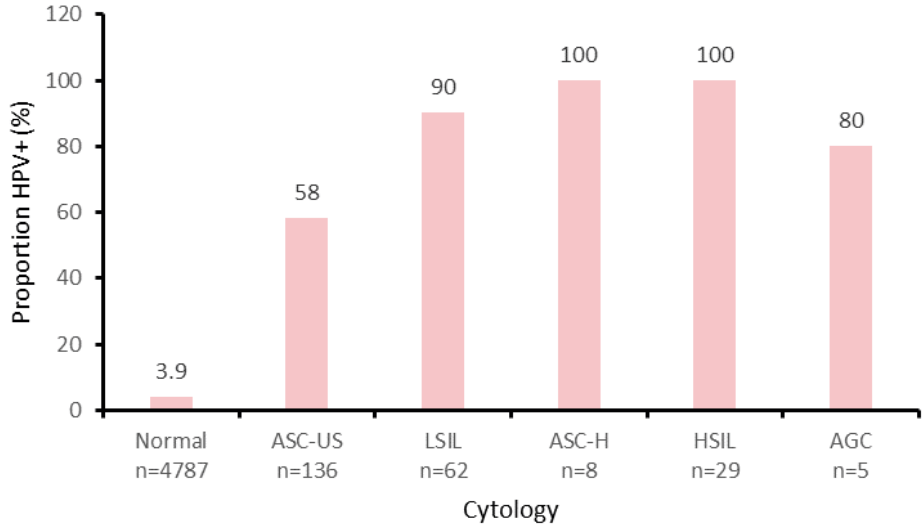


Figure 13. Proportion of HPV positive women 40-42 years old (n=5 039) stratified by cytology diagnosis.

The number of cytology assessments decreased markedly with the introduction of primary HPV screening, since only HPV positive samples were assessed. During 2017, 7% of the screening samples were HPV positive, resulting in a 93% reduction of cytology assessments. But since women 40-42 were "double-tested", an extra 4675 (n = 5039 minus the 364 samples with a positive HPV result) was added to the 4 433 HPV positive screening samples. Thus, 9108 cytology assessments were performed, 14% of 63 055, while 86% of the samples were only HPV tested.

Women with a positive HPV test and cytology ASCUS+ were referred for colposcopy follow up. In 2017, 1842 women 30-65 years (3.7%) had cytology ASCUS+. For obvious reasons, all were HPV positive. In 2016, 1618 women had cytology ASCUS+. But before the introduction of primary HPV screening, women with ASCUS and LSIL were only followed up if they were also HPV positive. Based on the percentage of HPV negative ASCUS and LSIL in 2016, 57% and 14 % respectively (data not shown) 513 women were subtracted from the total number of 1618 women with cytology ASCUS+ in 2016. Thus, the estimated colposcopy rate for women 30-65 years increased by 54%, from 1105 in 2016 to 1842 in 2017, assuming follow up according to the screening program.

Study II

The FAM19A4/miR124-2 methylation test gave a valid result for 106 of 113 (94%) tested LBC samples. The rest of the samples were invalid due to a too high Ct value for the control gene ACTB (β-actin). In total 54 samples (51%) were methylation positive. Most of the positive samples were positive for methylation of FAM19A4 while only about half were positive for methylation of miR124-2. Methylation of only FAM19A4 was seen in 28 samples, methylation of only miR124-2 in three samples and methylation of both markers in 23 samples. Of the samples from healthy women, 90% (28 of 31) were methylation negative (95% CI: 74%-98%).

For all analysed samples from women who developed high grade dysplasia or cancer (CIN3+) the agreement between the cytology result and the methylation result was 58%, Kappa 0.157 (slight agreement) (Table 4). The positivity rate for methylation, 51%, was significantly higher than the 31% positivity rate for cytology ASCUS+ (p=0.005). Methylation was detected in 45% (33/73) of normal cytology samples, while cytology ASCUS+ was detected in 36% (12/33) methylation negative samples.

Table 4. Agreement between cytology ASCUS+ and methylation prior to CIN3+. Results for LBC samples collected 4 months – 8 years before histology CIN3+. Concordant results are shown in blue.

Cytology	Methylation positive	Methylation negative	Total
ASCUS+	21	12	33 (31%)
Normal	33	40	73 (69%)
Total	54 (51%)	52 (49%)	106 (100%)

Cytology samples from women with subsequent CIN3, AIS, SCC and ADC histology were methylation positive in 39% (95% CI: 23-57%), 51% (95% CI: 34-68%), 61% (95% CI: 39-80%) and 70% (95% CI: 35-93%) of the cases, respectively (Table 5, Figure 14). The proportion of methylated samples increased significantly with the severity of the dysplasia (chi-square for linear trend p=0.036).

Table 5. Frequency of methylation and cytology ASCUS+ prior to CIN3+ among methylation tested samples. Frequency of ASCUS+ among *all* 1 225 cytology samples from from May 2007 to January 2012 from women who later developed CIN3+ is shown for comparison with the frequency of ASCUS+ in samples selected for methylation analysis.

Histology	Methylation positive/tested samples (%)	Cytology ASCUS+/tested samples (%)	Cytology ASCUS+/all samples (%)
CIN3	14/36 (39)	4/36 (11)	589/1094 (54)
AIS	19/37 (51)	17/37 (46)	22/45 (49)
SCC	14/23 (61)	12/23 (52)	18/32 (56)
ADC	7/10 (70)	0/10 (0)	2/18 (11)
Other cancers	NA	NA	6/36 (17)
all	54/106 (51)	33/106 (31)	637/1225 (52)

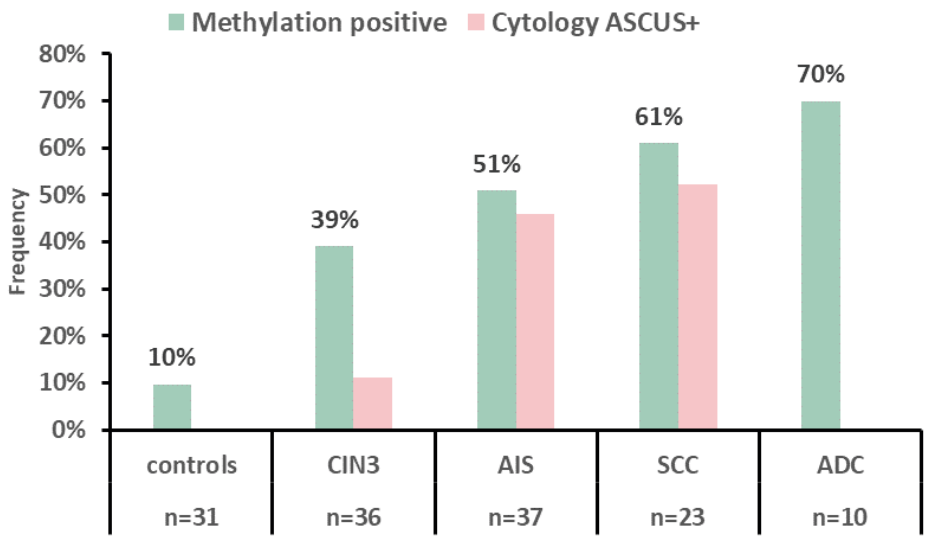


Figure 14. Frequency of methylated LBC samples 4 months-8 years before CIN3+ histology. Result for healthy women (n=31) is shown together with the results for the women with subsequent CIN3+ (n=106). The frequency of cytology ASCUS+ for the methylation tested samples is shown for comparison. For abbreviations of histology diagnoses, please see the list of abbreviations.

As mentioned, the methylation rate for samples collected prior to CIN3 histology was 39%. However, the analysed samples had a higher percentage of normal cytology (88%) than the entire group of cytology samples (46%) taken before CIN3 histology. Only 11% (4/36) of the analysed samples had ASCUS+ cytology, whereas 54% (589/1 094) of all cytology samples collected prior to CIN3 had abnormal cytology (Table 5). For the other histology diagnoses, the methylation tested samples were more representative for all cytology samples (n=1225) regarding the rate of cytology ASCUS+.

Prior to SCC, 61% (14/23) of analysed cytology samples were methylation positive and 52% (12/23) had cytology ASCUS+ (Table 5). All samples collected before ADC had normal cytology, but 70% (7/10) were methylation positive. In total, before development of invasive cancer (SCC, ADC), 64% (21/33) of the cytology samples were methylated, of which 36% (12/33) had ASCUS+ and 64% (21/33) had normal cytology. Some samples were negative for methylation but positive for cytology ASCUS+, while others were methylation positive despite normal cytology (Table 6). The methylation assay identified more cases of cancer than cytology did (p=0.048).

Table 6. Agreement between cytology ASCUS+ and methylation prior to cancer. Results for LBC samples collected 4 months – 8 years before cancer (SCC n =23, ADC n=10). Concordant results are shown in pink.

Cytology	Methylation positive	Methylation negative	Total
ASCUS+	9	3	12 (36%)
Normal	12	9	21(64%)
Total	21 (64%)	12 (36%)	33(100%)

Samples collected up to two years before histological CIN3+ diagnosis were methylation positive in 55% (30/55) of the cases and had cytology ASCUS+ in 55% (30/55) of the cases. Two to five years before the diagnosis 64% (18/28) were methylation positive, but only 7% (2/28) had cytology ASCUS+ ($p<0.0001$) (Table 7).

Table 7. Result of methylation test and cytology for samples collected at different time spans before histological CIN3+ diagnosis.

Time to histology CIN3+	Methylation		Cytology ASCUS+		Number of samples
	+	-	+	-	
4-24 months	30	25	30	25	55
25-60 months	18	10	2	26	28

As mentioned above, 51% (54/106) of cytology samples collected prior to CIN3+ cytology were methylation positive (Table 5). ASCUS+ cytology was seen in 31% (33/106) of the methylation tested samples. 61% (66/106) were positive for either methylation of *FAM19A4/miR124-2*, cytology ASCUS+ or both.

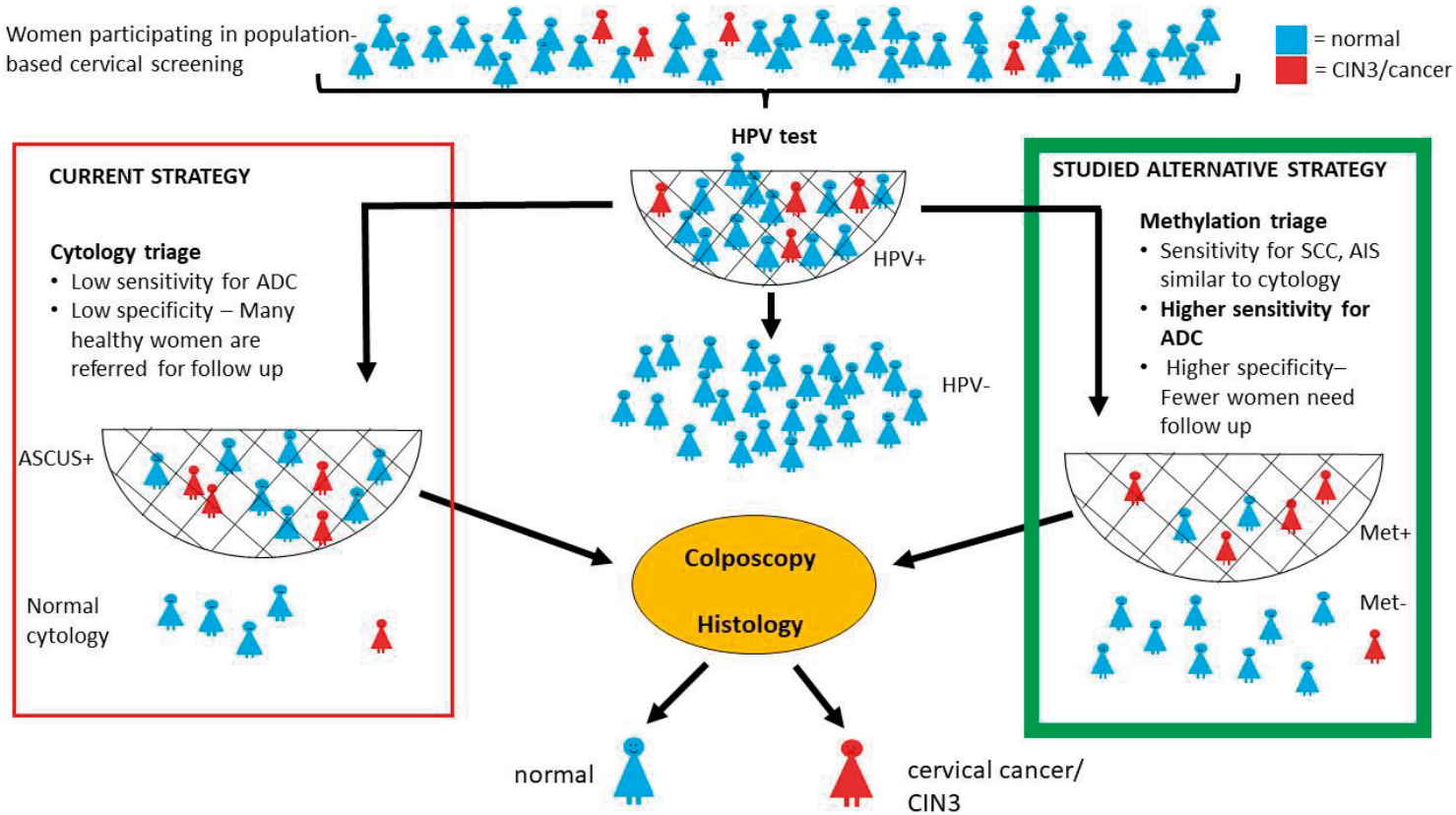


Figure 15. Graphical abstract illustrating Study II.

Study III

During 2017, 4 227 women 30 years and older who took part in the population-based screening program tested positive in the Aptima HPV mRNA assay. They made up 6.8% of the total 62 231 who took part in the screening in 2017, after the introduction of primary HPV testing (from 23 Jan to December 31). The average age of the HPV positive women was 45.5 years (range 30-78). Approximately half, 46%, had cytology ASCUS+, while the rest had normal cytology. Women with normal cytology were offered a new HPV test in 2020. Results from HPV testing in 2020 were identified for 55% of the women with normal cytology in 2017. In 2020 63% of tested women had cleared the HPV-infection, while 37% were still HPVpositive (Figure 16).

As shown in Figure 16, before the end of 2020, 617 (14.5%) of the HPV positive women had been diagnosed with HSIL, histology diagnosis, in a biopsy or cone. Histology diagnosis AIS, SCC and ADC were seen in 38 (0.9%), 13 (0.3%) and 12 (0.3%), respectively. In total 16% of the HPV positive women were diagnosed with HSIL+ within 3-4 years. 88% (601/680) of women with HSIL or cancer had been identified through abnormal reflex cytology in the 2017 screening sample, but 12% had normal cytology in the screening sample. The positive predictive value for HSIL or cancer (histology diagnosis) was 31% (95% CI: 29-33%) for cytology ASCUS+.

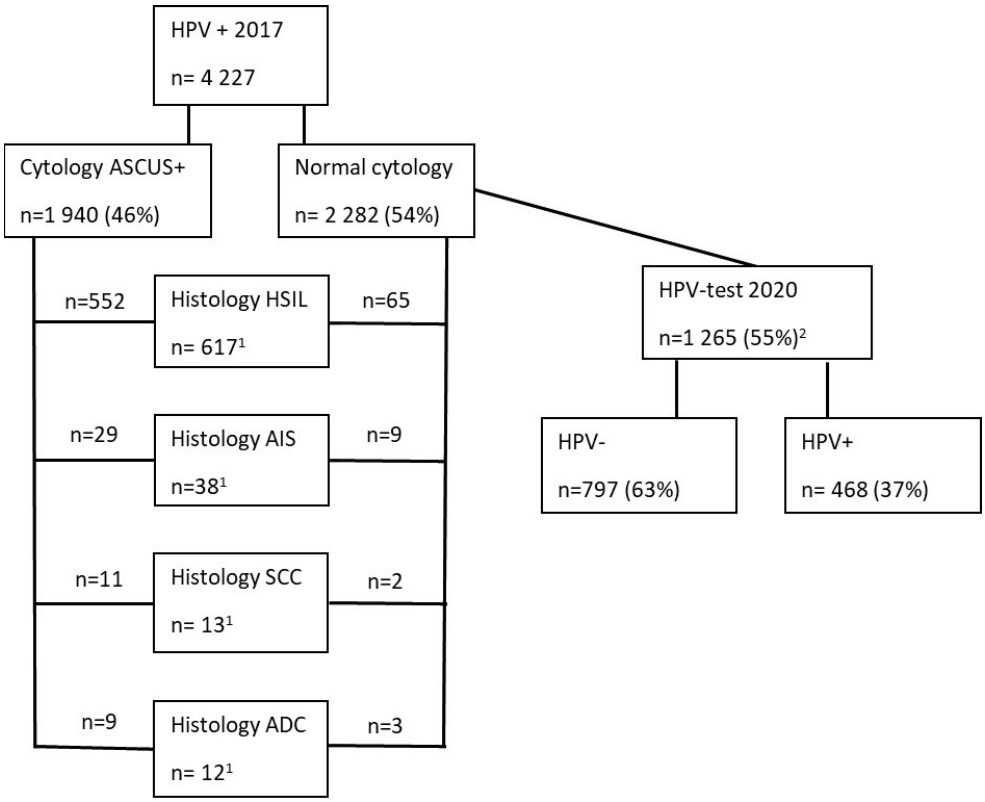


Figure 16. Overview of the women 30 years of age and older who took part in the cervical cancer screening program in 2017 with a positive HPV result.

Of the 4 227 HPV positive women, 54% had normal cytology and were followed up with a new HPV test three years later. Before the end of 2020, 617 women developed HSIL, 38 AIS, 13 SCC and 12 ADC, diagnosed by histology of a biopsy or cone. The number of women with ASCUS+ and the number with normal cytology in the screening sample is shown in the figure. [1]A subset of the cytology samples from 2017 from these women were tested for methylation. [2]Women tested 2018-2019 or after 2020 are not included in this number. For abbreviations of histology diagnoses, please see the list of abbreviations. Figure from Paper III.

In total 268 HPV positive cytology samples from 2017 were analysed for methylation of *FAM19A4/miR124-2*, including samples from women with development of HSIL or cancer before 2021 (n=135), samples from women with SCC diagnosis 2021-2022 (n=4), samples from women with AIS 2021-2022 (n=5), normal cytology samples from women with negative HPV test in 2020 (n=58) and samples from women with ASCUS in 2017 and a negative HPV test in 2020 (n=65). Valid results were obtained for 238 samples (89%). The agreement between cytology ASCUS+ and methylation was 60% (kappa=0.22 (95% CI: 0.11-0.33)).

Methylation results for cytology samples collected prior to histology diagnosis of HSIL, AIS or cancer 2017-2020 are shown in Table 8. Unfortunately, only 9/13 samples from women with SCC, 8/12 samples from women with ADC and 29/38 samples from women with AIS could be retrieved from the biobank. Of the tested samples, 8 samples collected prior to SCC and 6 samples collected prior to ADC gave valid results. In total 122 valid and 13 invalid results were obtained from 135 analysed samples.

Table 8. Frequency of methylation and cytology ASCUS+ for cytology samples collected in 2017 from women with histology HSIL, AIS or cancer before 2021. 122 valid methylation results were obtained from 135 tested samples.

Histology	Methylation positive/tested samples with valid result (%)	Cytology ASCUS+/tested samples (%)
SCC	7/8 (88)	8/9 (89)
ADC	5/6 (83)	5/8 (63)
AIS	19/28 (68)	24/29 (86)
HSIL	44/80 (55)	65/89 (73)

Samples collected before development of SCC and ADC showed a methylation rate at least as high as the rate of cytology ASCUS+. The frequency of methylation positive cytology samples prior to AIS and HSIL was 68% (95% CI: 48-84%) and 55% (95% CI: 43-66) respectively. No significant difference was seen between methylation and cytology for AIS (p=0.1) but for HSIL, cytology detected significantly more cases (p=0.009).

As mentioned, the correlation between methylation of *FAM19A4/miR124-2* and cytology ASCUS+ was not so strong. Some samples were methylation positive but cytology negative and vice versa. For our cohort, 100% (9/9) of women with SCC were either positive for cytology ASCUS+, methylation or both in the previous cytology sample. For ADC and AIS, corresponding number was 100% (8/8) and 93% (27/29),

Normal cytology samples from HPV positive women with no records of abnormal histology and a negative HPV test in 2020, were positive for methylation in 12% (95% CI: 4.5-24%, 6/50) of the cases (invalid results not counted). The corresponding rate for HPV positive women with only ASCUS cytology (not ASCUS+!) in 2017 but clearance of the HPV-infection by 2020 was 27% (95% CI: 16-40%, 15/56).

Study IV

In total 706 HPV infections were detected in the 446 women before treatment, of which 542 were high-risk types, 72 were potential high-risk types and 92 were low-risk types. After treatment, the total number of HPV-infections had decreased to 248. Type specific persistency, defined as detection of the same HPV type in a woman before and after treatment, was more often seen for the low-risk types. 14% (76/542) of high-risk HPV-infections and 34% (31/92) of low-risk HPV-infections persisted after treatment ($p < 0.001$). The potential high-risk HPV-infections persisted in 8% of the cases (6/72) (Figure 17).

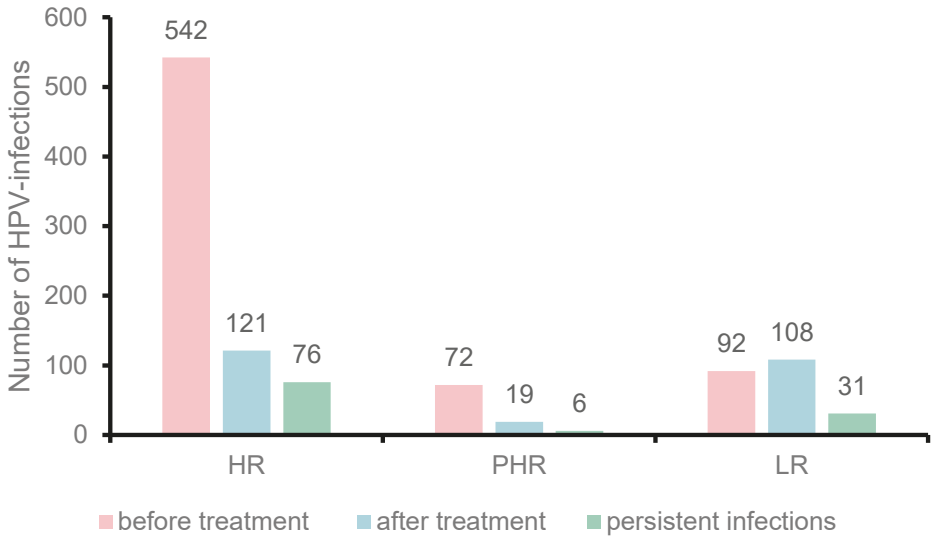


Figure 17. Number of HPV-infections before and after treatment
The number of HPV-infections among 446 women before and after treatment. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 were classified as high-risk types (HR). HPV 26, 53, 66, 67, 73 and 82 were denoted potential high-risk types (PHR). The low-risk types (LR) included HPV 6, 11, 30, 40, 42, 43, 54, 61, 70, 74, 81, 83, 86, 87,89, 90, 91 and 114). Note that multiple HPV types could be detected. “After treatment” is the total number of HPV-infections after treatment, regardless of which type within the category HR, PHR or LR that was detected. “Persistent infections” is the number of HPV-infections where the same type was detected in samples from the same woman before and after treatment.

At least one HPV type, high-risk, low-risk, or potential high-risk, was detected in 440 (99%) of the women before treatment. After treatment, 179 (40%) were still positive for at least one HPV type.

Before treatment of CIN2+ 91% (404/446) of the women were positive for at least one high-risk HPV type (Figure 18). After treatment, 23% (102/446) were positive for at least one high-risk type. Persistency of at least one high-risk type was seen among 16% (72/446).

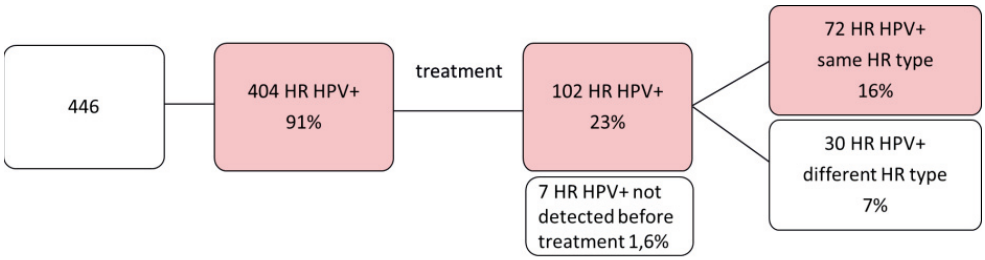


Figure 18. Number of women infected with HR HPV before and after treatment
Of the 446 eligible women, 404 were positive for at least one high-risk HPV type (HR HPV). After treatment, 102 were still HR HPV positive, and 72 had at least one type detected also before treatment. The other 30 had switched to a different HR type. Seven women not positive for HR HPV before treatment were positive after treatment. (% of 446 women),

Low-risk types were detected in 76 of the 446 women before treatment (17%) (Figure 19). Almost half, 36 women, still carried low-risk HPV after treatment and 27 had at least one low-risk type which had been detected before treatment. Of the 27 women with persistent low-risk HPV, 19 were infected with high-risk HPV before treatment but not after treatment. While 40 women had lost low-risk HPV after treatment, low-risk HPV was detected in 48 other women who were negative before treatment.

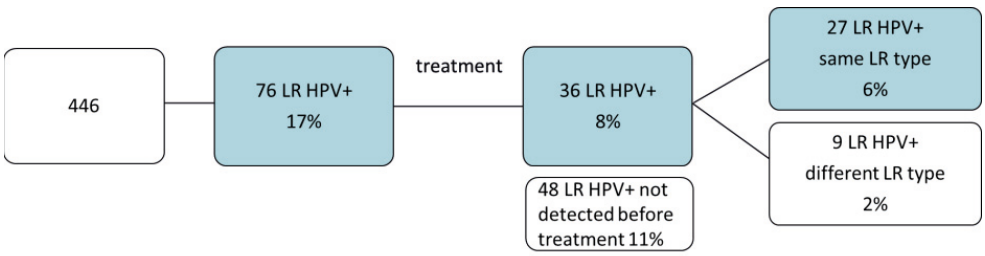


Figure 19. Number of women infected with LR HPV before and after treatment
Of the 446 eligible women, 76 were positive for at least one low-risk HPV type (LR HPV). After treatment, 36 were still HR HPV positive, and 27 had at least one type detected also before treatment, while nine had different LR types. 48 women not positive for LR HPV before treatment were positive after treatment. (% of 446 women).

The most prevalent high-risk type before treatment was HPV16, 40% of the women were infected. The second most common was HPV 31 (18%) followed by HPV52 (13%), HPV18 (11%), HPV33 (8%), HPV51 (6%), HPV45 and HPV 39 (both 5%). After treatment, the most common type was still HPV16 (6.5%), followed by HPV52 (4%), HPV31 and HPV33 (3%), and HPV45, HPV51 and HPV56 (2%).

Cytology result, for the first sample taken after treatment, in relation to HPV status is shown in Table 9. After treatment 83% of the women had normal cytology. Samples with HSIL cytology were positive for high-risk HPV in 11 of 12 cases. High-risk HPV testing of the first cytology sample after treatment thus showed a sensitivity of 92% (95% CI: 62-100%) for detection of residual HSIL. The corresponding specificity was 313/372, 84% (95% CI: 80-88%) and the negative predictive value was 336/337, 99.7% (95% CI: 98.2-100%). The OR for HSIL cytology when high-risk HPV is detected after treatment was 37.7 (95% CI: 4.8-296) and for *persistent* high-risk HPV-infection after treatment, 67.3 (95% CI: 8.5-530).

Table 9. Cytology diagnoses after treatment. Abbreviations: Atypical squamous cells of undetermined significance (ASCUS), Low-grade squamous intraepithelial lesion (LSIL), High-grade squamous intraepithelial lesion (HSIL). High-risk HPV type (HR HPV). [1] Persistency of at least one high-risk HPV type.

Cytology after treatment	All 446 women (%)	Any HR HPV after treatment	Persistent HR HPV after treatment[1]	No HR HPV after treatment
Normal cytology	372 (83)	59	35	313
ASCUS, LSIL or unclassified atypia	62 (14)	39	26	23
HSIL	12 (2.6)	11	11	1
Total number	446	109	72	337

Discussion

Study I

The HPV prevalence of 7.0% for women 30-70 years taking part in the population-based screening was in line with other studies. In Örebro 6.9% of women 30-67 years of age were HPV positive with the Aptima mRNA assay in 2016-2018 (83) and in the east part of the Netherlands, mRNA positivity was seen in 7.5% of an unselected screening population aged 29-63 (113). 7.8% of women 30-65 were HPV positive in our study. In the Venice area, 5.7% of women 35-64 years and 7.0% of women 25-64 years were HPV mRNA positive in the Aptima test (86). In southern Germany 4.9% of women 30-60 years in the routine screening were Aptima positive (114). A study from the USA showed a rate of 9.1% Aptima positive among screened women in Denver, Colorado, with a median age of 45.0 (115). A meta-analysis by de Sanjosé et al. gave an estimate of 8.1% HPV prevalence in European women with normal cytology (116). The results of the present study thus align with those of other comparable investigations.

As already shown in previous studies and confirmed by later studies (36, 113, 117) the HPV prevalence was higher among younger women. This, in combination with a high clearance rate in young women, was the reason not to introduce HPV-based screening for women under 30. However, starting during the covid pandemic, HPV-screening of self-collected vaginal samples is now the principal screening method in Skåne, also for women below 30.

No cases of HPV negative HSIL or ASC-H and only one case of HPV negative AGC (atypical glandular cells) was detected in the co-tested 40–42-year-old women. Thus, HPV testing is reliable not to miss cases of severe cytology. This has later been confirmed through continued follow up of this group until December 2020, which showed that only 2 of 18 132 HPV negative women had histologically confirmed HSIL (118). In a report published by the Swedish National Cervical Screening Registry (NKCx) in 2024, only 1% of women with HSIL histology in Skåne, as well as 1% of all women in Sweden with HSIL histology, had a negative HPV test within one screening interval (defined

as five years for women under 50 and seven years for women over 50 years) (55), confirming the sensitivity of HPV-based screening.

The prevalence of cytology ASCUS+ was approximately half of the prevalence of high-risk HPV (Figure 10). Not only did the HPV prevalence decrease by age, but also the proportion of abnormal cytology among HPV positive women. Since it is not reasonable to believe that older HPV-infected women are less prone to develop dysplasia then young HPV-infected women, a more likely explanation could be a larger number of false negative cytology samples in older women. Low sensitivity for cytology in older women has been shown in several studies (119-121). HPV testing is less affected by the sample quality, a few mRNA copies from a few exfoliated cells may be enough, while adequate cytology samples are often difficult to obtain from post-menopausal women. In conclusion, the prevalence of HPV in the older women is probably closer to the truth than the prevalence of ASCUS+ cytology.

A disadvantage of primary HPV-screening is the increased need for follow up, our estimation of 54% extra colposcopies is slightly lower than in the study from Örebro (83), where 74% extra colposcopies were performed after the introduction of primary HPV screening. A higher increase was seen in a Dutch study where a three-fold increase of referral rates was seen after introduction of primary HPV-screening by use of DNA-detection (PCR Cobas 4800, Roche) (84). One reason could be the higher specificity of mRNA testing compared to DNA testing (85). However, in an Italian study a duplication of colposcopies was the result when HPV mRNA-testing replaced cytology in the screening program (86).

The fact that all ASCUS and LSIL detected in 2017 were HPV positive, and that the proportion of ASCUS and LSIL cytology was largely unchanged after the introduction of primary HPV screening (Figure 12) means that the rate of HPV positive ASCUS and LSIL was higher in 2017 than in 2016. In 2016, 57% of ASCUS and 14 % of LSIL were HPV negative, and these women were not referred for immediate follow-up, but were scheduled for a new test one year later. The question is whether these higher proportions of HPV positive ASCUS and LSIL represent cases that would be missed by cytology based screening, or if the cytotechnicians are biased when they know the HPV status, which some studies suggest (122, 123).

What really is interesting is if the primary HPV screening could prevent more cases of cervical cancer. A weakness of the present study is the lack of histology data from follow up of the HPV positive women with abnormal cytology. Bergengren et al. found that HPV-based screening in fact detected

fewer HSIL+ cases (histology diagnosis) per 1000 screened women than the cytology screening (83). However, like in our study, two different time periods were compared, one before and one after the introduction of primary HPV screening. It could therefore be that the true HSIL+ prevalence in the population had decreased, independently of the change in screening strategy.

Study II

Testing for methylation of the human genes *FAM19A4/miR124-2* is a potential method for triage of HPV positive women in population based cervical cancer screening. This assay may be an alternative or complement to cytology. Possibly, methylation testing could identify more cases of cancer and severe dysplasia and decrease the number of women who need follow up after a positive HPV test.

In our study, methylation of the human genes *FAM19A4/miR124-2* identified more cases of cancer (SCC, ADC) than cytology did, in samples collected 4 months to 8 years before the diagnosis. The methylation assay was superior to cytology particularly for identification of adenocarcinoma (ADC). Therefore, analysis for methylation of *FAM19A4/miR124-2* can be considered in scenarios where cytology is not established. However, cytology identified cases that were negative in the methylation test, which makes switching to methylation as a triage tool difficult in laboratories with high quality cytology. Adding methylation as a complementary test for samples with normal cytology would identify more women who will develop severe dysplasia or cancer. While increasing the sensitivity, such a strategy would increase the cost for testing and would increase the number of women referred for colposcopy. Implementation of primary HPV screening, with cytology as the triage method, increased the colposcopy rate by approximately 50%, why a more specific triage method is warranted.

The methylation analysis could identify cases of future CIN3+ up to five years before the diagnosis in more than half of the cases, while normal cytology was seen in 24 of 26 samples collected two to five years before the diagnosis (Table 7). The reason why so few samples have abnormal cytology more than two years before the CIN3+ diagnosis, is that if the cytology would have been

assessed as abnormal, the women would have been followed up directly, and the CIN3+ would have been diagnosed sooner.

One concern is whether the methylation analysis has enough sensitivity for detection of CIN3. In the present study, the sensitivity for detection of CIN3 was not investigated enough, since mainly samples with normal cytology were analyzed. Still, a large proportion (39%) of the samples showed methylation of *FAM19A4* and /or *miR124-2.* A large European multicenter study found a sensitivity of 77% for detection of CIN3 and a sensitivity of 95% (19/20) for detection of cancer (124).

Surprisingly few cytology samples showed abnormal cytology prior to CIN3+ histology (Table 5). The reason could be that the cytology samples were collected up to eight years earlier. However, the average time between the cytology sample and the CIN3+ diagnosis was 2.8 years. For use as triage for follow up of HPV positive women, a test should be able to identify women at risk for high grade dysplasia or cancer within three to five years. Currently, HPV positive women with normal cytology will be retested after 18 months if positive for HPV16/18/45 and after three years if positive for other high-risk types. In this context, the fact that methylation could be detected up to five years before the diagnosis of high-grade dysplasia or cancer is promising.

Study III

Study II showed that methylation of *FAM19A4/miR124-2* had a higher sensitivity than cytology for detection of cancer (SCC, ADC) in cytology samples collected four months to eight years earlier. The assay gave a positive result for 10% of HPV negative women with normal cytology, indicating a specificity of at best around 90%. Since relatively few samples were tested in study II, and the samples were heterogenous in respect to the timespan from collection to histology diagnosis, we performed a study with samples from the first year of primary HPV screening, 2017. Preferably, to study the performance of the methylation test for triage of HPV positive women, all, or a representative selection of HPV low

positive women with a distribution of cytology diagnoses reflecting that of all screened women, should be tested for methylation of the 2017 cytology sample. In a prospective study, the specificity and sensitivity for detection of HSIL+ could be determined. Due to high cost of such test, we have instead focused on investigating the sensitivity for detection of HSIL, AIS and cancer, and to estimate the specificity by testing HPV positive women with normal cytology and histology and clearance of the HPV-infection.

Three years after a positive HPV test, 37% of women with normal cytology were still HPV positive. Since genotyping was not performed, there was no way to know if the same HPV persisted or if one HPV-infection had replaced another. Neither could we know if a woman had multiple infections or if she had cleared one infection and later become reinfected. A type-specific clearance rate of 43% within six months and 65% within 18 months for high-risk HPV has previously been reported for women with normal cytology (43). However, the high-risk types HPV16 and HPV31 had a lower clearance rate of 49% and 50% respectively. A 91% clearance rate of HPV (low-risk and high-risk) within 24 months for women with ASCUS or LSIL cytology has also been reported (125). Factors affecting the clearance rate is the age of the women, where young women clear the infection faster, and presence of abnormal cytology, which is correlated to decreased clearance rate (43, 126). Infections that have persisted for more than 18 months tend to persist longer than newly acquired infection, Possible explanations for the relatively low clearance rate for the HPV positive women with normal cytology in our study, could be acquirement of new HPV infections, either with a different genotype or reinfection with the same genotype, or a high prevalence of HPV types with low clearance rates, such as HPV16.

Increased colposcopy rate has been described to be a consequence of primary HPV screening. One reason is the tendency to upgrade the cytology when the HPV status is known (122, 123). When reviewing the 2017 screening program, we found that 31% of HPV positive women with cytology ASCUS+ in the screening were diagnosed with histological HSIL or cancer by 2020. Despite a large proportion of cytology ASCUS+ (46%) among HPV positive women, the PPV for HSIL and cancer was high, and a large proportion of colposcopies were performed on women who really needed follow up.

We hypothesized that methylation testing would be more specific than cytology, thus decreasing the need for colposcopy follow up. Women with clearance of the HPV-infection by 2020, without development of dysplasia were methylation positive in 12% (95% CI: 4.5-24%) of normal cytology samples and in 27% (95% CI: 16-40%) of samples with ASCUS (not

ASCUS+). Given that about one half of HPV positive women have normal cytology and one quarter have ASCUS (Paper III, Figure 2), while the rest have more severe cytology, the methylation rate for all HPV positive women could be expected to be at least lower than the rate of cytology ASCUS+, which was 46%.

Study II showed that the methylation test detected more cases of cancer than cytology did, in samples collected four to eight years before the diagnosis. This result was confirmed in Study III, where the rate of methylation positive was at least not less than the rate of abnormal cytology in samples collected in 2017 and histology verified SCC or ADC by 2020. Before SCC, 7/8 (88%) samples were methylation positive and 8/9 (89%) had cytology ASCUS+. For ADC 5/6 (83%) samples were methylation positive and 5/8 had cytology ASCUS+. In study II, all tested samples had normal cytology prior to ADC. In the 2017 screening, cytology had identified 9/12 women with development of ADC (Figure 16). The cytology samples in study III were in average collected shorter time before the histology diagnosis ADC, which could explain the higher proportion of samples with ASCUS+.

More cases of HSIL were identified by cytology than through methylation testing. Since most cases (88% of HPV positive women in the screening, Figure 16) of cancer and high-grade dysplasia are identified through cytology, it is not possible to make an objective comparison between the methods. Other women who would have a positive methylation test may still be undiagnosed because their cytology samples were (apparently) normal. It has been suggested that methylation can point out cases of HSIL that will progress (127, 128). Increased methylation in high-grade lesions with long duration has also been described (129). But there is not yet enough evidence for this, and the Swedish national guidelines recommend excision of HSIL in HPV positive women 30 years and older (130).

Samples with cytology ASCUS+ were often methylation negative and vice versa. As illustrated by the low kappa-values and as seen in Table 4 and Table 6, the agreement between cytology and methylation result was quite low. A possible explanation could be that the epigenetic changes take place before the appearance of the cells' morphology change. Methylation could possibly be detected when most of the cells still have normal epigenetic properties. The fact that methylation and cytology often give different results speaks for the use of both for triage of HPV positive women, to increase the sensitivity. Unfortunately, that would increase the cost for testing and follow-up.

A relatively high rate of invalid methylation results was seen, 6% in Study II and 10% in Study III. The analysis was invalid if the Ct for ACTB was above 26.4. The probable reason was too low DNA concentration in the samples. We used a fixed volume of extracted sample for the bisulfate reaction, which was the maximum that could be used given that we could only use 200 uL from each biobanked sample. (We could have used the double amount, but we wanted to have a second chance to analyze each sample in case the analysis failed). In the beginning we measured the DNA concentration by Qubit® (Invitrogen), but since the maximum input volume gave the best result, we continued to use maximum input without measurement of the DNA concentration. In a real-life situation, a larger part of the sample and a different extraction method could increase the amount of DNA used in the assay and thereby the validity rate. In a large multicenter study (124), between 3% and 27% invalid FAM19A4/miR124-2 methylation tests were seen in the participating laboratories, where different sample collection media and different DNA extraction platforms were used, indicating that the number of invalid samples can be minimized during the right conditions.

The sensitivity for detection of HSIL is somewhat discouraging. Maybe an alternative methylation method should be evaluated? The WID™ -qCIN test, which assess methylation of three human genes by PCR, has shown a sensitivity of 83% for detection of CIN3+ in women over 30 years of age, and a 100% sensitivity for detection of cancer, with a specificity of 90% (131). However, for women under 30 years, the sensitivity for detection of CIN3+ was moderate, 65%, and the sensitivity for detection of cancer 83%.

A comparison of the FAM19A4/miR124-2 methylation test, QIAsure®, and the GynTect® assay showed that GynTect® had lower sensitivity for detection of CIN2 and CIN3 (132). GynTect®, which is based on methylation on five different genes, is thus not a promising alternative to the FAM19A4/miR124-2 methylation.

Not all studies have given convincing support for methylation testing in cervical cancer screening. Dick et al. report a comparable cumulative CIN3+ incidence for HPV positive women with a negative FAM19A4/miR124-2 methylation test and HPV positive women with a negative cytology triage test, 16.3% and 15.6%, respectively. The cumulative CIN3+ incidence of methylation positive and cytology positive women were 39.8% and 46.5% (133). Despite promising results, methylation was not proved to be better than cytology for predicting CIN3+.

Furthermore, analysis of more than 500 samples for methylation of FAM19A4/miR124-2 in a study from Örebro, found a methylation rate of 28% in samples from women with no evidence of disease (including ≤LSIL histology) and a rate of 67% in samples from women with HSIL+ histology within three years (134). Thus, 33% of women were methylation negative prior to HSIL+. Interestingly, an increased methylation rate with increasing age, in the age span 30-59 year was seen in this study.

A pilot study of 55-59 year old women showed that cytology was superior to both genotyping and FAM19A4/miR124-2 methylation, for triage of HPV positive women in this age group (135), even though cytology is known to be less sensitive for detection of HSIL+ in older women (120, 136).

Research has been performed where methylation of specific CpG sites is studied, to obtain higher sensitivity and specificity for detection of CIN3+ (137). It is also possible to perform whole genome methylation profiling (138). However, more work is needed before implementation of these tests in clinical use.

In summary, before introducing methylation testing in the screening program, more research is needed.

Study IV

Cervical cancer screening is successful only if the follow up of women with abnormal screening results is appropriate. Women treated for CIN3 have an increased risk of invasive cervical cancer up to 25 years after treatment (139).

We found that treatment of CIN2 and CIN3 by conization in the Malmö area in 2010-2012 resulted in a negative high-risk HPV test in 73% of the follow up samples taken in average seven months later. The cytology was normal at follow up in 83% of the women, and 70% had both a normal cytology and a negative high-risk HPV result.

A larger study of more than 8 000 women in Skåne who underwent conization between 2015 and 2021 found a rate of cure, defined as negative HPV test and normal cytology, of 69.7% after eight months (140), the same rate as we found

in our study after in average seven months. The clearance of HPV was 79.6% after eight months in the large study, which is consistent with our result of 75%.

In our study we were able to distinguish between persistent infections and HPV-infections that either were not present or were not detected previously. Only 18 % of high-risk HPV types detected before treatment were still detected after conization, while the remaining 82% were eliminated. Previous studies have shown that *type-specific* HPV persistence predict residual disease, and genotyping can thus improve the specificity when using HPV testing in follow up after treatment (43, 141). As a matter of fact, in our study, all cases of HSIL cytology after treatment were seen in women with persistent HPV-infections (Table 9) and the OR for HSIL cytology was higher for persistent high-risk HPV infections compared to any high-risk infection, 67 vs 38. However, presence of any high-risk type after treatment was strongly associated with residual disease and it is thus reasonable to follow up these women regardless of genotyping results.

Co-testing for HPV and cytology as a test of cure six months after conization has been recommended in Skåne since 2010. We showed a high negative predictive value for HSIL cytology with a negative (high-risk) HPV testing, 99.7%. This is consistent with a previous study from Skåne which showed a negative predictive value of 99.2% (142). HPV testing has shown to be more sensitive than cytology for detection of residual or recurrent disease after treatment of CIN2+, while co-testing had a marginally higher sensitivity than HPV testing alone (143). A Danish study, found that cytology added very little extra sensitivity to HPV test as a test of cure for women with free resection margins (144). As a matter of fact, in the next guidelines, a new cytology sample is not required when endocervical cells are missing in follow up sample after treatment of dysplasia, if the sample is HPV negative.

Low-risk HPV seemed to be affected by the conization to a less extent than high-risk HPV, since 34% the low-risk infections persisted, compared to 14% of the high-risk types. The number of women with low-risk types did not change significantly after treatment (p=0.46). 19 of 27 women with persistent low-risk infections after conization had cleared a concomitant high-risk infection, suggesting that the low-risk infection was not restricted to the excised tissue. The low-risk HPV infection could have involved the outside of the cervix, the upper part of the endocervical canal or the vagina. When vaginal self-sampling has been used in cervical cancer screening, which is now the routine in Skåne, less than half of the women positive in the self-collected sample are also HPV positive when followed up by a mid-wife collected

cervical sample (145). Thus, there seems to be a discrepancy between HPV detected in the vagina and HPV-infection of the cervix.

However, the number of low-risk HPV-infections before treatment was lower than the number of high-risk-infections. The high number of high-risk infections can be explained by the fact that the studied cohort were women with confirmed CIN2 or CIN3 histology. In fact, it is surprising that not all, but only 91%, were high-risk HPV positive before treatment. The number of women with low-risk infections is approximately the same before and after treatment, 76 vs 84. After treatment, about a third of the women with low-risk types have persistent infections, while the rest have new infections (or previously undetected infections).

Of the 446 women, 37 were positive after treatment for high-risk types not detected before treatment and 57 were positive for previously not detected low-risk types. The reason could either be new infections or that the treatment "unmasked" HPV types hidden behind other types with a higher viral load. There could also be a bias in the MGP PCR, favouring detection of certain HPV types. If we trust the results of the PCR before and after treatment, more women acquired new low-risk HPV infections than new high-risk HPV infections, 57 of 446 vs 37 of 336, although the difference was quite small ($p=0.038$). The acquirement of new infections has previously been shown to be the same for oncogenic and non-oncogenic HPV (42, 126). One of the signums of high-risk HPV is their ability to cause persistent infections unnoticed by the immune system, while low-risk types more often cause transient infections (40-42).

A recent publication by Wang et al. (36) lists the prevalence of different HPV types in women with cervical cancer in Sweden between 2002 and 2011 as well as the prevalence of different HPV types in the population. The most common types in screened women with cervical cancer was HPV16 (51%), HPV18 (19%), HPV45 (6%), HPV31 (3.5%) and HPV33 (3%). In the general female population, the most common types were HPV16 (7.7%), HPV31 (1.6%), HPV45 (1.5%), HPV52 (1.4%) and HPV 51 (1.1%). Thus, there are different distributions of types found in cancer and in the population.

In our study, the most prevalent high-risk type before treatment of CIN2+ were HPV16 (40%), HPV 31 (18%), HPV52 (13%), HPV18 (11%), HPV33 (8%), HPV51 (6%), HPV45 and HPV 39 (both 5%). After treatment, the most prevalent types were HPV16 (6.5%), HPV52 (4%), HPV31 and HPV33 (3%), and HPV45, HPV51 and HPV56 (2%). Even though some women had persistent infection with HPV16 after treatment, the percentage of HPV16-

positive was not greater than in the general population. Interestingly, HPV18 is unusual in the general population as shown by Wang et al. (36) as well as in the women in our study after treatment of CIN2+, but one of the most common types in women with cancer. Before treatment, HPV18 was seen in 11% of the women in our study, which is slightly less than among women with cervical cancer. It seems reasonable to believe that the prevalence of HPV18 in women with CIN2+ should be somewhere in between the prevalence in women with cancer and the prevalence in the general population. Surprisingly many women with HPV31 and HPV52 before treatment were seen in our study. These types are common in the population but less common than HPV18 among women with cancer. Possible explanations could be local variations of HPV type distributions in Sweden, different HPV types in cancer and CIN2+ or different methods for HPV detection and typing.

Conclusions

- Primary HPV screening by use of HPV mRNA detection identified the same proportion of ASCUS+ cytology as cytology-based screening.
- 7.0% of women ≥30-70 years in the screening program were HPV positive.
- No cases of high-grade cytology were seen among HPV negative women 40-42 years old in the screening program.
- Primary HPV screening by use of HPV mRNA detection is safe and reliable.
- Testing for methylation of *FAM19A4/miR124-2* showed the same or better sensitivity as cytology, for prediction of SCC and ADC.
- The detection rate of HSIL was lower for methylation than for cytology.
- More studies are needed before introduction of methylation testing in the screening program.
- After treatment of HSIL, 16% of the women had at least one persistent high-risk HPV type.
- Persistent low-risk HPV infections were more common than persistent high-risk infections after treatment of HSIL.
- High-risk HPV testing after treatment showed a sensitivity of 92% for detection of residual HSIL and a negative predictive value of 99.7%. HPV testing is considered reliable as a test of cure.

Future perspectives

Since the planning of the studies included in this thesis, the cervical cancer screening has gone through major changes: HPV testing of self-collected vaginal samples has become the principal screening method for women, starting at age 23, genotyping has been more emphasized, and the oncogenic potential of some HPV types has been downgraded (124). In the National guidelines for cervix cancer screening from 2022, full genotyping is recommended. In 2017 we only tested women for presence of any high-risk HPV type, with a test which also gave a positive result for some low oncogenic types and non-oncogenic types such as HPV66. Therefore, evaluation of a screening program which includes extended genotyping, as well as evaluation of methylation testing in combination with genotyping, would be an appropriate next step.

The first cohort of vaccinated girls in Sweden reached screening age in 2023. We can expect few of these women to be positive for HPV16 or HPV18. Within a project for concomitant HPV vaccination and screening for elimination of HPV and cervical cancer, 2 152 women born in 1999, the first cohort offered school-based vaccination, were tested in 2021-2022 (146). HPV16 was detected in 15 (0.7%) of these women, and only one single woman (0.05%) was positive for HPV18. Among women born 1994-1999, where catch up vaccine had been offered to women born 1994-1998, 2% were HPV16 positive and 0.5% were positive for HPV18. With our current assay for detection of high-risk HPV mRNA and reflex testing with the Aptima GT test for identification of HPV16/18/45, most HPV positive samples from women born after 2000 are likely to fall in the category “other high-risk types than HPV16, 18 or 45”. A more precise assessment of these women will be necessary, since they can either be infected with a HPV of the mid-oncogenic group, such as HPV31, or with HPV66 which is no longer considered a high-risk type, and this will have great effect on the risk for cancer. A combination of methylation and genotyping could be worth to investigate.

Another application of methylation analysis is to triage HPV positive women with cytology ASCUS and LSIL. In a Dutch study, risk-stratification of these

women with a combination of genotyping and methylation was estimated to decrease the colposcopy rate by approximately 25%, with a CIN3+ after a negative result of 2.8% (147).

Self-collected vaginal samples have largely replaced midwife collected cervical samples in the screening. Currently, HPV positive women need to go to a midwife to take a follow-up sample from the cervix. If genotyping and methylation testing performed directly on the self-collected samples could give reliable information on the risk for HSIL and cancer, direct colposcopy referral will be favoured, and fewer women would be lost to follow up. HPV genotypes found in CIN lesions have shown to be detected also in self-collected vaginal samples (148).

For the next version of the Swedish National guidelines for cervical cancer prevention, direct colposcopy without cytology is suggested for women with HPV16, HPV18 or HPV45. This policy has been implemented in the Stockholm Region in 2024, resulting in only a small increase in colposcopy referrals, due to the low prevalence of HPV16 and HPV18 in vaccinated women (55). Direct referral of HPV16/18/45 positive women makes it possible to omit the midwife-collected follow-up sample.

Since the youngest cohort have a low prevalence of HPV16 and HPV18, thanks to vaccination, full genotyping of self-collected samples is warranted. For women with less oncogenic HPV types, methylation testing could possibly help to decide who needs follow up. Methylation appears to be as frequent in CIN3+ lesions caused by high-oncogenic HPV types as in CIN3+ lesions caused by other high-risk types (148), and could thus give additional information beside the genotype result.

Testing for *FAM19A4/miR124-2* methylation has been studied also for self-collected samples (105). A Dutch group found an increased sensitivity for detection of HSIL in self-collected samples when miR124-2 was replaced by two other genes, *ITGA4* and *ASCL1* (149). A student project is currently performed at Clinical microbiology, Lund, to investigate methylation testing of self-collected samples in our setting.

Women with HSIL don't always need treatment, since these lesions can regress spontaneously. Together with a gynecologist, we are planning to study if methylation can help to distinguish lesions that will regress from lesions that will progress. There is some date indicating that methylation can predict progression (128, 150) but more studies are warranted.

Acknowledgements

First, I want to thank my main supervisor, **Ola Forslund**, for accepting to become my PhD supervisor and showing me how routine diagnostics can be expanded to research projects. You have always been friendly and available for questions. You have shown a great persistency in keeping up research work at Clinical Microbiology, although resources have been scarce at times.

Blenda Böttiger, my co-supervisor, thank you for introducing me to clinical virology, for being a role model as a senior laboratory physician and for your support both in the "clinical" work and to encourage me to get involved in research.

Thank you, **Christer Borgfeldt**, my co-supervisor, for introducing me to your group of former and current PhD students. I have had many fulfilling online evening meetings with research active gynecologists. Thank you also for helpful advice on practical aspects of the PhD process, publishing, writing and presentation, and for sharing your subject expertise.

Former head of Clinical Microbiology, Ingvar Eliason, and current head of the medical staff, Sara Karlsson Söbirk, thank you for encouraging research at the laboratory. Thank you also to the current head of Clinical Microbiology, Richard Davidsson for supporting continued development in this direction.

Thank you, all my colleagues at Clinical Microbiology. You are a great team of smart and kind people, all residents not least! I especially want to thank Torgny for reminding me to book a date for my dissertation.

The staff at Clinical Microbiology, including laboratory technicians, administrators and caretakers: thank you for creating a friendly atmosphere in our workplace. I specially want to thank the technicians who helped with the methylation assay: Tim, Jacob, Louise, Tove and Nahla.

The staff at Clinical Pathology, especially Evgenia Khan and Kristina McNeish, thank you for helping me to identify the samples analyzed in study III.

Thank you, gynecological pathologist Anna Måsbäck, for showing me around in the pathology lab!

Thank you, gynecologist Arturas Dobilas, for letting me follow you work during colposcopy examinations.

My old friends Lovisa, Mia, Anna-Karin and Sandra, thank you for keeping in contact for so long and for being such sensible and honest women. I appreciate to have wise discussion partners also outside work.

My extended family, including among others Vidar, Lotta, Håkan, Ida, Simon, Karin, Charlotte, Nelly, Julius, Maria and Håkan, Meit and Christer, and my cousins Sara, Olof and Åsa with families. Thank you for being there.

Thank you to my mother Anne for all love and care during my upbringing. Good job!

My husband, Mats, you have been my best friend and soulmate for 26 years.

My daughters Kajsa, Elsa and Saga, I love you! You have always been a lot more important to me than my work. Lisa, thank you for being my guardian angel during the dissertation.

The Royal Physiographic Society of Lund, thank you for grants for methylation reagents.

Thank your Region Skåne Research Fund, for supporting my research. Thanks to being able to take time off from my regular work, I was able to complete my PhD studies.

References

1. Johnson KM, Kines RC, Roberts JN, Lowy DR, Schiller JT, Day PM. Role of heparan sulfate in attachment to and infection of the murine female genital tract by human papillomavirus. J Virol. 2009;83(5):2067-74.
2. Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proc Natl Acad Sci U S A. 2009;106(48):20458-63.
3. Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, et al. The biology and life-cycle of human papillomaviruses. Vaccine. 2012;30 Suppl 5:F55-70.
4. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond). 2006;110(5):525-41.
5. Doorbar J. The papillomavirus life cycle. J Clin Virol. 2005;32 Suppl 1:S7-15.
6. Dyson N, Guida P, Munger K, Harlow E. Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. J Virol. 1992;66(12):6893-902.
7. DeCaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, Piwnica-Worms H, et al. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell. 1989;58(6):1085-95.
8. Devoto SH, Mudryj M, Pines J, Hunter T, Nevins JR. A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. Cell. 1992;68(1):167-76.
9. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science. 1990;248(4951):76-9.
10. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990;63(6):1129-36.
11. zur Hausen H. Papillomaviruses in the causation of human cancers - a brief historical account. Virology. 2009;384(2):260-5.
12. Pett M, Coleman N. Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis? J Pathol. 2007;212(4):356-67.

13. Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, et al. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature. 1985;314(6006):111-4.
14. Graham SV. Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies. Future Microbiol. 2010;5(10):1493-506.
15. Jeon S, Allen-Hoffmann BL, Lambert PF. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. J Virol. 1995;69(5):2989-97.
16. Gray E, Pett MR, Ward D, Winder DM, Stanley MA, Roberts I, et al. In vitro progression of human papillomavirus 16 episome-associated cervical neoplasia displays fundamental similarities to integrant-associated carcinogenesis. Cancer Res. 2010;70(10):4081-91.
17. Yim EK, Park JS. The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. Cancer Res Treat. 2005;37(6):319-24.
18. Antonsson A, Forslund O, Ekberg H, Sterner G, Hansson BG. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J Virol. 2000;74(24):11636-41.
19. Antonsson A, Karanfilovska S, Lindqvist PG, Hansson BG. General acquisition of human papillomavirus infections of skin occurs in early infancy. J Clin Microbiol. 2003;41(6):2509-14.
20. Bravo IG, de Sanjose S, Gottschling M. The clinical importance of understanding the evolution of papillomaviruses. Trends Microbiol. 2010;18(10):432-8.
21. Bernard HU, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology. 2010;401(1):70-9.
22. de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology. 2004;324(1):17-27.
23. Doorbar J, Egawa N, Griffin H, Kranjec C, Murakami I. Human papillomavirus molecular biology and disease association. Rev Med Virol. 2015;25 Suppl 1(Suppl Suppl 1):2-23.
24. Munoz N, Bosch FX, de Sanjose S, Tafur L, Izarzugaza I, Gili M, et al. The causal link between human papillomavirus and invasive cervical cancer: a population-based case-control study in Colombia and Spain. Int J Cancer. 1992;52(5):743-9.
25. Bosch FX, Munoz N, de Sanjose S, Izarzugaza I, Gili M, Viladiu P, et al. Risk factors for cervical cancer in Colombia and Spain. Int J Cancer. 1992;52(5):750-8.
26. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003;348(6):518-27.

27. Durst M, Gissmann L, Ikenberg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U S A. 1983;80(12):3812-5.
28. Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. 1984;3(5):1151-7.
29. Schiffman M, Glass AG, Wentzensen N, Rush BB, Castle PE, Scott DR, et al. A long-term prospective study of type-specific human papillomavirus infection and risk of cervical neoplasia among 20,000 women in the Portland Kaiser Cohort Study. Cancer Epidemiol Biomarkers Prev. 2011;20(7):1398-409.
30. Smelov V, Elfstrom KM, Johansson AL, Eklund C, Naucler P, Arnheim-Dahlstrom L, et al. Long-term HPV type-specific risks of high-grade cervical intraepithelial lesions: a 14-year follow-up of a randomized primary HPV screening trial. Int J Cancer. 2015;136(5):1171-80.
31. Altekruse SF, Lacey JV, Jr., Brinton LA, Gravitt PE, Silverberg SG, Barnes WA, Jr., et al. Comparison of human papillomavirus genotypes, sexual, and reproductive risk factors of cervical adenocarcinoma and squamous cell carcinoma: Northeastern United States. Am J Obstet Gynecol. 2003;188(3):657-63.
32. Kjaer SK, Munk C, Junge J, Iftner T. Carcinogenic HPV prevalence and age-specific type distribution in 40,382 women with normal cervical cytology, ASCUS/LSIL, HSIL, or cervical cancer: what is the potential for prevention? Cancer Causes Control. 2014;25(2):179-89.
33. Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. Int J Cancer. 2007;121(3):621-32.
34. International_Agency_for_Research_on_Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 2012. Available from: https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Biological-Agents-2012.
35. International_Agency_for_Research_on_Cancer. Cervical cancer screening 2022. Available from: https://publications.iarc.fr/Book-And-Report-Series/Iarc-Handbooks-Of-Cancer-Prevention/Cervical-Cancer-Screening-2022.
36. Wang J, Elfstrom KM, Lagheden C, Eklund C, Sundstrom K, Sparen P, et al. Impact of cervical screening by human papillomavirus genotype: Population-based estimations. PLoS Med. 2023;20(10):e1004304.
37. Swedish_Regional_Cancer_Centers. Swedish national guidelines for cervical cancer prevention. 2022 [Available from: https://kunskapsbanken.cancercentrum.se/diagnoser/livmoderhalscancerprevention/vardprogram/.

38. Brown DR, Shew ML, Qadadri B, Neptune N, Vargas M, Tu W, et al. A longitudinal study of genital human papillomavirus infection in a cohort of closely followed adolescent women. J Infect Dis. 2005;191(2):182-92.
39. Nobbenhuis MA, Helmerhorst TJ, van den Brule AJ, Rozendaal L, Voorhorst FJ, Bezemer PD, et al. Cytological regression and clearance of high-risk human papillomavirus in women with an abnormal cervical smear. Lancet. 2001;358(9295):1782-3.
40. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet. 2007;370(9590):890-907.
41. Schiffman M, Herrero R, Desalle R, Hildesheim A, Wacholder S, Rodriguez AC, et al. The carcinogenicity of human papillomavirus types reflects viral evolution. Virology. 2005;337(1):76-84.
42. Goodman MT, Shvetsov YB, McDuffie K, Wilkens LR, Zhu X, Thompson PJ, et al. Prevalence, acquisition, and clearance of cervical human papillomavirus infection among women with normal cytology: Hawaii Human Papillomavirus Cohort Study. Cancer Res. 2008;68(21):8813-24.
43. Bulkmans NW, Berkhof J, Bulk S, Bleeker MC, van Kemenade FJ, Rozendaal L, et al. High-risk HPV type-specific clearance rates in cervical screening. Br J Cancer. 2007;96(9):1419-24.
44. de Sanjose S, Brotons M, Pavon MA. The natural history of human papillomavirus infection. Best Pract Res Clin Obstet Gynaecol. 2018;47:2-13.
45. Hibma MH. The immune response to papillomavirus during infection persistence and regression. Open Virol J. 2012;6:241-8.
46. Halec G, Scott ME, Farhat S, Darragh TM, Moscicki AB. Toll-like receptors: Important immune checkpoints in the regression of cervical intra-epithelial neoplasia 2. Int J Cancer. 2018;143(11):2884-91.
47. Norenhag J, Edfeldt G, Stalberg K, Garcia F, Hugerth LW, Engstrand L, et al. Compositional and functional differences of the vaginal microbiota of women with and without cervical dysplasia. Sci Rep. 2024;14(1):11183.
48. Mitra A, MacIntyre DA, Ntritsos G, Smith A, Tsilidis KK, Marchesi JR, et al. The vaginal microbiota associates with the regression of untreated cervical intraepithelial neoplasia 2 lesions. Nat Commun. 2020;11(1):1999.
49. Kyrgiou M, Moscicki AB. Vaginal microbiome and cervical cancer. Semin Cancer Biol. 2022;86(Pt 3):189-98.
50. Molina MA, Leenders WPJ, Huynen MA, Melchers WJG, Andralojc KM. Temporal composition of the cervicovaginal microbiome associates with hrHPV infection outcomes in a longitudinal study. BMC Infect Dis. 2024;24(1):552.
51. The_Swedish_National_Board_of_Social_Affairs_and_Health. Cancer i siffror 2023. Available from: https://www.socialstyrelsen.se/globalassets/sharepoint-dokument/dokument-webb/statistik/cancer-i-siffror-2023.pdf.
52. Swedish_Regional_Cancer_Centers, editor. Swedish national guidelines for cervical cancer prevention. 2017.

53. Dillner J, Sparen P, Andrae B, Strander B. [Cervical cancer has increased in Sweden in women who had a normal cell sample]. Lakartidningen. 2018;115.
54. Edvardsson H, Wang J, Andrae B, Sparen P, Strander B, Dillner J. Nationwide Rereview of Normal Cervical Cytologies before High-Grade Cervical Lesions or before Invasive Cervical Cancer. Acta Cytol. 2021;65(5):377-84.
55. Swedish_National_Cervical_Screening_Registry. Swedish National Cervical Screening Registry Analysis [Available from: https://www.nkcx.se/.
56. World_Health_Organization_(WHO). Global strategy to accelerate the elimination of cervical cancer as a public health problem 2020. Available from: https://www.who.int/publications/i/item/9789240014107.
57. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999;189(1):12-9.
58. Arroyo Muhr LS, Lagheden C, Lei J, Eklund C, Nordqvist Kleppe S, Sparen P, et al. Deep sequencing detects human papillomavirus (HPV) in cervical cancers negative for HPV by PCR. Br J Cancer. 2020;123(12):1790-5.
59. International_Collaboration_of_Epidemiological_Studies_of_Cervical_Cancer. Cervical carcinoma and reproductive factors: collaborative reanalysis of individual data on 16,563 women with cervical carcinoma and 33,542 women without cervical carcinoma from 25 epidemiological studies. Int J Cancer. 2006;119(5):1108-24.
60. International_Collaboration_of_Epidemiological_Studies_of_Cervical_Cancer. Cervical carcinoma and sexual behavior: collaborative reanalysis of individual data on 15,461 women with cervical carcinoma and 29,164 women without cervical carcinoma from 21 epidemiological studies. Cancer Epidemiol Biomarkers Prev. 2009;18(4):1060-9.
61. International Collaboration of Epidemiological Studies of Cervical C, Appleby P, Beral V, Berrington de Gonzalez A, Colin D, Franceschi S, et al. Carcinoma of the cervix and tobacco smoking: collaborative reanalysis of individual data on 13,541 women with carcinoma of the cervix and 23,017 women without carcinoma of the cervix from 23 epidemiological studies. Int J Cancer. 2006;118(6):1481-95.
62. Andrae B, Kemetli L, Sparen P, Silfverdal L, Strander B, Ryd W, et al. Screening-preventable cervical cancer risks: evidence from a nationwide audit in Sweden. J Natl Cancer Inst. 2008;100(9):622-9.
63. Jansson A, Gustafsson M, Wilander E. Efficiency of cytological screening for detection of cervical squamous carcinoma. A study in the county of Uppsala 1991-1994. Ups J Med Sci. 1998;103(2):147-54.
64. Stelzle D, Tanaka LF, Lee KK, Ibrahim Khalil A, Baussano I, Shah ASV, et al. Estimates of the global burden of cervical cancer associated with HIV. Lancet Glob Health. 2021;9(2):e161-e9.

65. Sundqvist A, Moberg L, Dickman PW, Hogberg T, Borgfeldt C. Time Trends for Incidence and Net Survival of Cervical Cancer in Sweden 1960-2014-A Nationwide Population-Based Study. Cancer Epidemiol Biomarkers Prev. 2022;31(8):1572-81.
66. Dahlstrom LA, Ylitalo N, Sundstrom K, Palmgren J, Ploner A, Eloranta S, et al. Prospective study of human papillomavirus and risk of cervical adenocarcinoma. Int J Cancer. 2010;127(8):1923-30.
67. Hellsten C, Holmberg A, Astrom J, Forslund O, Borgfeldt C. Cervical cancer in Region Skane, Sweden 2017-2020 after the implementation of primary HPV screening: A quality assurance audit. Acta Obstet Gynecol Scand. 2024;103(1):129-37.
68. McCredie MR, Paul C, Sharples KJ, Baranyai J, Medley G, Skegg DC, et al. Consequences in women of participating in a study of the natural history of cervical intraepithelial neoplasia 3. Aust N Z J Obstet Gynaecol. 2010;50(4):363-70.
69. Tainio K, Athanasiou A, Tikkinen KAO, Aaltonen R, Cardenas J, Hernandes, et al. Clinical course of untreated cervical intraepithelial neoplasia grade 2 under active surveillance: systematic review and meta-analysis. BMJ. 2018;360:k499.
70. Kyleback K, Ekeryd-Andalen A, Greppe C, Bjorkenfeldt Havel C, Zhang C, Strander B. Active expectancy as alternative to treatment for cervical intraepithelial neoplasia grade 2 in women aged 25 to 30 years: ExCIN2-a prospective clinical multicenter cohort study. Am J Obstet Gynecol. 2022;227(5):742 e1- e11.
71. Cox JT, Schiffman M, Solomon D, Group A-LTS. Prospective follow-up suggests similar risk of subsequent cervical intraepithelial neoplasia grade 2 or 3 among women with cervical intraepithelial neoplasia grade 1 or negative colposcopy and directed biopsy. Am J Obstet Gynecol. 2003;188(6):1406-12.
72. Moscicki AB, Shiboski S, Hills NK, Powell KJ, Jay N, Hanson EN, et al. Regression of low-grade squamous intra-epithelial lesions in young women. Lancet. 2004;364(9446):1678-83.
73. Castle PE, Schiffman M, Wheeler CM, Solomon D. Evidence for frequent regression of cervical intraepithelial neoplasia-grade 2. Obstet Gynecol. 2009;113(1):18-25.
74. Moscicki AB, Ma Y, Wibbelsman C, Darragh TM, Powers A, Farhat S, et al. Rate of and risks for regression of cervical intraepithelial neoplasia 2 in adolescents and young women. Obstet Gynecol. 2010;116(6):1373-80.
75. Wiik J, Karrberg C, Nilsson S, Strander B, Jacobsson B, Sengpiel V. Associations between cervical intraepithelial neoplasia during pregnancy, previous excisional treatment, cone-length and preterm delivery: a register-based study from western Sweden. BMC Med. 2022;20(1):61.
76. LIF. FASS 2024 [Available from: https://www.fass.se/.

77. Papanicolaou GN. A New Procedure for Staining Vaginal Smears. Science. 1942;95(2469):438-9.
78. Gustafsson L, Adami HO. Cytologic screening for cancer of the uterine cervix in Sweden evaluated by identification and simulation. Br J Cancer. 1990;61(6):903-8.
79. Apgar BS, Zoschnick L, Wright TC, Jr. The 2001 Bethesda System terminology. Am Fam Physician. 2003;68(10):1992-8.
80. Ronco G, Dillner J, Elfstrom KM, Tunesi S, Snijders PJ, Arbyn M, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. Lancet. 2014;383(9916):524-32.
81. Cuzick J, Szarewski A, Terry G, Ho L, Hanby A, Maddox P, et al. Human papillomavirus testing in primary cervical screening. Lancet. 1995;345(8964):1533-6.
82. Naucler P, Ryd W, Tornberg S, Strand A, Wadell G, Elfgren K, et al. Efficacy of HPV DNA testing with cytology triage and/or repeat HPV DNA testing in primary cervical cancer screening. J Natl Cancer Inst. 2009;101(2):88-99.
83. Bergengren L, Ryen L, Flodstrom C, Fadl H, Udumyen R, Karlsson MG, et al. Effectiveness and costs of an implemented primary HPV cervical screening programme in Sweden - A population based cohort study. Prev Med Rep. 2022;25:101675.
84. Aitken CA, van Agt HME, Siebers AG, van Kemenade FJ, Niesters HGM, Melchers WJG, et al. Introduction of primary screening using high-risk HPV DNA detection in the Dutch cervical cancer screening programme: a population-based cohort study. BMC Med. 2019;17(1):228.
85. Haedicke J, Iftner T. A review of the clinical performance of the Aptima HPV assay. J Clin Virol. 2016;76 Suppl 1:S40-S8.
86. Maggino T, Sciarrone R, Murer B, Dei Rossi MR, Fedato C, Maran M, et al. Screening women for cervical cancer carcinoma with a HPV mRNA test: first results from the Venice pilot program. Br J Cancer. 2016;115(5):525-32.
87. Gustavsson I, Aarnio R, Berggrund M, Hedlund-Lindberg J, Sanner K, Wikstrom I, et al. Randomised study of HPV prevalence and detection of CIN2+ in vaginal self-sampling compared to cervical specimens collected by medical personnel. Int J Cancer. 2019;144(1):89-97.
88. Gyllensten U, Sanner K, Gustavsson I, Lindell M, Wikstrom I, Wilander E. Short-time repeat high-risk HPV testing by self-sampling for screening of cervical cancer. Br J Cancer. 2011;105(5):694-7.
89. Bosch FX, Burchell AN, Schiffman M, Giuliano AR, de Sanjose S, Bruni L, et al. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine. 2008;26 Suppl 10:K1-16.

90. Asciutto KC, Ernstson A, Forslund O, Borgfeldt C. Self-sampling with HPV mRNA analyses from vagina and urine compared with cervical samples. J Clin Virol. 2018;101:69-73.
91. Meijer CJ, Berkhof J, Castle PE, Hesselink AT, Franco EL, Ronco G, et al. Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older. Int J Cancer. 2009;124(3):516-20.
92. Heideman DA, Hesselink AT, van Kemenade FJ, Iftner T, Berkhof J, Topal F, et al. The Aptima HPV assay fulfills the cross-sectional clinical and reproducibility criteria of international guidelines for human papillomavirus test requirements for cervical screening. J Clin Microbiol. 2013;51(11):3653-7.
93. Arbyn M, Snijders PJ, Meijer CJ, Berkhof J, Cuschieri K, Kocjan BJ, et al. Which high-risk HPV assays fulfil criteria for use in primary cervical cancer screening? Clin Microbiol Infect. 2015;21(9):817-26.
94. Arbyn M, Simon M, de Sanjose S, Clarke MA, Poljak M, Rezhake R, et al. Accuracy and effectiveness of HPV mRNA testing in cervical cancer screening: a systematic review and meta-analysis. Lancet Oncol. 2022;23(7):950-60.
95. Soderlund-Strand A, Carlson J, Dillner J. Modified general primer PCR system for sensitive detection of multiple types of oncogenic human papillomavirus. J Clin Microbiol. 2009;47(3):541-6.
96. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol. 1995;76 (Pt 4):1057-62.
97. Robertson KD. DNA methylation and human disease. Nat Rev Genet. 2005;6(8):597-610.
98. Herzog C, Jones A, Evans I, Raut JR, Zikan M, Cibula D, et al. Cigarette Smoking and E-cigarette Use Induce Shared DNA Methylation Changes Linked to Carcinogenesis. Cancer Res. 2024;84(11):1898-914.
99. Moqri M, Herzog C, Poganik JR, Biomarkers of Aging C, Justice J, Belsky DW, et al. Biomarkers of aging for the identification and evaluation of longevity interventions. Cell. 2023;186(18):3758-75.
100. Papanicolau-Sengos A, Aldape K. DNA Methylation Profiling: An Emerging Paradigm for Cancer Diagnosis. Annu Rev Pathol. 2022;17:295-321.
101. Hesselink AT, Heideman DA, Steenbergen RD, Coupe VM, Overmeer RM, Rijkaart D, et al. Combined promoter methylation analysis of CADM1 and MAL: an objective triage tool for high-risk human papillomavirus DNA-positive women. Clin Cancer Res. 2011;17(8):2459-65.
102. Lorincz AT, Brentnall AR, Scibior-Bentkowska D, Reuter C, Banwait R, Cadman L, et al. Validation of a DNA methylation HPV triage classifier in a screening sample. Int J Cancer. 2016;138(11):2745-51.

103. De Strooper LM, Meijer CJ, Berkhof J, Hesselink AT, Snijders PJ, Steenbergen RD, et al. Methylation analysis of the FAM19A4 gene in cervical scrapes is highly efficient in detecting cervical carcinomas and advanced CIN2/3 lesions. Cancer Prev Res (Phila). 2014;7(12):1251-7.
104. Luttmer R, De Strooper LM, Dijkstra MG, Berkhof J, Snijders PJ, Steenbergen RD, et al. FAM19A4 methylation analysis in self-samples compared with cervical scrapes for detecting cervical (pre)cancer in HPV-positive women. Br J Cancer. 2016;115(5):579-87.
105. De Strooper LMA, Verhoef VMJ, Berkhof J, Hesselink AT, de Bruin HME, van Kemenade FJ, et al. Validation of the FAM19A4/mir124-2 DNA methylation test for both lavage- and brush-based self-samples to detect cervical (pre)cancer in HPV-positive women. Gynecol Oncol. 2016;141(2):341-7.
106. Lai HC, Ou YC, Chen TC, Huang HJ, Cheng YM, Chen CH, et al. PAX1/SOX1 DNA methylation and cervical neoplasia detection: a Taiwanese Gynecologic Oncology Group (TGOG) study. Cancer Med. 2014;3(4):1062-74.
107. Wilting SM, van Boerdonk RA, Henken FE, Meijer CJ, Diosdado B, Meijer GA, et al. Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. Mol Cancer. 2010;9:167.
108. Zhu S, Hu X, Bennett S, Mai Y, Xu J. Molecular Structure, Expression and Role of TAFA4 and its Receptor FPR1 in the Spinal Cord. Front Cell Dev Biol. 2022;10:911414.
109. Steenbergen RD, Ongenaert M, Snellenberg S, Trooskens G, van der Meide WF, Pandey D, et al. Methylation-specific digital karyotyping of HPV16E6E7-expressing human keratinocytes identifies novel methylation events in cervical carcinogenesis. J Pathol. 2013;231(1):53-62.
110. De Strooper LMA, Berkhof J, Steenbergen RDM, Lissenberg-Witte BI, Snijders PJF, Meijer C, et al. Cervical cancer risk in HPV-positive women after a negative FAM19A4/mir124-2 methylation test: A post hoc analysis in the POBASCAM trial with 14 year follow-up. Int J Cancer. 2018;143(6):1541-8.
111. Vink FJ, Meijer C, Clifford GM, Poljak M, Ostrbenk A, Petry KU, et al. FAM19A4/miR124-2 methylation in invasive cervical cancer: A retrospective cross-sectional worldwide study. Int J Cancer. 2020;147(4):1215-21.
112. Faul F, Erdfelder E, Buchner A, Lang AG. Statistical power analyses using G*Power 3.1: tests for correlation and regression analyses. Behav Res Methods. 2009;41(4):1149-60.
113. Huijsmans CJ, Geurts-Giele WR, Leeijen C, Hazenberg HL, van Beek J, de Wild C, et al. HPV Prevalence in the Dutch cervical cancer screening population (DuSC study): HPV testing using automated HC2, cobas and Aptima workflows. BMC Cancer. 2016;16(1):922.
114. Iftner T, Becker S, Neis KJ, Castanon A, Iftner A, Holz B, et al. Head-to-Head Comparison of the RNA-Based Aptima Human Papillomavirus (HPV) Assay and the DNA-Based Hybrid Capture 2 HPV Test in a Routine Screening Population

of Women Aged 30 to 60 Years in Germany. J Clin Microbiol. 2015;53(8):2509-16.
115. Chorny JA, Frye TC, Fisher BL, Remmers CL. Human papillomavirus detection with genotyping by the cobas and Aptima assays: Significant differences in HPV 16 detection? Diagn Cytopathol. 2018;46(7):568-71.
116. de Sanjose S, Diaz M, Castellsague X, Clifford G, Bruni L, Munoz N, et al. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect Dis. 2007;7(7):453-9.
117. Bonde JH, Sandri MT, Gary DS, Andrews JC. Clinical Utility of Human Papillomavirus Genotyping in Cervical Cancer Screening: A Systematic Review. J Low Genit Tract Dis. 2020;24(1):1-13.
118. Borgfeldt C, Leksell A, Forslund O. Co-testing in cervical screening among 40- to 42-year-old women is unreasonable. Acta Obstet Gynecol Scand. 2022;101(3):374-8.
119. Gyllensten U, Lindell M, Gustafsson I, Wilander E. HPV test shows low sensitivity of Pap screen in older women. Lancet Oncol. 2010;11(6):509-10; author reply 10-1.
120. Aarnio R, Wikstrom I, Gustavsson I, Gyllensten U, Olovsson M. Diagnostic excision of the cervix in women over 40 years with human papilloma virus persistency and normal cytology. Eur J Obstet Gynecol Reprod Biol X. 2019;3:100042.
121. Hermansson RS, Olovsson M, Hoxell E, Lindstrom AK. HPV prevalence and HPV-related dysplasia in elderly women. PLoS One. 2018;13(1):e0189300.
122. Bergeron C, Giorgi-Rossi P, Cas F, Schiboni ML, Ghiringhello B, Dalla Palma P, et al. Informed cytology for triaging HPV-positive women: substudy nested in the NTCC randomized controlled trial. J Natl Cancer Inst. 2015;107(2).
123. Doxtader EE, Brainard JA, Underwood D, Chute DJ. Knowledge of the HPV status biases cytotechnologists' interpretation of Pap tests originally diagnosed as negative for intraepithelial lesion or malignancy. Cancer Cytopathol. 2017;125(1):60-9.
124. Bonde J, Floore A, Ejegod D, Vink FJ, Hesselink A, van de Ven PM, et al. Methylation markers FAM19A4 and miR124-2 as triage strategy for primary human papillomavirus screen positive women: A large European multicenter study. Int J Cancer. 2021;148(2):396-405.
125. Plummer M, Schiffman M, Castle PE, Maucort-Boulch D, Wheeler CM, Group A. A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion. J Infect Dis. 2007;195(11):1582-9.
126. Castle PE, Schiffman M, Herrero R, Hildesheim A, Rodriguez AC, Bratti MC, et al. A prospective study of age trends in cervical human papillomavirus

acquisition and persistence in Guanacaste, Costa Rica. J Infect Dis. 2005;191(11):1808-16.
127. Louvanto K, Aro K, Nedjai B, Butzow R, Jakobsson M, Kalliala I, et al. Methylation in Predicting Progression of Untreated High-grade Cervical Intraepithelial Neoplasia. Clin Infect Dis. 2020;70(12):2582-90.
128. Kremer WW, Dick S, Heideman DAM, Steenbergen RDM, Bleeker MCG, Verhoeve HR, et al. Clinical Regression of High-Grade Cervical Intraepithelial Neoplasia Is Associated With Absence of FAM19A4/miR124-2 DNA Methylation (CONCERVE Study). J Clin Oncol. 2022;40(26):3037-46.
129. Bierkens M, Hesselink AT, Meijer CJ, Heideman DA, Wisman GB, van der Zee AG, et al. CADM1 and MAL promoter methylation levels in hrHPV-positive cervical scrapes increase proportional to degree and duration of underlying cervical disease. Int J Cancer. 2013;133(6):1293-9.
130. Swedish_Regional_Cancer_Centers. Swedish National Guidelines for treatment of cervical cancer [Available from: https://kunskapsbanken.cancercentrum.se/globalassets/cancerdiagnoser/gynekologi/livmoderhalscancer/varprogram/nationellt-vardprogram-livmoderhalscancer-cervixcancer-vaginalcancer.pdf.
131. Herzog C, Sundstrom K, Jones A, Evans I, Barrett JE, Wang J, et al. DNA methylation-based detection and prediction of cervical intraepithelial neoplasia grade 3 and invasive cervical cancer with the WID-qCIN test. Clin Epigenetics. 2022;14(1):150.
132. Dippmann C, Schmitz M, Wunsch K, Schutze S, Beer K, Greinke C, et al. Triage of hrHPV-positive women: comparison of two commercial methylation-specific PCR assays. Clin Epigenetics. 2020;12(1):171.
133. Dick S, Kremer WW, De Strooper LMA, Lissenberg-Witte BI, Steenbergen RDM, Meijer C, et al. Long-term CIN3+ risk of HPV positive women after triage with FAM19A4/miR124-2 methylation analysis. Gynecol Oncol. 2019;154(2):368-73.
134. Kaliff M, Lillsunde Larsson G, Helenius G, Karlsson MG, Bergengren L. Full genotyping and FAM19A4/miR124-2 methylation analysis in high-risk human papillomavirus-positive samples from women over 30 years participating in cervical cancer screening in Orebro, Sweden. PLoS One. 2022;17(9):e0274825.
135. Helenius G, Lillsunde-Larsson G, Bergengren L. Molecular triage of cervical screening samples in women 55-59 years of age: a pilot study. Infect Agent Cancer. 2023;18(1):31.
136. Asciutto KC, Borgfeldt C, Forslund O. 14-type HPV mRNA test in triage of HPV DNA-positive postmenopausal women with normal cytology. BMC Cancer. 2020;20(1):1025.
137. Molano M, Machalek DA, Phillips S, Tan G, Garland SM, Hawkes D, et al. DNA methylation at individual CpG-sites of EPB41L3, HTERT and FAM19A4 are useful for detection of cervical high-grade squamous intraepithelial lesions

(HSIL) or worse: Analysis of individual CpG-sites outperforms averaging. Tumour Virus Res. 2024;18:200288.
138. Boers R, Boers J, de Hoon B, Kockx C, Ozgur Z, Molijn A, et al. Genome-wide DNA methylation profiling using the methylation-dependent restriction enzyme LpnPI. Genome Res. 2018;28(1):88-99.
139. Strander B, Andersson-Ellstrom A, Milsom I, Sparen P. Long term risk of invasive cancer after treatment for cervical intraepithelial neoplasia grade 3: population based cohort study. BMJ. 2007;335(7629):1077.
140. Sundqvist A, Nicklasson J, Olausson P, Borgfeldt C. Post-conization surveillance in an organized cervical screening program with more than 23,000 years of follow-up. Infect Agent Cancer. 2023;18(1):81.
141. Soderlund-Strand A, Kjellberg L, Dillner J. Human papillomavirus type-specific persistence and recurrence after treatment for cervical dysplasia. J Med Virol. 2014;86(4):634-41.
142. Asciutto KC, Henic E, Darlin L, Forslund O, Borgfeldt C. Follow up with HPV test and cytology as test of cure, 6 months after conization, is reliable. Acta Obstet Gynecol Scand. 2016;95(11):1251-7.
143. Cuschieri K, Bhatia R, Cruickshank M, Hillemanns P, Arbyn M. HPV testing in the context of post-treatment follow up (test of cure). J Clin Virol. 2016;76 Suppl 1:S56-S61.
144. Bruhn LV, Hyldig N, Schledermann D. HPV Test as Test of Cure After Conization for CIN2+: A Nationwide Register-Based Cohort Study. J Low Genit Tract Dis. 2022;26(4):287-92.
145. Hellsten C, Ernstson A, Bodelsson G, Forslund O, Borgfeldt C. Equal prevalence of severe cervical dysplasia by HPV self-sampling and by midwife-collected samples for primary HPV screening: a randomised controlled trial. Eur J Cancer Prev. 2021;30(4):334-40.
146. Arroyo Muhr LS, Gini A, Yilmaz E, Hassan SS, Lagheden C, Hultin E, et al. Concomitant human papillomavirus (HPV) vaccination and screening for elimination of HPV and cervical cancer. Nat Commun. 2024;15(1):3679.
147. Dick S, Vink FJ, Heideman DAM, Lissenberg-Witte BI, Meijer C, Berkhof J. Risk-stratification of HPV-positive women with low-grade cytology by FAM19A4/miR124-2 methylation and HPV genotyping. Br J Cancer. 2022;126(2):259-64.
148. Leeman A, Ebisch RMF, Kasius A, Bosgraaf RP, Jenkins D, van de Sandt MM, et al. Defining hrHPV genotypes in cervical intraepithelial neoplasia by laser capture microdissection supports reflex triage of self-samples using HPV16/18 and FAM19A4/miR124-2 methylation. Gynecol Oncol. 2018;151(2):311-8.
149. de Waard J, Bhattacharya A, de Boer MT, van Hemel BM, Esajas MD, Vermeulen KM, et al. Methylation Analysis to Detect CIN3+ in High-Risk Human Papillomavirus-Positive Self-Samples From the Population-Based Cervical Cancer Screening Program. Mod Pathol. 2024;37(8):100528.

150. Vink FJ, Meijer C, Hesselink AT, Floore AN, Lissenberg-Witte BI, Bonde JH, et al. FAM19A4/miR124-2 Methylation Testing and Human Papillomavirus (HPV) 16/18 Genotyping in HPV-Positive Women Under the Age of 30 Years. Clin Infect Dis. 2023;76(3):e827-e34.