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Identification and clinical implementation of biomarkers for cervical cancer

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Abstract

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Introduction of organised screening programs and prophylactic vaccination against human papilloma virus (HPV) have successfully reduced the incidence of cervical cancer globally. In Sweden, the incidence has been reduced by about 50 % since the introduction of the national screening programme in the late 1960's. Despite these efforts, cervical cancer is still a major cause of cancer deaths globally.

In order to reduce cervical cancer, the screening program should have a high participation rate and be based on a sensitive and specific screening test. About 20 % of women in Sweden do not participate in the organised screening program, and during the last years we have also seen a rise in cervical cancer cases in Sweden among women who participate in the screening program. Thus, there is a need to develop improved screening strategies that result in a higher participation rate, and are based on tests that more precisely identify women with high risk of developing cervical cancer. This includes searching for novel biological markers (biomarkers) that can be used to more accurately identify women with a high risk of developing cervical cancer.

By offering women self-sampling for HPV analysis through direct mailing of sample kits with a chemically treated paper card, the FTA card, we were able to increase the participation rate in the screening program. We also found that the use of repeated self-sampling for women that were HPV positive in the primary screening sample increased the number of women detected with higher risk of cervical cancer (Paper II). Self-sampling was shown to be non-inferior to assisted sampling by midwife (Paper III). Using this sample collection device, we further investigated the association between increased risk of cervical cancer and HPV viral load (Paper V) as well as the vaginal microbiota (Paper VI). We also showed that proteins in the vaginal fluid can be studied using self-sampling and the FTA card (Paper I). Lastly, we identified plasma proteins that are associated with cervical cancer and could represent future biomarkers (Paper IV).

This thesis has provided novel aspects on the present screening strategy, explored opportunities to increase the participation rate as well as examined possible future biomarkers for screening of cervical cancer.

Keywords: Cervical cancer, Human papilloma virus, Screening, Self-sampling, Biomarkers, Vaginal microbiota

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. Reprints were made with permission from the respective publishers.

I **Berggrund, M.**, Ekman, D., Gustavsson, I., Sundfeldt, K., Olovsson, M., Enroth, S., and Gyllensten, U. (2016). Protein Detection Using the Multiplexed Proximity Extension Assay (PEA) from Plasma and Vaginal Fluid Applied to the Indicating FTA Elute Micro Card. *Journal of Circulating Biomarkers*, 5, 9

II Gustavsson, I., Aarnio, R., **Berggrund, M.**, Hedlund-Lindberg, J., Strand, A.S., Sanner, K., Wikstrom, I., Enroth, S., Olovsson, M., and Gyllensten, U. (2018). Randomised study shows that repeated self-sampling and HPV test has more than two-fold higher detection rate of women with CIN2+ histology than Pap smear cytology. *British Journal of Cancer*, *118*(6), 896

III Gustavsson, I., Aarnio, R., **Berggrund, M.**, Hedlund-Lindberg, J., Sanner, K., Wikstrom, I., Enroth, S., Olovsson, M., and Gyllensten, U. (2019). Randomised study of HPV prevalence and detection of CIN2+ in vaginal self-sampling compared to cervical specimens collected by medical personnel. *International Journal of Cancer*, *144*(1):89-97

IV **Berggrund, M.**, Enroth, S., Lundberg, M., Assarsson, E., Stålberg, K., Lindquist, D., Hallmans, G., Grankvist, K., Olovsson, M., and Gyllensten, U. (2019). Identification of Candidate Plasma Protein Biomarkers for Cervical Cancer Using the Multiplex Proximity Extension Assay *Molecular & Cellular Proteomics*, 18(4):735-43

V **Berggrund, M.**, Gustavsson, I., Aarnio, R., Hedlund-Lindberg, J., Sanner, K., Wikstrom, I., Enroth, S., Olovsson, M., and Gyllensten, U. HPV viral load in self-collected vaginal fluid samples as predictor for presence of cervical intraepithelial neoplasia. *Virology Journal*, 16(1):146

VI **Berggrund M.**, Gustavsson I., Aarnio R., Hedlund-Lindberg J., Sanner K., Wikström I., Enroth S., Bunikis I., Olovsson M., Gyllensten U. (2020). Temporal changes in the vaginal microbiota in self-samples and its association with persistent HPV16 infection and CIN2+, *Submitted*

Related publications

Enroth, S., Maturi, V., Berggrund, M., Enroth, S.B., Moustakas, A., Johansson, A., and Gyllensten, U. (2018). Systemic and specific effects of antihypertensive and lipid-lowering medication on plasma protein biomarkers for cardiovascular diseases. *Scientific Reports*, 8(1), 5531.

Enroth, S., Berggrund, M., Lycke, M., Lundberg, M., Assarsson, E., Olovsson, M., Stalberg, K., Sundfeldt, K., and Gyllensten, U. (2018). A two-step strategy for identification of plasma protein biomarkers for endometrial and ovarian cancer. *Clinical Proteomics*, 15, 38.

Enroth, S., Berggrund, M., Lycke, M., Broberg, J., Lundberg, M., Assarsson, E., Olovsson, M., Stalberg, K., Sundfeldt, K., and Gyllensten, U. (2019). High throughput proteomics identifies 484 high-accuracy plasma protein biomarker signatures for ovarian cancer. *Communications biology,* 2:221.

Contents

Abbreviations

AIDS	Acquired immune deficiency syndrome
ASCUS	Atypical squamous cells of undetermined significance
CIN	Cervical intraepithelial neoplasia
CIN2+	Cervical intraepithelial neoplasia grade 2 or worse
CIN3+	Cervical intraepithelial neoplasia grade 3 or worse
DNA	Deoxyribonucleic acid
EU	European Union
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSIL	High-grade squamous intraepithelial lesion
LSIL	Low-grade squamous intraepithelial lesion
mRNA	Messenger ribonucleic acid
NKCx	Swedish National Cervical Screening Registry
NPV	Negative predictive value
NPX	Normalized protein expression
PCR	Polymerase chain reaction
PEA	Proximity extension assay
PPV	Positive predictive value
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
SCJ	Squamo-columnar junction
STI	Sexually transmitted infection
URR	Upstream regulatory region
V1–9	Variable region 1–9
WHO	World Health Organization
qPCR	Quantitative polymerase chain reaction

Introduction

For diseases that are hard to cure and have severe consequences for those affected, prevention is the key to reduce suffering. Cervical cancer is the most common gynaecological cancer. The World Health Organization (WHO) estimated that 570 000 women would be diagnosed with cervical cancer in 2018 and that cervical cancer would cause 311 000 deaths [1].

There are substantial global differences in incidence. Cervical cancer ranks as the 6th most common cancer among women in countries with a higher development index. In low income settings, cervical cancer is the 2nd most common cancer among females, with regards to both incidence and mortality. It is the most common cancer in 28 countries and the cancer that claims the most lives in 42 countries [1]. The Director-General for the World Health Organization (WHO), Tedros Ghebreyesus, has repeatedly declared the ambition of global elimination of cervical cancer and have called for global coordinated action [2].

Research on cervical cancer is of utter importance and it was the foundation for the Nobel Prize in Physiology or Medicine for Harald zur Hausen in 2008. Harald zur Hausen received the prize after presenting and confirming the hypothesis that cervical cancer is the result of a viral infection with the human papilloma virus (HPV) [3, 4]. Approximately 13 % of all cancer cases can be attributed to infectious agents, with the main four pathogens being *Helicobacter pylori*, hepatitis B virus, hepatitis C virus and the HPV. HPV accounts for over 30 % of the cancer cases caused by infectious agents [5].

There are national screening programs for cervical cancer in a number of countries. These screening programs have had considerable impact: it has been estimated that the screening program has reduced the mortality rate of cervical cancer with 53 % in Sweden [6]. However, since the initial disease reduction there has been limited progress in the number of cervical cancer cases. Between 2010 and 2014, there was a yearly incidence of approximately 473 cervical cancer diagnoses in Sweden causing approximately 193 deaths each year [7].

The limitations of the screening program can be attributed to two main issues. One is the participation rate and number of women screened. A report from the Swedish National Cervical Screening Registry (NKCx) showed that on average 30 % of women who were invited to screening had not attended within one year after invitation. Also, looking at screening coverage after 5 years follow-up time, approximately 20 % of women had not attended the

organised screening program [8]. The second limitation is the screening test used to identify individuals at increased risk of disease. The screening test used for cervical cancer has traditionally been a Papanicolaou-stain, or Pap test. It is a cytology test, usually collected by a clinician and inspected visually in microscopy by trained personnel. The performance of this test varies, but the sensitivity of the cytology test is approximately 40–70 % depending on the severity of the cervical lesion [9, 10].

The limited sensitivity of the cytology test has led to the introduction of a new method of screening for cervical cancer and the stages of cervical dysplasia that precede cervical cancer. To test for HPV infection itself shows a higher sensitivity for finding women with disease. However, it suffers from a lower specificity, due to the fact that infection with HPV is a common event during a lifetime and most individuals will clear the infection without any intervention or side effects of the virus. Still, screening for HPV infection has been estimated to have 60–70 % greater protection for cervical cancer than cytology-based screening [11]. Since 2015, the Swedish screening guidelines recommend HPV testing as a screening test for women between 30–64 years of age. However, not all county councils in Sweden have implemented these guidelines [7].

This thesis aims to explore two main issues. First, to investigate the possibilities of self-sampling for HPV testing. This could increase participation rates in the existing screening program and offer women a possibility to independently plan their own health-care. Secondly, to search for new markers for cervical cancer and preceding cervical dysplasia. Such markers could improve the possibility for early detection of cervical cancer. Progress on the two main issues in this thesis could contribute to further development of the organised screening program for cervical cancer.

Background

The HPV infection

Infection with the human papilloma virus (HPV) is a common event that affects most people during their lifetime. The infection is mostly asymptomatic or causes mild disease, such as skin warts or condyloma. Some HPV types can infect mucus membrane and in rare cases cause malignant diseases, such as cancer in e.g. the oropharynx or cervix.

HPV is transmitted via contact and is one of the most common sexually transmitted infectious agents. Contrary to many other sexual transmitted infections (STI), due to mode of transition, use of condom only reduces the risk of infection rather than prevents it [12, 13]. Over a third of women will have acquired an HPV infection within 2 years of their sexual debut [14].

There are over 200 known types of HPV, and a number of these are considered to be high-risk types for cervical cancer [15]. HPV16 is considered the cause of the major part of all cervical cancer cases [16].

HPV is a naked double stranded DNA virus. It is approximately 8000 base pairs and contains 8 genes and an upstream regulatory region (URR) (Figure 1). HPV's oncogenic properties lays within the genes E6 and E7 [17]. The viral protein E6 activates telomerase [18] and the SRC kinases [19] which leads to increased cell growth. E6 also inhibits the p53 protein and the BAK protein which inhibits apoptosis and increases chromosomal instability [20]. The E7 protein degrades the tumor suppressor retinoblastoma protein [21]. This should by extension increase apoptosis, but it is bypassed by E6 inhibition of programmed cell death. E7 also stimulates the DNA replication stage in the cell cycle [21]. This can cause aneuploidy, abnormal number of chromosomes, in a cell which contributes to the tumorigenesis.

Infection is established by the virus entering micro-abrasion in the epithelium and infecting keratinocytes in the basal layer. Initial infection is maintained at a low copy number, only increasing through differentiation of the cell. This also leads to the expression of the capsid protein, which is necessary for building new virions [16]. The induced cell growth causes the cells to build up, and can visually be seen as a wart or, in the case of cervical infection, a lesion. The initial low copy number and the lack of lytic cell-death in the HPV viral cycle contribute to the virus escaping the immune system [15]. Also, there are indications that the low inflammatory response in initial disease progression is due to downregulation of innate immune receptors responsible for

initiating immune response [22]. Integration of HPV into the human genome drives cancerogenic development, but it is not a part of the viral life cycle as it does not lead to the production of new virions. E6 and E7 expression are often upregulated in tumours where the HPV genome is integrated into the host genome. This increases the cells properties with proliferation and apoptosis regulation, giving them a growth advantage [23].

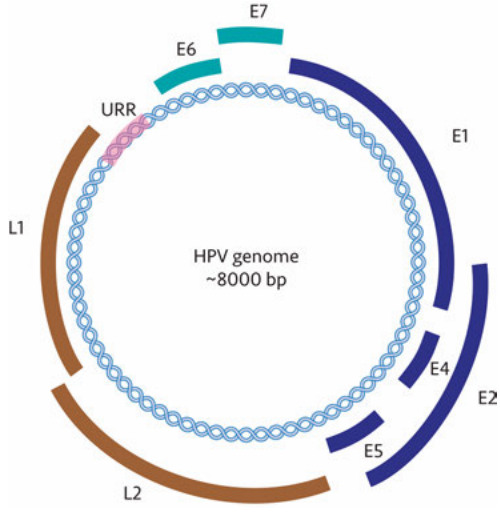

Figure 1. Schematic drawing of the Human Papilloma virus (HPV) genome. The genome is approximately 8000 base pairs long, with some variation between HPV types. E represents early genes and L late genes. The upstream regulatory region is marked as URR.

HPV tissue tropism and origin of cervical cancer

The papilloma viruses are species-specific. Different HPV types may also have different tissue tropism, which means that they infect different epithelial sites and require a certain cellular origin to establish an infection [24]. For cervical cancer the majority of cases arises in the squamo-columnar junction (SCJ) in the cervix [25]. The cervix is located in the lower part of the uterus and connects the uterus with the vagina (Figure 2). It is divided into two parts, the endocervix at the top against the uterus and the ectocervix that is visible in a vaginal examination. The endocervix consists of a columnar epithelium and the ectocervix is lined with a squamous epithelium. The junction between these two epithelium types is known as the SCJ. The SCJ changes its positions depending on hormonal status and age [26].

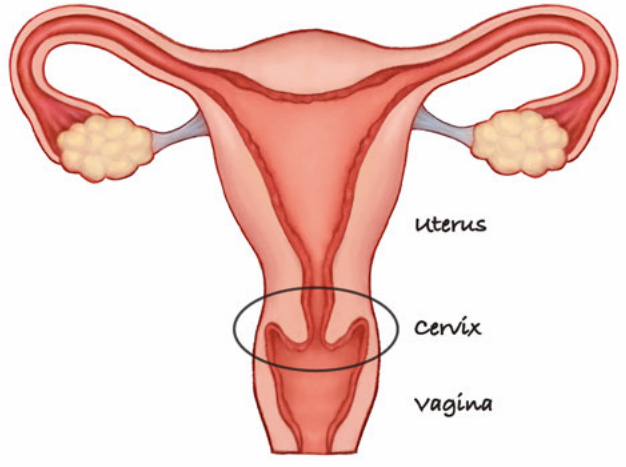

Figure 2. Schematic drawing of the female reproductive system. The cervix is located at the border between the vagina and the uterus. Illustration by Erik Bünger.

Development of cervical cancer

Cervical dysplasia, or lesions, in the cervix can be classified as cervical intraepithelial neoplasia (CIN) 1, 2 or 3, with 1 being milder and 3 more severe [27]. An alternative classification is the Bethesda classification of cervical lesions with atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) [28] (Figure 3).

Even if an infection with HPV develops into a persistent infection and causes a CIN, the immune system can still clear the infection and the lesion can regress spontaneously. Within 2 years around 40 % of detected CIN2 will have regressed spontaneously without intervention [29]. Regression rate in young women is estimated to be even higher, up to 60–70 % [30-32]. Women with CIN2+ and HPV16 infection are less likely to regress [29, 33]. The regression rate is increased for women with HPV16 infection whose partner practice consistent condom use [34].

In contrast to many other cancers, that mainly develop later in life, most women affected by cervical cancer are in their midlife. The world mean age at cervical cancer diagnosis is 53 years and the mean age at death by cervical cancer is 59 years. In countries with a high development index a maximum of cervical cancer incidence is reached at around 40 years [35].

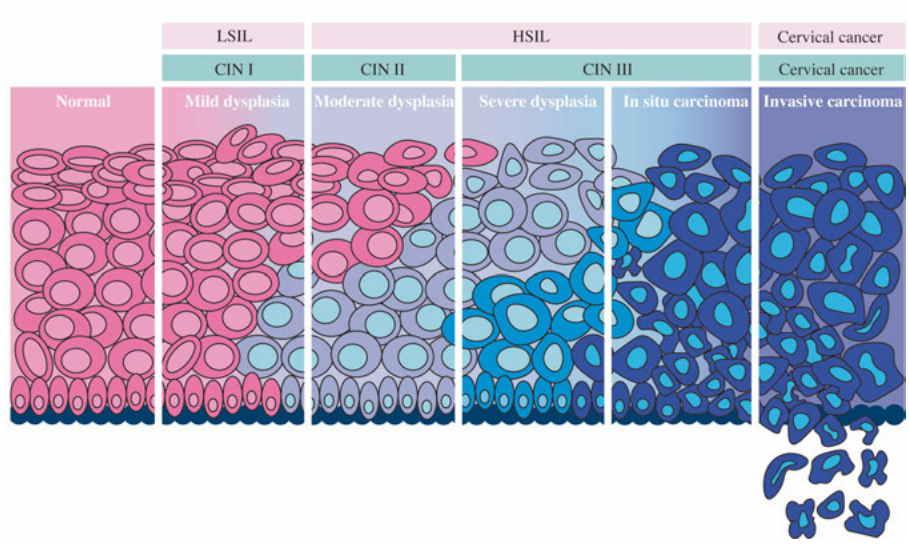

Figure 3. Development of cervical dysplasia. HPV infection starts in the basal layer of the epithelium. Headings in pink describes the Bethesda classification system with atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). Blue heading represents the cervical intraepithelial neoplasia (CIN) classification system, with a scale of 1, 2 or 3, with 1 being milder and 3 more severe.

Risk factors for cervical cancer

Risk factors for HPV infection and cervical cancer can be classified into viral and non-viral factors. Viral factors are HPV type and viral load. HPV16 is, for example, considered the cause of the major part of all cervical cancer cases [16]. Non-viral factors are, among others, smoking and use of oral contraceptive, as both these factors are reported to increase risk for HPV infection as well as development of cervical cancer [13, 36, 37]. There are also associations with sexual behaviour such as number of sexual partners and previous STI [38-40]. Several genetic variations in regions relating to the immune response have been associated with an increased risk of HPV infection and cervical cancer development [41, 42]. The risk of cervical cancer is increased in immunosuppressed individuals, both individuals with HIV/AIDS [43, 44] and in patients who have received bone marrow transplants [43]. Not participating in the screening program or irregular participation is also associated with both an increased risk for cervical cancer and cervical cancer death [45].

Disease prevention strategies

Disease prevention strategies can be ordered into three intervention levels. The primary level of intervention targets the disease development itself by avoiding exposure to hazards that can lead to the disease. For cervical cancer this can be achieved by e.g. information campaigns about sexual habits or prophylactic HPV vaccination [46]. The WHO recommends that all countries provide nationwide vaccination for HPV [47]. In the year of 2012, a vaccine against two high-risk types (HPV16 and -18) and two of the HPV types that cause condyloma (HPV6 and -11) was introduced in school-based vaccination program for young girls in Sweden [7]. The WHO recommendations stipulate that the vaccination should be gender-neutral if feasible [48], and the Public Health Agency of Sweden released a report in 2017 recommending that boys should be included in the HPV vaccination program in Sweden [49]. In a press release in 2019 the Swedish Ministry of Health and Social Affairs announced the plan of introducing a gender-neutral vaccination program for HPV in Sweden during the fall of 2020 [50].

An early evaluation of the vaccination programs shows a significant reduction in condyloma cases among both girls and boys. The first significant reduction in CIN2+ is now starting to show as the early vaccinated cohorts are entering the screening programs [51]. The largest effect of the HPV vaccination is yet to be shown as vaccinated cohorts gradually will come to the age where they enter the screening program.

Secondary prevention aims to halter the impact of disease. Cervical cancer has clearly defined stages of dysplasia, CIN (Figure 3), which can be detected by screening [46]. In Sweden, organised screening with cytology was introduced in 1967 and offered nationwide in 1973 [7]. From 2015, the Swedish screening guidelines recommend HPV testing as a screening test for women between 30–64 years of age (Table 1) [52].

Lastly, tertiary prevention seeks to impact the already ongoing course of disease. For cervical cancer that is often achieved by surgical removal of tumours and involved lymph nodes. Radiation and chemotherapy are also in use [46]. The importance of early disease detection is reinforced by the age demographic pattern of cervical cancer. Major surgical intervention that may be necessary at late diagnosis can obstruct the reproductive ability in young women or increase their risk for adverse events during pregnancy [53, 54].

This thesis will focus on secondary disease prevention strategies for cervical cancer.

National screening program and recommendations

Since the year of 2015 the European Union (EU) recommends HPV test for primary screening of cervical cancer [55]. The same year, the Swedish

National Board of Health and Welfare recommended HPV test rather than cytology for women between age 30 and 64 (Table 1) [52]. However, not all county councils in Sweden have implemented these guidelines [7]. Failure of implementing current guidelines is a recognized problem and in 2018 the Swedish government appointed a public inquiry to address issues concerning following evidence-based care and equality in the healthcare system, including the adherence to national guidelines [56]. The report is scheduled to be released during the summer of 2020.

The WHO recommends that if countries have the necessary funding and infrastructure, they should establish a national screening system for cervical cancer with a special effort for women between 30–49 years of age with HPV DNA testing or visual inspection (VIA), instead of the traditional cytology. The WHO does not recommend to set up new screening programs with cytology due to the difficulties of meeting quality indicators with this method. Cytology requires solid training of personnel due to the specialized skills required to interpret cytology samples [57]. HPV DNA testing has a higher sensitivity for screening of cervical cancer and CIN than cytology and can therefore identify more women at risk of cervical cancer. However, due to the fact that HPV is a common STI and the likelihood of women clearing the infection without any intervention, the HPV DNA test has a lower specificity compared to cytology [58, 59]. This means that the HPV DNA test identifies not only women at risk of cervical cancer, but also a substantial proportion of women who will eventually clear their infection without developing any CIN or cancer. Additional biological markers would therefore be valuable in order to identify women at risk of cervical cancer, while avoiding overtreatment of women not at risk.

Table 1. *Screening recommendations for cervical cancer in Sweden [52].*

Age	**Recommendations**
23–29	Cytology, every 3rd year
30–49	HPV test every 3rd year and co-testing with cytology at 41 years of age
50–64	HPV test every 7th year
All ages	Yearly reminder to non-participants Liquid-based test collection to facilitate cytology triage analysis Triage of HPV positive with cytology Cytology positive referred to colposcopy Cytology negative offered new HPV test after 3 years. Continued HPV positive referred to colposcopy

Biomarkers and triage tests

WHO has defined biological markers, or biomarkers, as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [60]. Biomarkers should be objective markers of health or disease and whose measurements should be accurate and reproducible. An individual's experience of their health status may or may not correspond with the biomarker measurement. This is separate from clinical endpoints that directly characterize the actual health or disease status in an individual [61].

In the context of cervical cancer, the biomarkers used in screening today are mainly ASCUS or worse observed using cytology [62] or detection of HPV DNA. Several other biomarkers have been investigated such as mRNA from HPV [63-65], microRNA [66, 67], methylation of both human and HPV genes [68-71], HPV viral load, p16/Ki67 staining of cytology samples [72] and host genetic markers [73].

Triage is a secondary step after screening to identify which individuals to proceed with in diagnostic evaluation. This is mostly used to compensate for a lack in sensitivity or specificity in the primary screening test. A triage test should aim to reduce overtreatment and lower the number of diagnostic tests performed. For cervical cancer screening this can be achieved through adding a cytology sample after confirming HPV positivity, as in the Swedish recommendations (Table 1). The triage could however also be performed with other methods, such as colposcopy to decide whether an diagnostic histology sample should be retrieved or not [74] or repeated HPV test to target HPV persistence [75]. Triaging with p16/Ki67 staining of cytology samples has been shown to have similar sensitivity as a triage test as cytology, but with a higher specificity [72].

Self-sampling as a strategy to increase participation rates

Even with the best of biomarkers and screening tests, a screening program needs a high population coverage to be successful. The majority of cervical cancer cases occur among women that have not participated in the organised screening program [76]. The main reason for women choosing not to attend the organised screening program is either that they report feeling uncomfortable with the procedure (collection of vaginal samples by a clinician) or have practical barriers (as attending an appointment) [77-79].

The first study presenting self-sampling was published in year 1993, showing concordant results between self-sampling and clinician-assisted sampling [80]. There is generally a high acceptance level for self-sampling among women and a majority of women prefer self-sampling as compared to assisted

sampling for HPV testing [81, 82]. Fewer women experience discomfort and pain with self-sampling for HPV analysis compared to cytology sampling at a clinic [83]. The patient organisation for gynaecological disease in Sweden have introduction of self-sampling as one of their highest priorities [84]. Self-sampling can improve participation rates in cervical screening programs in all age groups [85] and is an effective way of reaching women who does not usually attend the screening program [86, 87]. Self-sampling can therefore increase the coverage of the screening program.

Direct mailing of sample-kits is effective compared to invitations to order self-sampling kit, but with regional variations [88]. Women report it to be harder to find time to participate in the clinic-based screening compared to self-sampling [83].

There have been reports of a higher frequency of fright of test result among women performing self-sampling compared to cytology testing [83]. This emphasizes the need for thoroughly communicated information regarding HPV infection and risk of cervical cancer.

There have been successful attempts to incorporate self-sampling for HPV into existing HIV management programs, where it can be used to reach high-risks groups in rural settings [89]. Self-sampling can also significantly improve participation rates in lower socioeconomic groups [90].

Methodology

Study design and ethical approval

A randomised intervention study is a rigorous method to estimate the effect of an intervention and an outcome. The study participants are randomly assigned to either the intervention group or the control group to minimise confounding effects. The study is often blinded, meaning that the participant and/or the ones conducting the study are unaware of which arm the participant belongs to. This is not always possible, depending on the method of intervention. A randomised intervention study provided data for paper II and III and the vaginal fluid samples on the FTA card analysed in paper I, V and VI all derived from this study.

In the randomised intervention study, the participants were randomly allocated to the intervention group, with self-sampling for HPV test, or the control group, with cytology sample collected by midwife. The study was performed in Uppsala County, Sweden, between the years of 2013 and 2015. The study concerned women between the age of 30 and 49 years at the date of invitation, who had no current pregnancy and no hysterectomy record. An exclusion criterion was if the woman had previous clinical test relating to cervical cancer screening (cytology, HPV test or histology) within a year before the date of invitation. This was to avoid inclusion of women already in clinical follow-up relating to cervical cancer. The follow-up period was 18 months after date of invitation.

All samples included in this thesis, self-sampled vaginal fluid as well as blood plasma, were collected with minimal invasive methods. All analyses and statistical calculations have been performed on group level and no individual data were analysed or presented. No results can be connected to specific individuals. The collection of samples in paper I falls under the ethical permission approved by the Regional Ethics Committee in Uppsala (Dnr 2012/099). No specific permission for the protein analysis itself was obtained since there was no connection to personal information or disease outcome. The collection and analysis of samples in study II, III, V and VI fall under the ethical permission approved by the Regional Ethics Committee in Uppsala (Dnr 2012/099). The samples included in study IV fall under the ethical permission from the Regional Ethics Board in Uppsala, Sweden (Dnr 2016/145) and the Regional Ethics Board in Umeå, Sweden (Dnr 2013-314-32 M and 2012-229-31M).

Self-sampling of vaginal fluid

Self-samples of vaginal fluid analysed in paper I, II, III, V and VI were collected through direct mailing of sample kits to the women. The kit contained a sample brush, an FTA card, a postage paid return envelope and information on how to perform the sample collection (Figure 4). A letter of reminder was sent to the women who did not return their sample to the laboratory within 3 weeks. The women received their result within 2 weeks from the sample's arrival at the laboratory. HPV positive women were invited to repeat their sample within 3–6 months and encouraged to contact a gynaecologist if they experienced any symptoms within this time period. The women who cleared their infection within this time frame, and hence were HPV negative in their follow-up sample, were returned to the screening program. The women who were HPV positive in their follow-up sample were referred to colposcopy for further investigation.

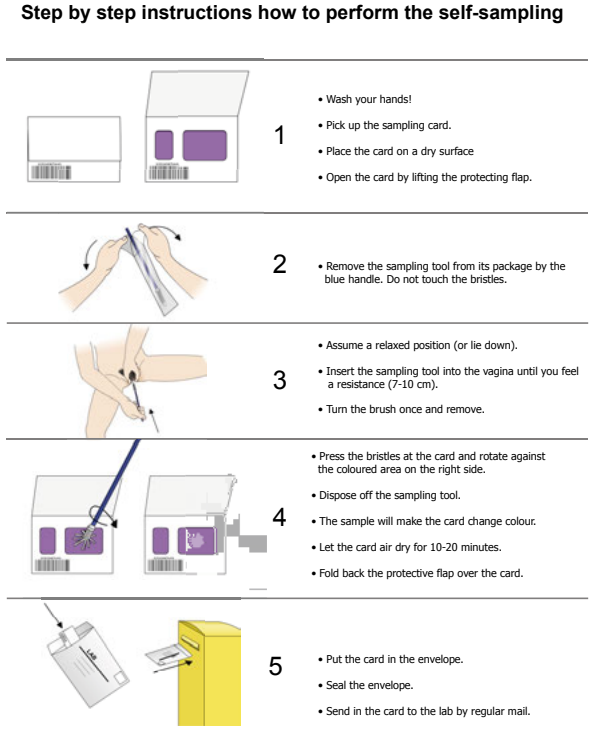

Figure 4. Instruction for self-sampling of vaginal fluid for HPV DNA testing.

Histology diagnosis and clinical endpoint

A common procedure to examine the status of the cervix is a visual inspection of the cervix, vagina and vulva by a clinician. This procedure is known as a colposcopy. An acetic acid solution is often applied on the cervix to visualize white areas with punctuation, which imply atypical regions suitable for biopsies [91]. Lesions seen in the cervix are classified as cervical intraepithelial neoplasia (CIN) 1, 2 or 3, with 1 being milder and 3 more severe [27] or with the Bethesda classification of cervical lesions with atypical squamous cells (ASCUS), low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) [28] (Figure 3).

The defined CIN that precede cervical cancer can be used as clinical endpoints. When studying cervical cancer biomarkers, this is a major advantage compared to some other tumour diseases where well-defined preceding stages are missing. Which CIN that is used as endpoint vary between different studies and will affect the interpretation of the biomarker performance and predictive value. In our studies, we relate our results to the clinical endpoint CIN2+, because of its relevance for guiding clinical intervention in the course of disease.

Screening test performance

The performance of a screening test can be evaluated according to sensitivity and specificity, or the combined term, accuracy. One could also use the terms positive predictive value (PPV) or negative predictive value (NPV), which describes the tests value for predicting disease outcome. New tests are often compared to another test or no testing, to estimate the added value of the new test.

To make these comparisons patients are grouped according to disease status or condition of interest. Those who are diseased and are correctly identified as such by the test are referred to as true positives. Those who are healthy but identified as diseased by the test are referred to as false positives. A healthy individual which is identified as such by the test is a true negative. Lastly, a diseased person that is identified as healthy are referred to as a false negative.

Sensitivity describes the proportion of individuals with disease who are accurately diagnosed as diseased. It is calculated as the number of true positives over the sum of true positives and false negatives. Specificity describes the test ability to correctly identify a healthy individual as such. It is calculated as the number of true negatives over the sum of true negatives and false positives. Accuracy is a combined term which is calculated as number of correct assessments over the number of all assessments and describes the test's ability to identify an individual's true disease status (Table 2).

The predictive value of a test, i.e. with what certainty could the test result be interpreted, can be described with PPV and NPV. The PPV describes the

probability that the ones that are classified as having the disease, also truly have the disease. It is calculated as true positives over the number of true positives and false positives, i.e. all patients that are classified as diseased by the test. The NPV describes with what certainty the ones that are classified as healthy by the test are truly healthy. NPV is calculated as the true negatives over the true negatives and false negatives, i.e. all patient that are classified as healthy by the test. The PPV and NPV are dependent on the prevalence of the disease, which makes it necessary to consider the context in which the test was applied (Table 2).

Table 2. *Test performance variables.*

Variable	**Description**	**Calculation**
Sensitivity	Proportions of individuals with disease accurately identified as such.	True positives / Sum of true positives and false negatives
Specificity	Proportions of healthy individuals accurately identified as such.	True negatives / Sum of true negatives and false positives
Accuracy	Test's ability to identify individual's true disease status.	Number of correct assessments / Sum of all assessments
Positive predictive value (PPV)	Probability that an individual classified as diseased actually is diseased.	True positives / Sum of true positives and false positives
Negative predictive value (NPV)	Probability that an individual classified as healthy actually is healthy.	True negatives / Sum of True negatives and false negatives

HPV DNA testing and quantification

There are multiple assays for HPV DNA testing, based on both quantitative and qualitative methods. Variations in sensitivity and specificity between HPV DNA assays can partly be attributed to method of HPV DNA testing. PCR-based methods show a higher sensitivity than signal-based assays and it is suggested that a PCR-based HPV DNA test is the preferable choice in order to implement self-sampling for HPV testing in routine screening [92, 93]. The recommendations for HPV testing systems in Sweden is that it should measure HPV types classified as high-risk and should measure HPV16 and -18 separately [94].

In study I, II, III, V and VI the HPV DNA measurement was performed with the HPVIR assay, a qPCR-based system which has been clinically validated [95]. It measures seven high-risk HPV types separately (16, 31, 35, 39, 51, 56, 59) and additionally five high-risk HPV types in two groups (18/45 and 33/52/58) [96, 97].

Proximity extension assay

The samples used in study I and IV were analysed with the proximity extension assay (PEA). This method is based on using a pair of oligonucleotide-labelled antibodies that bind to a target protein. If both of the antibodies are able to bind to their target region they will be in close proximity to each other and a PCR target sequence can be constructed by a proximity-dependent DNA polymerization event. The method offers a high specificity, as it requires two conserved epitopes on the protein. The amplicons that are produced can be detected and quantified using qPCR (Figure 5). The PEA offers a high-throughput multiplex-method. It is provided in panels with 92 pre-selected proteins and requires only 1 µl input material per panel. The protein abundance is reported as normalized protein expression levels (NPX), a relative quantification unit [98, 99]. The low volume of required input material makes this method suitable for material from biobanks where amount of material can be scarce.

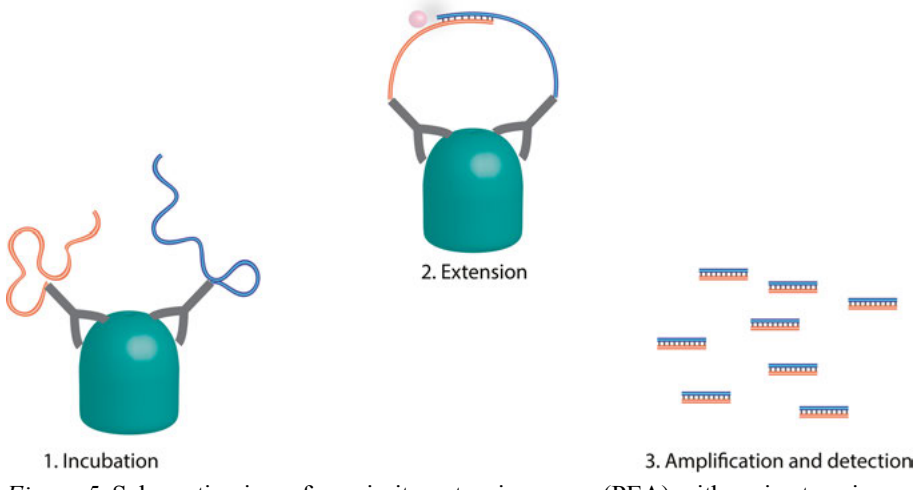

Figure 5. Schematic view of proximity extension assay (PEA) with main steps included. 1. Incubation where the two DNA oligo-labelled antibodies bind to the targeted protein. 2. Extension of the oligos. If the sequence of the oligos do not match, there will be no hybridization. When they do match, a DNA polymerase will extend the amplicons. 3. The amplicons are amplified and detection readout is possible by qPCR.

16S rRNA analysis

The 16S rRNA gene in bacteria is a genetic region used to study bacterial phylogeny. It contains nine variable subregions, or variable domains (V1-V9) used to identify bacterial species. Using high-throughput next-generation

sequencing methods, it is possible to characterize the bacterial composition of a sample. The sequencing output is aligned against databases with known bacterial species to identify from what bacteria the sequencing output originated from. Results from sequencing of the 16S rRNA gene in bacteria can be affected by technical variations such as choice of variable regions of the 16S gene [100] and sequencing platform [101]. In paper VI the Ion 16S Metagenomics Kit (Life Technologies, Carlsbad, CA, United States) was used to characterize the vaginal microbiota from FTA cards deriving from the study presented in paper I. This kit amplifies seven of the nine variable regions using six amplicons (Figure 6.)

Figure 6. Schematic view of the 16S rRNA gene and the primer targets of the Ion 16S Metagenomics Kit (Life Technologies, Carlsbad, CA, United States). Black arrows indicate primer and dotted line the amplified amplicon. The seven variable regions, V2, V3, V4, V6, V7, V8 and V9 are targeted by this assay. Region V6 and V7 are amplified as one amplicon.

Statistical methods

We generally use a statistical test to determine if there are associations or differences in our data. Statistical tests generate a *p*-value, in order to assess the relevance of the study results and its impact. The *p*-value is often considered to be significant if the *p*-value < 0.05, which means that the probability of obtaining results as extreme as the observed result is less than 5 %. However, performing numerous statistical tests, there is a risk of generating false significant *p*-values. To correct for this, a multiple testing correction method can be used to minimize the risk of overestimating significant results. There are numerous multiple correction methods such as false discovery rate (fdr) or Bonferroni. The Bonferroni adjustment method is a stringent method for multiple testing correction. In short, Bonferroni adjustment means multiplying the generated *p*-values with the number of statistical tests performed in the data set. The adjusted *p*-value is given as a *q*-value. The Bonferroni adjustment method was applied to the analyses in papers I–VI.

If there is a distribution of cases and controls in a data set, a cut-off value for a test can be decided to predict if a given sample belongs to either of the outcomes. As discussed previously, how relevant the cut-off is can be estimated with parameters as sensitivity and specificity. Additionally, the trade-off between sensitivity and specificity can be visualized with a receiver operating characteristic (ROC) curves. In a ROC curve, sensitivity and specificity

of a whole range of cut-offs are visualized for binary outcomes. Area under curve (AUC) can be used to summarize the ROC curve characteristic. The AUC range between 0 and 1 where 1 is perfect classification and 0.5 would be the result achieved by guessing.

Because the data set is always limited (we can rarely include all individuals in a population) there is a risk of overfitting a model to the data set itself. Overfitting means that the model is adapted to the specific characteristic of the data set used to train the model, and will not necessarily preform the same in another data set. This can be addressed by cross-validation to detect overfitting or by dividing the samples into a test and validation set, if the sample size is large enough. Estimating the performance in an independent data set gives a better estimate of the model performance overall.

Relevance and aim

Cervical cancer is the most common gynaecological cancer. The Swedish screening program has been hugely successful in reducing the mortality rate by half, but now it seems that the previous triumph of the screening program has been haltered.

The studies described in this thesis focus on two main issues that are relevant to prevent cervical cancer. The first is the participation rate of women in the organised screening program. The second is the limitations of screening test performance.

This thesis aims to explore if it is possible to develop the screening program for cervical cancer, both through broadened test collection opportunities and by identification of novel biomarkers for cervical cancer and preceding CIN stages. The main focus is biomarkers studied in vaginal fluid because of its suitability for self-sampling and screening.

Summary of included papers

Paper I: Protein detection using the multiplexed proximity extension assay (PEA) from plasma and vaginal fluid applied to the indicating FTA elute micro card™

The FTA card can be used as a material for collection of vaginal fluid from women performing self-sampling for HPV testing [102]. This card has previously been used in forensic science, where it is used to collect post-mortem samples [103-105]. The card is developed to cross-link proteins which inactivates potential infectious agents, making it suitable to study infectious agents such as influenza [106], rabies [107] and bacteria [108]. The FTA card also stabilizes nucleic acids, making them suitable for collection of DNA and RNA. It is especially well-suited for material collection in the field since it does not require freezing and it is used in veterinary medicine [109], food science [110] and environmental science [111, 112]. DNA extracted from the FTA card has been reported to perform equivalent to freshly extracted DNA [113]. The stability for nucleic acids for storage time in room temperature has been reported to be at least 16 years [114] and the FTA card performs better for storage of DNA compared to other paper cards [115]. In this paper we examined the possibility of measuring proteins in the vaginal fluid applied to the FTA card with the PEA method. The PEA method is highly sensitive and requires a small amount of material [99], which is an advantage when the abundance of proteins is unknown.

Aim

To investigate if it is possible to measure protein abundance with the proximity extension assay (PEA) in dried plasma or vaginal fluid applied to FTA elute micro card™.

Result & Discussion

In this paper we showed that it is possible to detect proteins in both plasma and vaginal fluid applied to the FTA card. The PEA method and the Proseek Multiplex Oncology Iv2 panel (Olink Bioscience AB, Uppsala, Sweden) was

used to analyse the samples. A comparison between liquid plasma and short-time storage of plasma on the FTA card (24 and 72 hours) was performed. As expected there was a lower abundance of protein from dried plasma on the FTA card. Still, 56 proteins (24 hours) and 52 proteins (72 hours) could be detected from the dried plasma compared to 87 proteins in the liquid plasma. A lowered abundance of proteins was as expected due the loss of material when spreading the material on a card and retrieving a one 3 mm punch from that area. All proteins had a high correlation (Spearman's rho, R2 = 0.89, *p*-value < 2.2×10^{-16}) when comparing short-time storage of plasma in 24 hours compared to 72 hours on the FTA card (Figure 7). Also, there was no significant loss in abundance of any protein between the two time points.

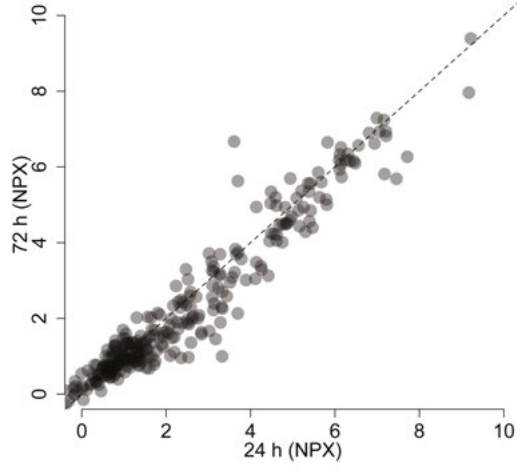

Figure 7. Correlation between NPX values for all observations with measurements above the limit of detection at two time-points from dried plasma stored on FTA card for 24 h (x-axis) and 72 h (y-axis), Spearman's rho, R2 = 0.89, *p*-value < 2.2×10^{-16}.

We also compared measurements from vaginal fluid applied on the FTA card and cervical-vaginal fluid in the transport media presently used in organised cervical cancer screening program in Sweden. These results showed a higher detection rate in samples from the FTA card. This is most likely due to the dilution of the cervical sample in 20 ml of transport media [95], whereas the FTA extraction was performed in 20 µl buffer. Several proteins present in the vaginal fluid have been associated with cervical cancer and CIN [116-119] and some of them are present in the protein panel used in this analysis, e.g. interleukin 8 (IL-8). These proteins were also detectable in the vaginal fluid samples on the FTA card, which shows that this could be a potential extension

of use when collecting screening samples for cervical cancer with this medium.

This study showed that even if the FTA card inactivates and cross-links proteins, some proteins either escape this cross-linking event or maintain the epitopes targeted by the antibodies in the PEA. Screening test collection with the FTA card opens up the possibility for use of an easy sample collection matrix that do not require freezing and needs limited storage space. The study shows that the FTA card can be used for studying DNA, RNA as well as proteins. This card is an appealing alternative for screening sample collection, since it could generate opportunities for a number of biological markers, including not only HPV DNA testing, but also protein markers or the microbiota, as illustrated in paper VI.

In summary, this paper has showed that it is possible to measure protein abundance from the FTA card, both for plasma and vaginal fluid. Going forward there is a need to study a larger number of both samples and protein panels to identify optimal biomarkers for cervical cancer in vaginal fluid, using these methods.

Paper II: Randomised study shows that repeated self-sampling and HPV test has more than two-fold higher detection rate of women with CIN2+ histology than Pap smear cytology

Cytology has since the implementation of cervical cancer screening been the standard screening test for cervical cancer and identification of CIN. More recent recommendations suggest that screening for presence of HPV infection should be introduced. The Swedish National Board of Health and Welfare changed their recommendations in 2015, calling for all county councils in Sweden to use HPV DNA test as primary screening method [52]. However, a screening program, regardless of screening test, will need to have a high participation rate to be effective. Also, introducing HPV testing as primary screening will lead to the need for stratification of HPV positive women due to the lower specificity of the test. One way to do this is to measure short-time persistence to reduce the number of women in need of follow-up [75].

Aim

This study aimed to compare the participation rate and the detection rate of CIN2+ among women performing repeated vaginal self-sampling for HPV test with women following the clinical practice with midwife-collected cytology test in Uppsala county between the years of 2013 and 2015.

Result and discussion

The results from this study showed a higher participation rate among women in the intervention group performing self-sampling and repeated HPV test compared to the control group with women performing midwife-collected cervical sampling with cytology test (47 % vs 39 %, *p*-value $<2.2 \times 10^{-16}$, two-sided binomial test). Some limitations in the study make it harder to evaluate the true difference in participation rate. Firstly, the county council charged a small fee for women in the control group. Secondly, women in the intervention group received a reminder after 3 weeks if they had not responded, whereas women in the control group received a reminder after 1 year. However, these effects seem unlikely to diminish the total difference in participation rate between the intervention group and the control group. The higher participation rate for women performing self-sampling is consistent with several other studies, showing that self-sampling has a high acceptance rate and is preferred by a majority of women [81, 82]. Some county councils in Sweden has now implemented trials with self-sampling for women who has failed to participate in the screening program for a longer time period. There is also a call for making self-sampling available by the Swedish patient organisation for cervical cancer [84]. Modelling shows that a strategy based on repeated HPV self-sampling could also be cost-effective within the Swedish health care [120].

The time required to receive diagnosis was shorter for women in the intervention group compared to the control group. The majority of this effect is most likely due to the rapidness of the HPV DNA test. Analyses of cytology samples are labour-intensive and require experienced personnel, which can prolong the time from sample collection to sample analysis. The shorter timespan between sample collection and return of information from test result is of high importance, not only for women who need further follow-up but also for women who are receiving a reassuring result of their health (HPV negative test). The time between sample collection and return of test result can be a stress factor and should be considered when discussing method for sample analysis.

The Swedish guidelines recommend using cytology test as triage method after confirmed HPV infection [52]. Repeated self-sampling and HPV test could be an alternative triaging method that does not require the woman to visit a clinic for sample collection, and therefore reduces burden on the healthcare system.

The repeated HPV test strategy in this study had a substantially higher PPV (0.45) than cytology (0.37). By using short-term HPV persistence as a triage method in this study, approximately 30 % of the women in the intervention group cleared their infection and this reduced the number of women who needed clinical follow-up. The short time span between the repeated HPV samples was due to the clinical practice and to the fact that this was a population that had not previously been screened with HPV testing. We do not know

the time of initial infection, but if HPV testing was to be implemented as a regular screening, it would be possible to identify a time span for when the infection occurred. This could make it possible to somewhat prolong the time between self-sampling for screening and repeated sampling, without risking the health of the woman.

There was a higher cumulative prevalence of CIN2+ among women performing self-sampling for HPV testing (20.2 per 1000 women) compared to women who performed a midwife-collected cytology test (10.8 per 1000 women). There was no difference for CIN3+, which is a less common event. A larger study population would therefore be needed to be able to reach the necessary power for detection of possible differences between CIN3+ diagnoses. This study was able to show that women were more likely to participate in screening offering self-sampling of vaginal fluid, and that the discovery rate of women with CIN2+ was two-fold higher as compared to using the cytology test as implemented in the organised screening in the clinic. This study also showed that repeated HPV testing has a higher PPV than screening with cytology.

Paper III: Randomised study of HPV prevalence and detection of CIN2+ in vaginal self-sampling compared to cervical specimens collected by medical personnel

Not participating in the organised screening is associated with increased risk of cervical cancer [76]. By providing the option of self-sampling, the population coverage could be increased and non-attenders could be encouraged to take part in the organised screening. Self-sampling often has a slightly different sampling location compared to samples provided by midwife (vaginal sampling vs cervical sampling). A crucial step in offering self-sampling is to make sure that it performs as well as sampling provided by medical personnel.

Aim

To investigate if the detection rate of CIN2+ in self-sampling of vaginal fluid for HPV DNA testing was noninferior compared to assisted sampling by medical personnel.

Result and discussion

No differences in HPV DNA test performance could be detected due to the mode of sample collection and there was no statistically significant difference in detection of CIN2+ between the two collection strategies. The results show that there is no difference in the detection rate between self-sampling and

sampling by medical personnel, and both procedures could be used as a strategy for sample collection in a screening program for cervical cancer. This is consistent with previous results [121]. There are, however, reports indicating that self-samples have an even higher detection rate of CIN2+ than clinician collected samples [122].

Self-sampling usually collects vaginal fluid rather than a cervical specimen. This could lead to variations between the results. This study was performed among women over the age of 50, from whom it is more difficult to retrieve a representative cervical specimen than from women of younger age. This is due to the biological changes after menopause where the SJC of the cervix often is located further up in the cervical canal. The use of FTA cards in self-sampling of vaginal samples is not as widespread as the liquid-based collection of vaginal fluid, but do show general good concordance with liquid-based test collection [123].

One could argue that providing HPV self-sampling could result in that women would not be attending follow-up. However, this does not seem to be the case [86]. Self-sampling for HPV testing would increase participation rates, even when accounting for loss to follow-up [124].

The limitation of this study is its sample size, which makes it hard to detect smaller differences in outcome. Also, we did not have statistical power to calculate the difference in detection of CIN3+. There was a significantly higher prevalence of HPV infection among women performing self-sampling in the first HPV test. However, this did not lead to a higher rate of referrals than in the controls, as they were more likely to clear their infection between sample rounds. This could possibly be due to the detection of infections with low viral load that are detectable in the vaginal fluid, but harder to detect with a cervical sample.

Paper IV: Identification of candidate plasma protein biomarkers for cervical cancer using the multiplex proximity extension assay

The HPV test represents the basis of the present cervical cancer screening programs. However, there is a limitation in the specificity of the HPV test. To increase the specificity would require additional biomarkers for cervical cancer, or rather – the preceding CIN stages. There have been several suggestions of new biomarkers for cervical cancer. However, there is a limitation in the knowledge of the window of information relative to the time of diagnosis. Development of cervical cancer is usually a slow process and have the benefit of defined CIN stages, which should make it optimal for identification of biomarker candidates. However, the number of samples collected before diagnosis is scarce, which makes evaluation of biomarkers challenging.

Aim

To discover plasma proteins that have biomarker potential for cervical cancer and validate them using samples collected at different time-points prior to cervical cancer diagnosis.

Result & Discussion

The abundances of 100 proteins were measured in plasma collected at the time of diagnosis among patients with invasive cervical cancer and in population controls. This study identified 80 proteins with increased levels in cases compared with healthy controls. A signature of 11 proteins distinguished cases and healthy controls with a high accuracy. The results were validated in a prospective cohort with samples collected before, at, or after diagnosis. These results showed that the protein markers were mainly informative at the time of cervical cancer diagnosis (Figure 8B).

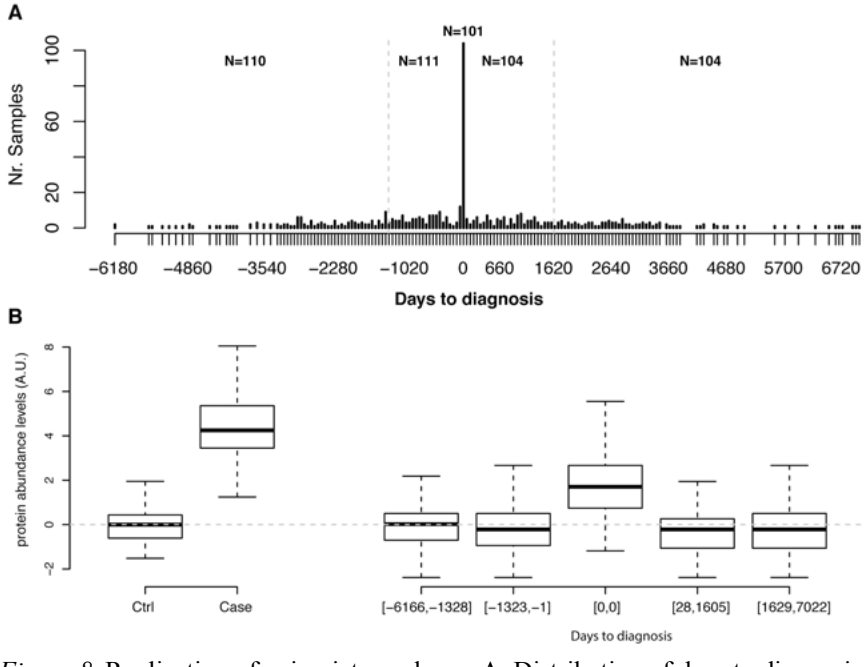

Figure 8. Replication of univariate analyses. A, Distribution of days to diagnosis for samples in the replication cohort. The grey dotted vertical lines indicate the cut-offs used in the binned analysis. B, Distribution of normalized protein abundance levels in the cohorts used for PTX3. The top and the bottom of the box represent the 25th and 75th percentile and the band inside the box the median value. The whiskers are calculated as 1.5× the interquartile range. Cohorts ordered as in (A). The dotted grey line indicates the mean of the groups used to determine normalization coefficients, e.g. the control samples from the discovery cohort and the samples from the replication cohort collected at least 3 years before diagnosis.

This study identified plasma protein markers relevant for identifying women with cervical cancer, but the results also indicated that further studies are needed to investigate these proteins informative value for finding women with CIN. In particular, even though a large number of women in the prospective cohort had been diagnosed with cervical cancer at different time-points after submitting a sample to the biobank, the time-span covered was very wide, and relatively few samples were available within a time period of 1 year or less prior to diagnosis (Figure 8A). Thus, the statistical power to detect trends in the levels of biomarker candidates was limited. The results highlight the difficulty in evaluating biomarker candidates, due to lack of samples collected prior to disease development.

Paper V: HPV viral load in self-collected vaginal fluid samples as predictor for presence of cervical intraepithelial neoplasia

There have been several studies regarding the effect of HPV viral load on risk for development of cervical cancer and its preceding CIN stages. Even though the effects of viral load on risk may be limited there is the possibility for using the information obtained from the HPV test for a more comprehensive interpretation of risk assessment, and possibly triaging.

Aim

To evaluate the use of HPV viral load as a biomarker for persistent HPV infection and CIN2+ in self-samples of vaginal fluid.

Result & Discussion

The HPV DNA test, HPVIR, used in the previous studies described is a qPCR-based assay which quantifies the amount of HPV copies for 12 HPV types, individually or in groups [95]. Viral load as a predictor for presence of CIN2+ was studied as HPV copies or HPV copies per cell, e.g. HPV titer. This study shows that women with a persistent HPV16 infection and CIN2+ have a higher viral load in their primary screening test, than women that have cleared their infection between the repeated test collections. The relationship is the same when comparing the total viral load for all HPV types included in the HPVIR test (Figure 8).

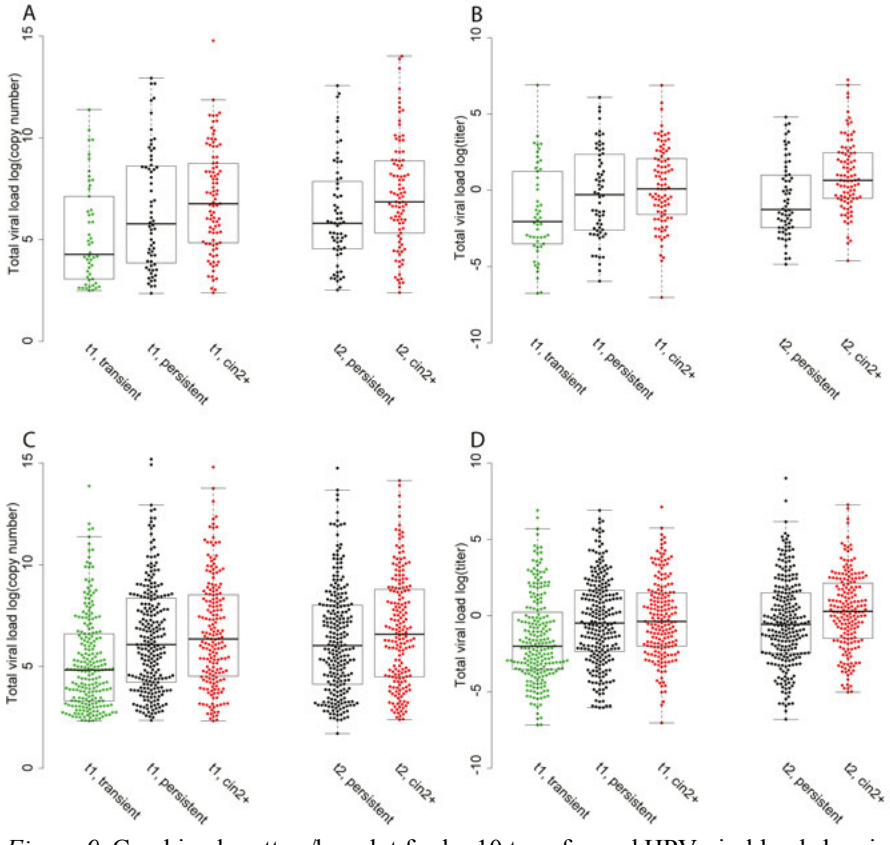

Figure 9. Combined scatter-/boxplot for log10 transformed HPV viral load showing the distribution for transient infections (green), persistent infections without CIN2+ lesions (black) and persistent infections with CIN2+ (red) in primary screening test (t1) and follow-up test (t2). The top and bottom of the box represent the 25th and 75th percentile and the band inside the box the median value. The whiskers are calculated as 1.5x the interquartile range. A. HPV16 copy number, B. HPV16 titer, C. Total hrHPV viral load copy number, D. Total hrHPV viral load.

The study also showed that a higher number of women with persistent HPV16 infection and CIN2+ had a higher viral load in their second test than in their first test compared to women with persistent infection but without CIN2+. This paper describes the possibility to measure the viral load of HPV from self-samples and its association with both HPV persistence and risk of CIN2+.

The main question in this study has been addressed in other studies, but this study is unique in that it is based on self-samples only, showing that the use of HPV viral load as a predictive biomarker is independent of the sampling strategy. As a triage method for HPV positive women HPV viral load does not perform as well as triaging with cytology [125]. However, in low income or rural settings it may not be feasible with cytology testing and viral load could there be an alternative for triaging. Lastly, this study shows that the FTA card

as collection medium for vaginal fluid allows not only for HPV detection, but also for HPV quantification.

Paper VI: Temporal changes in the vaginal microbiota in self-samples and its association with persistent HPV16 infection and CIN2+

The stabilization of nucleic acids on the FTA card makes it suitable not only for HPV testing but also for investigating genetic material from other microbes in the vaginal fluid. The vaginal microbiota is complex but has been reported to be associated with cervical cancer. Similar to the case of HPV viral load, adding additional analysis to the HPV test could possibly aid in the risk assessment regarding cervical cancer disease development.

Aim

To compare the vaginal microbiota in women with persistent or transient HPV16 infection in self-samples collected at two different time-points.

Result & Discussion

A selection of self-samples from the study described in paper II was analysed by sequencing of the 16S rRNA gene. The analysis contained three sample groups i) single samples from women that were HPV negative in their initial screening sample, ii) paired samples from women who had only HPV16 infection in their first screening sample and then cleared their infection before their repeated HPV test and iii) paired samples from women who had two consecutive HPV tests with only HPV16 in both and were diagnosed with CIN2+.

Lactobacillus sp. dominance in the vaginal flora has previously been associated with a healthy state of the vaginal microbiota and contributes to the low pH value in the vaginal environment, which itself seems to have a protective effect against infections [126]. Our study showed that there was a higher prevalence of *Lactobacillus sp.* dominance in HPV negative women, than in women with HPV16 infection (Figure 10).

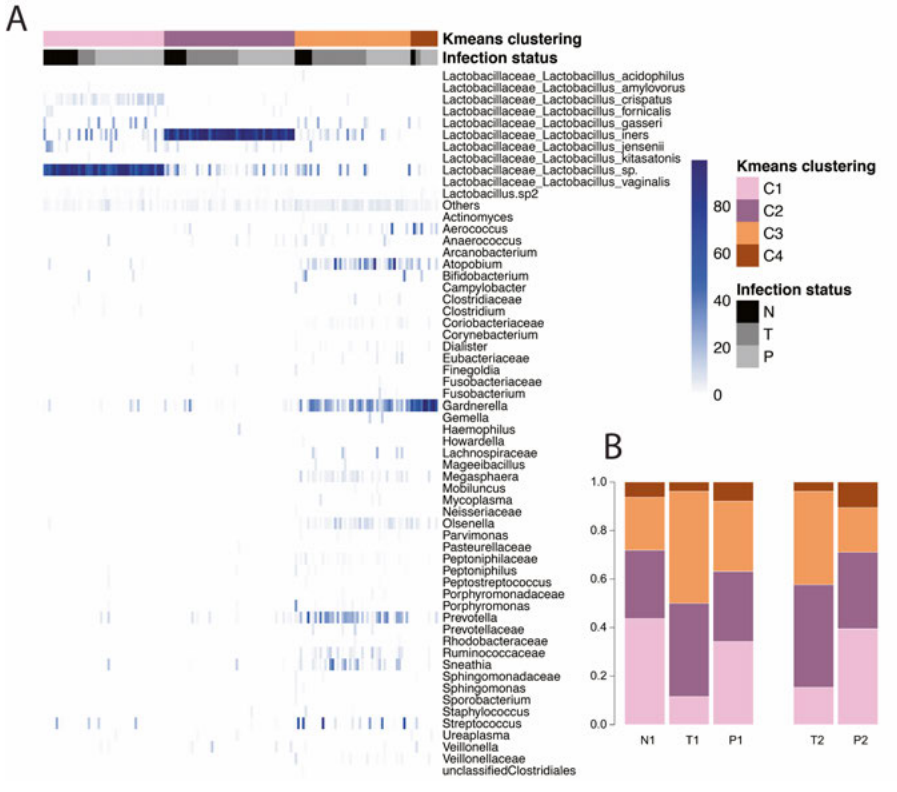

Figure 10. A. Heatmap of relative abundance of bacterial taxa. Each column represents a sample and each row a bacterial taxon. The top box indicates K-means clustering (C1–C4) and the second box from the top indicate infection group belonging (N = HPV negative, T = Transient HPV16 infection, P = Persistent HPV16 infection). *Lactobacillus sp.* are reported at highest possible taxonomic level and other bacteria are grouped at genus level if known, otherwise they are grouped on family level. Bacteria with lower abundance than 1 % are gathered in "Others" if they are non-*Lactobacillus* and in "Lactobacillus.sp2", if they belong to *Lactobacillus sp.*. B. Stacked barplot for each infection group and sampling round (N = HPV negative, T = Transient HPV16 infection, P = Persistent HPV16 infection, 1 = baseline sample, 2 = follow-up sample). Colors indicate K-means cluster groups (C1–C4)

The results also showed that there was a higher number of transitions between microbial profiles in women with persistent HPV16 infections and CIN2+ as compared to women who cleared the HPV16 infection between sampling rounds (Figure 11). The short time between sampling makes it likely that sampling should be in close proximity to clearance of the infection. The increased rate of transition between microbial profiles indicates that there is a disturbance in the microenvironment of the vagina that influence the bacterial flora. The increasing inflammatory response during CIN progression [15] could possibly affect stability of the vaginal microbiota.

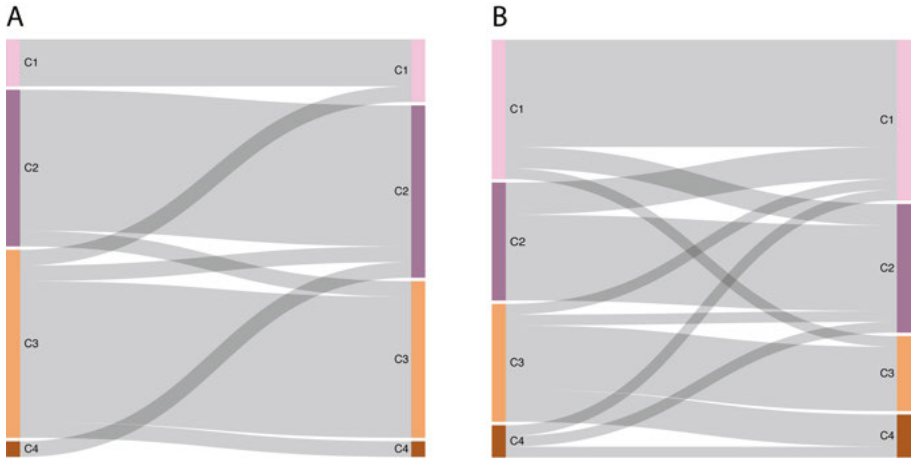

Figure 11. Sankey plots for transition events between baseline and follow-up sample for K-means cluster analysis. A. Women with transient HPV16 infection. Left column represents baseline sample and right column follow-up sample. Color and text indicate K-means cluster, B. Same as A, but for women with persistent HPV16 infection and CIN2+.

This study shows that it is possible to study the vaginal microbiota from vaginal self-samples collected on FTA cards and that there is there is a significantly higher proportion of transition events between microbial profiles in women with persistent HPV infection than in women clearing their infections.

Implications and future directions

This thesis has contributed to the knowledge on how to improve the screening opportunities for cervical cancer and CIN stages by providing a reliable procedure for self-sampling of vaginal fluid for HPV DNA testing. It has also explored the possibility of additional biomarkers for cervical cancer.

The screening programs for cervical cancer have saved many women from cancer disease and death. It is one of the most successful screening programs for cancer. However, we should continue to evaluate and strengthen the program with new and better methods. There are still so many women who are affected by cervical cancer, especially in low income settings. Self-sampling has an overall high acceptance level and are in rural setting maybe the only possible form of sample collection for cervical cancer screening. Self-sampling could and should be a part of the strategy to reach women who can't or won't attend screening at healthcare facilities.

The future health care system should invite patient to take part in the planning for their own health, on their own terms. Patient organisations has long called for opportunities for women to perform self-sampling for HPV testing. The most important reasons for women to perform self-sampling are convenience and self-control [127]. Offering self-sampling would reduce the threshold for participating in the screening program, regardless of if the reason is lack of time, distance to health care facilities, or attending a clinical procedure, which is many times perceived as exposed and stressful for the woman.

Implementation of HPV DNA based screening programs with its higher sensitivity provides a better possibility to prevent cervical cancer. However, it will require a system to separate those with a risk for cervical cancer from those who are likely to clear their infection without intervention. This is exemplified by the introduction of primary HPV screening in the Netherlands, which resulted in a higher detection rate of women with CIN2+ compared to the cytology every 5-year program, but at a cost of more referrals that may not be clinically justified [122].

Cytology testing has saved many women´s life since the implementation of the screening programs. In a Swedish context, it has been a success story and reduced the cervical cancer incidence with more than 50 %. However, there has been a stagnation of its effect, and for the last years, we have seen an increase of cervical cancer cases [128]. Most cervical cancer cases occur in women who have not attended the screening program. But it is noteworthy that most of these new cases are among women who have attended the

screening program. This means that the screening program performs less well than before. A report from the NKCx shows that the most recent increase appears to be connected to a higher rate of cervical cancer cases in women with a previous normal cytology. A re-evaluation of cytology screening samples was performed, and resulted in the identification of a high rate of re-classification (e.g. samples who were previously classified as normal were re-classified to abnormal) [129]. Cytology screening is labour intensive and requires experienced personnel. Even if cytology testing has contributed enormously to the screening program, is it feasible to maintain? Cytology is recommended for women under the age of 30 and as a triage method to HPV positive women over 30 years. However, if the quality of the cytology test cannot be maintained it may not be the best method for either screening or triage. The work in this thesis has highlighted other options for triage of HPV positive women. Triaging HPV positive women with repeated HPV testing targets women with persistent HPV infection without requiring the need for a visit to a clinic. It also offers a test with a higher PPV than primary cytology screening. The rapid turnaround of self-sampling and HPV test could also reduce the stress for women, as not having to wait for a test results can increase the well-being for women.

The FTA card has been shown to offer not only the ability to detect and quantify HPV DNA, but also to study the vaginal microbiota and measure levels of proteins in the vaginal fluid. Samples collected on FTA cards are also amendable to analysis of biomarkers that are not studied within this thesis, such as host genetic variation, early tumor mutations (somatic genetic variation), epigenetic markers such as methylation of HPV or human genes [130, 131] or detection of microRNA [132]. The samples do not require freezing for long-term storage and are therefore well-adapted for biobanking without the need of expensive freezing storage. This format could therefore be very suitable for low-income and rural settings.

As vaccinated women enter the screening program, the prevalence of cervical cancer will be reduced. A reduced prevalence of disease will lower the performance measure of PPV for screening tests [133], if the test sensitivity and specificity stay the same. A reduced prevalence of cervical cancer in the younger age groups will also change the age demographic pattern for women with the highest risk. There has already been a significant shift in the mean age of women who developed cervical cancer since the time before the implementation of the present screening program until now. Between the years of 1944 and 1957, 5.4 % of women treated for cervical cancer was over 69 years of age, while between 1990 and 2004 this group had increased to approximately 27 % [134]. According to current guidelines this age group is not included in the screening program. Recent studies have shown an HPV prevalence of approximately 4 % in women between 65 and 75 years of age [135, 136]. This indicates that the guidelines for cervical cancer screening may have

to be updated to include also this group of women, in order to effectively reduce the number of cervical cancer cases.

HPV is responsible for over 30 % of all infection-related cancers. The main part of this is cervical cancer, but the virus can also cause oropharyngeal cancers, anal cancer, penile cancer etc. [5]. The number of HPV positive oropharyngeal cancers have increased the last decades [137]. There could be a possibility for the HPV test to be used in screening programs for additional cancer types, mainly oropharyngeal cancer. However, oropharyngeal cancer is mainly caused by HPV16 [138] and with proper vaccination coverage, the incidence of these cancers should diminish.

A screening program that would be truly desirable would be one that includes all women, also those who wish to avoid physical examination. It should have safe and effective tests with biomarkers to stratify women to effectively find those who truly need the healthcare systems and clinical follow-up. This thesis presents openings to proceed with two major issues with the current screening program: availability of screening opportunities and novel biomarkers to strengthen the performance of the tests used in screening.

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