Bachelor Degree Project

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Oral and genital human papillomavirus (HPV) prevalence in young women and men attending a youth clinic in Stockholm, Sweden

-a follow up study after the introduction of HPV vaccination

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ABSTRACT

Background and Aim: In 2010 HPV vaccination was subsidized in Sweden and in 2012 a national vaccination program against HPV16, 18, 6 and 11 was launched for girls ages 10-12 years. In parallel was a catch-up vaccination program for young women. To investigate base line HPV cervical and oral prevalence in non-vaccinated youth two studies were performed at a youth clinic in Stockholm 2008-2011. This project initiated 2013 aimed to follow HPV prevalence in youth since the previous studies, in the same population.

Materials and Methods: 117 women, of which 73% were HPV catch-up vaccinated donated 93 cervical samples and 117 oral samples, and 54 unvaccinated men donated 54 oral samples and 47 urinary samples. All samples were tested for 27 HPV types with a PCR based system and the data was compared to that obtained in 2008-2011. The categorical Fishers exact test was used for statistical analysis due to HPV-positive samples being $n < 5$ for certain types.

Results and Conclusion: HPV16 cervical prevalence was significantly lower in the HPV vaccinated women compared to unvaccinated women (7% and 27% respectively, $p=0.033$) in the 2013 group. For HPV18 and HPV6 there was a significantly lower prevalence in the 2013 vaccinated group compared to the 2008-2010 unvaccinated group (1.5% vs. 10% respectively, $p=0.021$ and 1.5% vs. 8% respectively, $p=0.048$). Overall oral HPV prevalence for both genders, was lower in the 2013 group compared to that of 2009-2011, (2.3% and 9.1% respectively, $p=0.005$). Male urinary prevalence was low (6%) and not efficient to follow changes in specific HPV types. The data indicate that HPV catch-up vaccination was gradually exhibiting an effect, with significant decrease of cervical HPV16 prevalence.

POPULÄR SAMMANFATTNING

Syfte: Att efter den gradvisa introduktionen av vaccination mot humant papillomavirus (HPV) 2010, undersöka möjliga förändringar av olika HPV typer hos vaccinerade och ovaccinerade ungdomar på en ungdomsklinik i centrala Stockholm 2013.

Bakgrund: Det finns fler än 100 HPV typer och några HPV typer finns i visa tumörer, ex. Livmoderhalscancer, tonsill- och tungbascancer. I Sverige, så har HPV vaccination gradvis introducerats sedan 2010. Från 2012 så erbjöds 10-12 åriga flickor vaccination mot HPV typerna 16,18, 6 och 11 via skolvaccinationsprogrammet och dessutom fanns s.k. ”catch-up” vaccination för unga kvinnor upp till 26 års ålder i Stockholm. Två tidigare studier utfördes på en ungdomsklinik i Stockholm, Sverige mellan 2008 och 2011 för att innan HPV vaccination få en bild av baslinje HPV frekvensen i livmoderhalsen och i munhålan. Målet med denna studie 2013 var att följa upp HPV frekvens i livmoderhalsen och i munhålan efter det vaccinationsprogrammet gradvis påbörjats. Urinprover från HPV-ovaccinerade män undersöktes också för att undersöka om mer information om HPV typer hos män kunde fås.

Material och metoder: 117 kvinnor, där 73% var HPV vaccinerade men inte nödvändigtvis före sin sexdebut, donerade 93 prover från livmoderhalsen samt 117 prover från munhålan. Dessutom erhölls 54 prover från munhålan och 47 urinprover från 54 HPV ovaccinerade män. DNA extraherades från proverna och testades för 27 relevanta HPV typer med s.k. PCR teknik och jämfördes för vaccinerade och ovaccinerade för 2013 och med tidigare resultat från 2008-2011. Data var sedan analyserade statistiskt med ”categorical Fischers exact test”.

Resultat: 2013, var frekvensen av alla HPV typer gemensamt 60 % hos HPV-vaccinerade och 73% hos HPV ovaccinerade kvinnor och denna skillnad var inte statistiskt signifikant mellan dessa grupper eller jämfört med frekvensen (70%) hos ovaccinerade kvinnor från 2008-2010. Däremot var frekvensen av HPV16 i livmoderhalsen signifikant lägre hos vaccinerade jämfört med ovaccinerade kvinnor 2013 (7% respektive 27%, p=0.033). HPV18, 6 och 11 frekvens i livmoderhalsen var dock inte signifikant olika hos vaccinerade jämfört med ovaccinerade kvinnor 2013, men det fans en statistisk lägre frekvens hos vaccinerade kvinnor 2013 jämfört med ovaccinerade kvinnor 2008-2010 för HPV typ 18 (1.5% respektive 10%, p=0.021) och för HPV typ 6 (1.5% respektive 8% p=0.048), men inte för HPV typ 11. Dessutom, var frekvensen av HPV frekvensen totalt i prover från munhålan signifikant lägre 2013, jämfört med 2009-2011 (2.3% respektive 9.1%, p=0.005). Frekvensen HPV i urin hos män var låg med endast 6% positiva prover.

Slutsats: HPV typ 16 frekvensen minskade hos HPV vaccinerade jämfört med HPV ovaccinerade kvinnor 2013, medan för HPV typ 18 och HPV typ 6 men inte för HPV typ 11 så kunde en minskning noteras mellan vaccinerade kvinnor 2013 och ovaccinerade kvinnor 2008-2010. Frekvensen av HPV i munhålan minskade också mellan 2013 och 2009-2011. Data tyder på att HPV vaccination gradvis skyddat även hos kvinnor som vaccinerats med s.k. ”catch-up” vaccinering. Urinprover hos män hade låg HPV frekvens vilket inte är användbart för att genomföra studier för att undersöka förändringar av enskilda HPV typer.

CONTENTS

ABBREVIATIONS

DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleoside triphosphates
FFPE	Formalin-Fixed Paraffin-Embedded
HNSCC	Head Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
HPV^+	Human Papilloma Virus positive
HPV^-	Human Papilloma Virus negative
HR	High Risk
LR	Low Risk
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT	Room temperature
STD	Sexually Transmitted Diseases
STI	Sexually Transmitted Infections

INTRODUCTION

General background

Cervical cancer, where 90-99% of the cases are caused by human papilloma virus (HPV), is the second most common and fifth deadliest cancer type among women worldwide.[1] Cancer in several other sites, e.g. the anogenital area and oropharynx are likewise often caused by HPV.[2,3] In HPV-positive cervical cancer, HPV types 16 and 18, which are only two of the more than 100 existing HPV types, account for 70% of the cases.[2,3] HPV16 is also frequently observed in anogentital cancer and oropharyngeal squamous cell cancer.[3-5] The latter cancer type mainly affecting men has been increasing the past decades in many Western Countries.[5-8] Two HPV vaccines, Gardasil and Cervarix, both effective against genital infection by HPV 16 and 18 have been on the market for some years.[9] Gardasil also covers HPV types 6 and 11 that cause genital warts. In HPV-positive tonsillar and base of tongue cancer, 90-95% of the cases are caused by HPV16.[6,10] Recently, it was demonstrated that HPV vaccines can protect against oral infection by HPV.[11] Hence, it is likely that HPV vaccination can also prevent a large proportion of tonsillar and base of tongue cancer. In Sweden, HPV vaccination was available from 2006. From 2010 it was free of charge for young girls aged 10-12 years and at a reduced price for young women up to the age of 17 years. From 2012 in Sweden a public vaccination program was initiated with Gardasil for girls aged 10-12 years, through the school based program, and in Stockholm it became free of charge for women up to 26 years of age.[12]

Between 2008-2011, before the public vaccination program in Sweden was initiated, two studies investigating base-line HPV prevalence in youth were performed. In the first study (2008-2010), performed by Du et al 2012, cervical samples were obtained from 555 women aged 15-23 years, and in the second study oral samples were collected from 401 women and 82 men aged 15–23 years, and in addition, 179 women donated a cervical sample.[13,14] Both studies were carried out at a Stockholm youth clinic in central Stockholm, and a high cervical HPV prevalence was found (70-74%), while oral HPV prevalence was lower (10%).[13,14] This study was initiated in order to follow any changes in HPV prevalence in youth at the same clinic after HPV-vaccination was initiated. In addition, urinary samples were collected from men to examine whether similar HPV types were found genitally in men and women. Below is an introduction to the field.

The HPV-family

There are over 100 HPV types of which most are cutaneous and fewer mucosal (for reviews see Zur Hausen and Tommasino).[2,3] Cutaneous HPV types can cause warts. Mucosal HPV types are divided into "high-risk"(HR) that potential can cause tumours, intermediate types that more seldom cause tumours, and "low-risk"(LR) containing the HPV types that almost never cause tumours, but mainly condylomas, or respiratory papillomas. The HR types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 and putative HR types: 26, 53, and 66, are associated with cervical cancer, and anogenital cancer such as e.g. penile and vulvar cancer, and HPV16 is mainly observed in tonsillar and base of tongue cancer.[3] LR types:e.g. 6 and 11 can be associated with condyloma and respiratory papillomas.

HPV and Cancer

Cervical Cancer

Cervical cancer is the most well known of the cancers caused by HPV, and also the one HPV related cancer causing over 500 000 cases and almost 250 000 deaths annually.[15,16] Up to 75% of HPV-positive cervical cancers are caused by HPV16 and 18.[15] As with all other HPV related cancers, HPV positive cervical cancer is of the epithelial variety. Most information regarding HPV induced cancer development is derived from cervical cancer that has pre-stages. For some details the genes, gene regulation of HPV, and transmission, see below.

Vulvar, Vaginal and Penile Cancer

Vulvar, vaginal and penile cancers are rather rare, with a relatively low mortality rate, but if the cancer metastasizes in lymph nodes in the pelvis, mortality increases. Furthermore, genital cancers cause great suffering due to the area of placement. Penile cancer ranges from 0.3 to 1.0 per 100,000 men, accounting for 0.4–0.6% of malignancies in industrial countries.[16] HPV has been found in 30-90% of these cancers.[17]

Anal cancer

HPV infection is a major risk-factor for anal cancer, with an estimate of up to 90% of anal cancers being attributable to HPV.[16] Similar to vaginal and cervical cancer, the rapid cell turnover and abrasions associated with sexual intercourse in the rectum, correlates with greater risk of HPV transmission as well as increased risk of cancer. HIV infection and smoking also increases risk of anal cancer usually attributed to the immunosupressing effects of both, preventing the HPV infection to heal, or being supressed properly.[18]

Tonsillar and base of tongue cancer

In the 1990s there was a proposed association between HPV and head neck cancer and eventually it was unravelled that HPV was mainly associated to tonsillar and base of tongue cancer.[6,19] However, it was not until 2007, that the International Agency for Research for Cancer (IARC) declared HPV as a contributing factor also for oropharygneal squamous cell carcinoma, where tonsillar- and base of tongue squamous cell carcinoma dominate.[4,5] In addition, several studies have demonstrated a rise in the incidence of tonsillar and base of tongue cancer, in several countries including Sweden and the USA during the last 40 years.[5-8] Notably, HPV-positive tonsillar and base of tongue cancer were shown to be responsible for the increased incidence of these tumours, while instead the incidence of the corresponding HPV-negative tumours caused by smoking had declined.[5-8] Furthermore, HPV-positive tonsillar and base of tongue cancer had a much better clinical outcome than HPV-negative cancers of these sites, with about 80% compared to about 40% 5-year disease free and overall survival.[6] Knowing HPV status of a tumour before treatment could possibly be relevant for adjusting care for the patient, due to HPV cancer patients generally having a better prognosis. HPV16 is estimated today to be responsible for around 90% of all HPV-positive tonsillar and base of tongue cancer and these diseases mainly affect men.

The HPV genome and its encoded proteins

All HPV types are non-enveloped viruses, with a circular genome consisting of around 7900bp double-stranded DNA.[2,3] The genome is divided into three sections, a non-coding control regulatory region, and two coding regions, the early and late region. The early region mainly encodes proteins E1-E7, which have regulatory functions and each protein often has many functions.[2,3] In HR-HPV types the E6 and E7 proteins bind the tumour suppressor proteins p53 and Rb and deregulate cell-cycle control and this can lead to dysplasia and cancer development. The late region encodes the two late proteins L1 and L2 that are structural proteins responsible for the formation of the viral capsid.[2,3] However, some early proteins are in effect also expressed or upregulated in the later phase of expression making the distinction between late and early somewhat vague.[3] The major capsid protein L1 can self assemble under the right conditions and form virus like particles (VLPs) and this quality has been important for the development of todays vaccines, which are based on VLPs.[11]

HPV-transmission

HPV is assumed to gain access to the basal cells of the epithelia trough the stratum corneum, via microscopic wounds, making genital and oral transmission effective transmission routes since abrasions in these areas during sexual activity is common[20-22].

Common warts are most likely transmitted from skin to skin, or from skin to other surface areas and to skin again.

Mucosal HPV types are often transmitted through sexual intercourse and there is a correlation between a high prevalence of HR types and a high number of sexual partners.[21,22] It has also been argued that an early sexual debut presents a higher risk for cervical cancer, possibly due to that the transformation zone of the cervix is more exposed on the outside of the cervix in younger individuals. However, in studies of heterosexual monogamous couples, hand-genital contact was shown of some but of less importance than genital-genital contact in transmission of HPV.[22] Moreover, there is a correlation between HPV prevalence and anal cancer in male homosexual groups, making anal sex a likely route of transmission.[23] Open-mouth kissing has been shown to be an important source of oral HPV transmission.[24] In addition, debut age of open-mouth kissing was significantly earlier than first experience of oral sex. The number of oral sex partners was a more important factor for HPV prevalence, while the number of only vaginal partners did not affect the prevalence of oral HPV infection.[24] Condoms give some protection against HPV, but there is still. The same goes for hand-genital contact. Microtears in skin is probably more common in areas subjected to abrasion, and studies did find genital-genital contact to be the greatest risk factor.[25] One concern of HPV-vaccination program has been that vaccinated women will be less worried about sexually transmitted diseases (STDs), and stop using protection, ergo the "Peltzman effect", but studies have found this incorrect.[26]

Finally, prenatal infection also occurs, although rarely.[27] It has been observed that the otherwise mild low risk HPV types 6,11 can cause recurrent respiratory papillomatosis, a condition that causes problems with breathing, talking and swallowing. It requires extensive surgery and can lower quality of life.

HPV-vaccines and other protection against HPV infection

L1 in the form of VLPs are the main components of the vaccines Gardasil and Cervarix, which at the time of this study were the only vaccines on the global market. Cervarix and Gardasil both contain L1 (VLPs) from HPV16 and 18, however, they differ in that Gardasil additionally contains L1 from HPV type 6 and 11. Their adjuvants also differ, but the main clinical difference is the additional protection against HPV6 and 11 in Gardasil. Early trials 2002, on the quadrivalent "test-vaccine" against HPV16 demonstrated the effectiveness of capsid based HPV vaccines.[28] In addition, it has been shown that Gardasil induces immunological cross-protection against some additional high-risk HPV types e.g. HPV45. However, nonavalent (effective against 9 types) vaccines are now available.

According to the Public Health Agency of Sweden, since 2010, HPV vaccination are publicly funded in Sweden for girls born 1999 or later, in the 5th to 6th-grade in school (about 10-12 years of age).[12]

In theory, vaccination of women should lower HPV-positive cancer prevalence also among men through herd immunity.[29] Herd immunity is the notion that a vaccinated portion of a population reduces exposure to disease for non-vaccinated individuals.[29] In the case of sexually transmitted infections (STIs) the percentage of vaccinated population is not necessarily required to be very high, mainly depending on how much age groups mingle sexually, sexual activity amongst different groups, as well as numbers of partners.[29] How many individuals needed to be vaccinated continues to be discussed, and several countries take different stances, with Australia vaccinating both boys and girls, while Sweden subsidises only girls at present time.

A North American study found signs that some effect of herd immunity was already taking place, just 4 years of licensing the quadrivalent HPV vaccine. HPV-prevalence amongst women 13-26 years of age dropped from 30.2% prevalence amongst unvaccinated 2006-2007, to 15.4 % year 2011. Among vaccinated individuals, the drop-rates where 31.8 to 9.9 percent.[30] The study was however not fully applicable to the general population, since its participants were from selected high-risk groups. All subjects were African-American, which is a group that tend to suffer from low socio-economic status in the US, as well as higher risk of acquiring sexually transmitted infections (STIs).[30] Similar problems can arise when looking at youth centre populations. However, a great benefit of sampling youth centre patients is access qualified personnel and equipment. The patients are often already at the youth centre for cervical smears and other sampling and are treated individually. This is different from studies done in e.g. in a classroom setting, which is more public and where cervical samples are almost impossible to obtain.

AIM OF THE STUDY

The aim of the present study was to follow-up the HPV prevalence from the autumn of 2013, after a larger proportion of young women in Stockholm had been HPV catch-up vaccinated, and compare with previous data on cervical samples from 2008-2010 and oral samples from 2009-2011 from the same youth clinic in Stockholm.

Our goal was to examine if any changes in oral or genital HPV prevalence of vaccine or non-vaccine HPV types, could be detected in the female group. More specifically, if there were decreases in the vaccine HPV types, in HPV vaccinated women compared to un-vaccinated in the same group from 2013, but also compared to the unvaccinated group from 2008-2011. In addition, we wanted to investigate if oral HPV had decreased in males and females by female HPV vaccination. Finally, we wanted to examine whether it would be possible to detect several HPV types in urinary samples of men and compare these HPV types with those in the cervical samples of the women.

MATERIAL AND METHODS

Participants and collection of samples

In total, 117 young women (of which 73% were HPV vaccinated) and 54 young men (aged 15-23 years, median age 20 years) at a youth clinic in the centre of Stockholm, Sweden, participated in the study from October 1st 2013 until December 31st 2013. All patients were given an informal letter explaining the study, what would be taken as samples, what would happen to the samples, and that no changes would be done to the routine of their treatment, due to patient-identity being anonymous in the study. Therefore also the name of the clinic is not exposed in this project or in any of the publications. Furthermore no negative consequences for the participants were assessed to arise from sample collecting and processing. Also, the aim was to get as many samples to work with as possible for statistical power, but could not be estimated or defined beforehand.

The young women donated a total of 117 mouthwashes and 93 cervical swabs by self-test and the young men donated a total of 54 mouth washes and 47 urine samples during 2013. All samples stored in a fridge at the youth clinic were collected every 2-3 days. Maximally one sample of each type was collected from per person. The obtained data from the samples were then compared to data obtained from 544 cervical samples between 2008-2010[13] and 483 oral samples from 2009-2011[14]. The study was performed according to ethical permissions **2012/1756-31 and 2013/1427-32** from the regional ethical committee in Stockholm.

Mouthwash samples were collected by the patient rinsing his/her mouth with a 50/50 Listerine/water solution for 30 sec before spitting into a vial.

Cervical samples were collected by self-test at the youth clinic. In both cases the samples were collected with swabs and that were placed into tubes containing 5 ml Surepath® fluid, a preservation solution containing isopropanol, methanol and ethanol.

Urine samples were self-collected in plastic vials.

Storage and DNA extraction of samples

Mouthwash samples were stored at ($+4^0$ C), collected twice/week and centrifuged 10 minutes at 3000 g. The pellet was washed twice in PBS, followed by a new centrifugation in each wash. Lastly, the washed pellets were suspended in 3 ml PBS and divided into two aliquots and stored at -20^0 C. Upon defrosting, DNA was extracted using the Gentra Puregene Buccal Cell kit (QIAGEN AB, Sollentuna, Sweden) according to the manufacturer´s protocol.

Cervical samples containing Surepath® fluid included in the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics GmbG, Mannheim, Germany), were stored in a cold room ($+4^0$ C) and collected weekly and extracted according to the manufacturer´s protocol.

Urine samples were aliquoted into smaller vials and also frozen at -20^0 C. DNA was not extracted from these samples instead aliquots of 2.5µl and 5µl were used directly in the PCR reaction.

Multiplex HPV analysis via a Magpix system

PCR mix

Qiagen multiplex master mix (QIAGEN AB, Sollentuna, Sweden) was used for PCR amplification. This contains necessary polymerases, nucleotides etc. To this a set of GP5+/6+ primers, as described in Table 1, amplifying part of the HPV L1 gene from 27 different HPV types was added, thus including most mucosal HPV types known today. Primers for HPV16E6 were also added. The reason for inclusion of these primers is that the assay is used also for testing HPV in tumours, where some HPV16 L1 regions might be deleted in later stages of tumour development. Primers for the cellular gene beta-globin (Table 1) were also included to be sure that the sample contained amplifiable DNA. All reverse primers were coupled to biotin in order to allow for binding to a flourescent reporter at a later step for visualization of the PCR products in the MagPix analysis. For more details see Appendix A.

To this mix the DNA templates were added. For urine, 2 µl of sample was diluted in 8 µl of water and added to the mix. For cervical and mouthwash samples, 10 µl sample was added. 10 µl of distilled water was added as a negative control.

Table 1. Primers included in the Magpix assay (modified from Cecilia Nordfors PhD thesis[31]**).**

Forward

Name	sequence	gene
GP5+	5´-TTT GTT ACT GTG GTA GAT ACT AC-3´	L1
BSGP5+-2	5´-TTT GTT ACT GTT GTI GAT ACT AC-3´	L1
BSGP5+-3	5´-TTT GTT ACT GTT GTI GAT ACC AC-3´	L1
BSGP5+-4	5´-TTT GTT ACT TGT GTI GAT ACT AC-3´	L1
BSGP5+-5	5´-TTT TTA ACT GTT GTI GAT ACT AC-3´	L1
BSGP5+-6	5´-TTT GTT ACT GTG GTA GAC ACT AC-3´	L1
BSGP5+-7	5´-TTT GTT ACA GTI GTA GAC ACT AC-3´	L1
BSGP5+-8	5´-TTT GTT ACA GTI GTA GAT ACC AC-3´	L1
BSGP5+-9	5´-TTT GTT ACT GTG GTA GAT ACC AC-3´	L1
HPV16E6-1.F	5´- TCA AAA GCC ACT GTG TCC TGA -3´	HPV16 E6
HPV33E6.F	5´-TCG TTG GGC AGG GCG CTG TG-3´	HPV33 E6/E7
MS3.F	5´-AAT ATA TGT GTG CTT ATT TG-3´	β-globin[1]
Beta-globin1170.F	5´-GTA CAC ATA TTG ACC AAA TCA GGG TAA-3´	β-globin[1]

Reverse (5´ Biotinylated)

Bio-GP6+	5´-GAA AAA TAA ACT GTA AAT CAT ATT C-3´	L1
Bio-GP6+-b	5´-GAA AAA TAA ATT GTA AAT CAT ACT C-3´	L1
Bio-GP6+-c	5´-GAA AAA TAA ATT GCA ATT CAT ATT C-3´	L1
HPV16E6-3.R	5´- GCT GGG TTT CTC TAC GTG TTC -3´	HPV16 E6
HPV33E6.R	5´- CTC GTG TCC TCT CAT GGC GTT-3´	HPV 33 E6/E7
Bio-MS10.R	5´-AGA TTA GGG AAA GTA TTA GA-3´	β-globin[1]
Beta-globin1293.R	5´-GCC CTG AAA GAA AGA GAT TAG GGA AAG-3´	β-globin[1]

[1]In earlier studies MS3.F and Bio-MS10.R were used. These were later replaced by beta-globin1170.F and beta-globin1293.R with a higher annealing temperature and giving a shorter amplicon.

PCR amplification

The amplification was performed in a T100 PCR BioRad (Sweden) PCR thermal cycler. 15 min of denaturation was followed by 40 cycles of amplification. Each of the 40 amplification cycles consisted of 20 sec of denaturation at 94^{o} C, 90 sec annealing at 38^{o} C, followed by 80 sec of elongation at 71^{o} C. In the last of the 40 steps, the elongation step is extended to 5.3 min at 71^{o} C. The denaturation step is required for opening the DNA strands up for binding and for separating the template from newly synthesized amplicons, while during the annealing step the primers binds to the DNA.

Analysis of PCR products on a Magpix instrument

Bead mix:

After amplification, 5 µl of PCR products was mixed with 2 µl of beadmix, containing 29 different bead types, each type containing a specific L1 probe for one of 27 different HPV types, HPV16 E6 or beta-globin, as presented in Table 1. For more details see Appendix 1. The PCR product and bead mix were loaded into a 96-well plate, covered with adhesive tin foil and incubated on the thermal cycler for 10 minutes at 95^{0} C for denaturation of the sample DNA. After 1 min incubation on ice, the plate was placed in a thermomixer for 30 min at 41^{0} C, 500 rpm, in order to hybridize the amplicons to probes. Samples are then mixed and transferred to a filter plate where they are washed by vacuum-filtration and samples then vacuum-filtrated an additional time with 100 µl wash buffer. To visualize the amplicons, 70 µl conjugate, streptavidin R phycoerythin (Eugene OR, USA), diluted 1:300, was added to each well, followed by incubation at room temperature (RT) for 30 min on a thermomixer. This fluorescent conjugate binds to the biotin from the primers incorporated in the amplicons. Samples were then washed 3 times with 100 µl wash buffer, resuspended in 100 µl wash buffer and transferred to a new 96-well plate, and inserted into the Magpix instrument for analysis.

Statistics

Due to comparisons often having n > 5 for different subgroups, Fishers Exact test was used to compare group differences in HPV prevalence, and confidence level was set at 95%. The statistical program R, version 3.2.0[32] was used for statistical calculations. R language function "fisher.test" was used with default parameters.

RESULTS

Study population included during 2013

During the study period from October to December in 2013 samples were collected from 171 individuals aged 15-23 years, 117 young women aged 15-23 years of age (of which 85 were HPV vaccinated most likely with Gardasil, but not necessarily before their sexual debut and 32 were not vaccinated against HPV) and 54 young men aged 16-22 years of age, all non-HPV vaccinated.

In this report, the first sample was collected 2013-10-04, and the last sample collected before 2013-12-31. The study was initiated with my project in October 2013, however sample collection continued and was completed in the spring of 2015.

More specifically, during the period of this study 171 mouthwash samples were donated from 54 young men (all non-vaccinated) and from 117 young women, of which 85 women were HPV vaccinated and 32 women were not HPV vaccinated.

In addition, and 47 of the young men donated a urine sample, and 93 of the young women donated a cervical sample, and of these 93 women 67 were HPV vaccinated and 26 were not HPV vaccinated.

Cervical HPV prevalence in 2013 in vaccinated and non-vaccinated individuals

HPV positive cervical samples were found in 63% (59/93) of the women in the 2013 cohort. Separating the vaccinated and unvaccinated women. Totally, 60 % (40/67) of HPV vaccinated women and 73% (19/26) of non-HPV vaccinated women had overall HPV positive samples. There were thus no significant differences in overall HPV cervical prevalence between vaccinated and non-vaccinated women in 2013 ($p=0.34$).

Simultaneous infection with several HPV types was frequently observed. Of the women with an HPV positive cervical sample, 68% (40/59) tested positive for more than one HPV type, and more specifically 65% (26/40) in the HPV vaccinated group and 74% (14/19) in the non-HPV vaccinated group, again with no significant differences between the groups ($p=0.56$).

For details of the different the 27 examined HPV types see Fig 1. In total, all the 27 HPV types in the assay were detected, and of these 17, were as indicated in the figure HR and/or putative HR types (Fig 1). For differences between specific HPV types, see below in the next paragraph and Fig. 2.

Fig.1 shows that the most frequently found LR types were HPV42, 66, 53, 30 and 6 and that the most common frequently found HR types were HPV 51, 59, 56, 39, 52, 73 and 82.

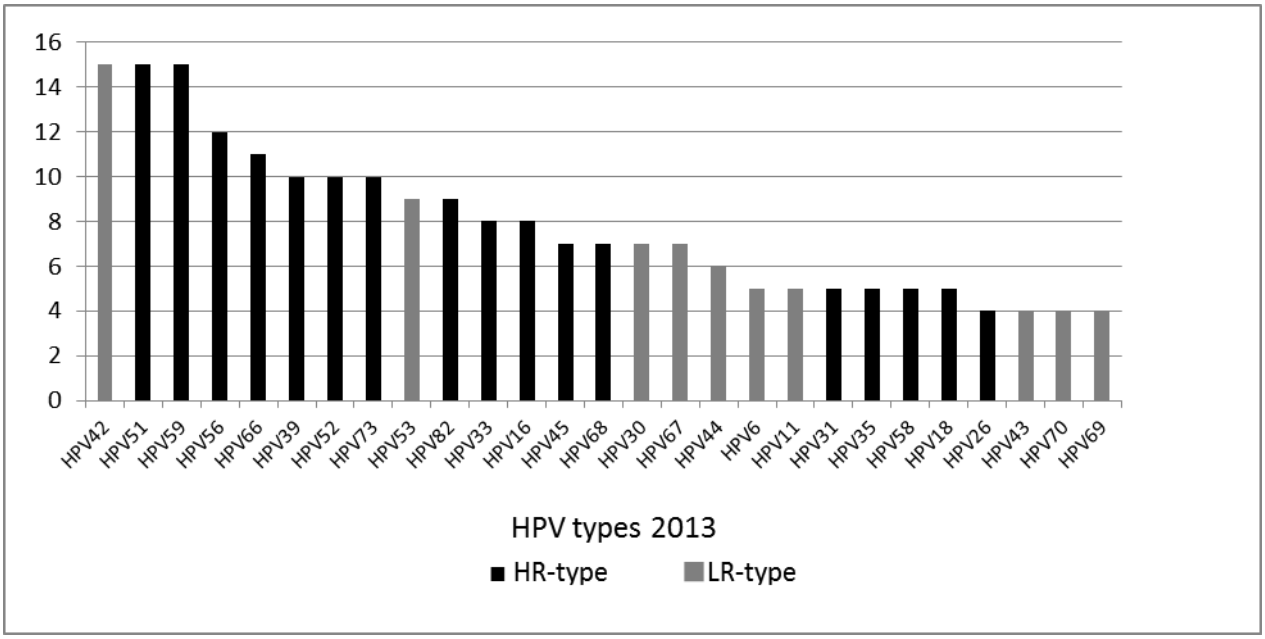

Fig. 1. Numbers of samples positive for different HPV types in the cervix of young women. The x-axis represents specific HPV types, while the y-axis represents the number of cases. In black high-risk/putative high-risk types (HR) and in grey low risk types (LR) are depicted.

Cervical HPV prevalence and vaccine HPV type prevalence in vaccinated and unvaccinated youth in 2013 compared to in unvaccinated youth 2008-2010

The was no significant difference in the proportion of HPV positive samples in the unvaccinated women cohort 2013, 73% (19/26) and the 2008-2010 cohort including 70% (387/544) (p=1).[13] There was also no significant difference in cervical overall HPV prevalence in the 2013 vaccinated group 60% (40/67) compared to that in the unvaccinated group 70% (381/544) of 2008-2010, p=0.094.

There were differences for some but not all vaccine HPV types (6, 11, 16 and 18), see below and Fig. 2.

HPV16 prevalence. In the 2013 cohort cervical HPV16 prevalence was significantly lower in vaccinated 7% (5/67) compared to 27% (7/26) in non-vaccinated women (p=0.033). Cervical HPV16 prevalence was however not significantly lower in 2013 unvaccinated individuals, 27% (7/26) as compared to 2008-2010 unvaccinated individuals 35% (190/544), (p=0.53).

HPV18 prevalence. HPV18 cervical prevalence was 1.5% (1/67) in the 2013 vaccinated group as compared to 4% (1/26) in the non-vaccinated group, p=0.48. However, there was significant drop in HPV18 prevalence between the 2013 vaccinated individuals 1.5% (1/67) compared to the 2008-2010 non-vaccinated group, 10% (44/544), p=0.021.

HPV6 prevalence. HPV6 cervical prevalence the 2013 vaccinated 1.5% (1/67) and non-vaccinated groups 8% (2/26) did not show significant differences, p=0.19. However, there was a significant drop in cervical HPV6 prevalence between 2013 vaccinated 1.5% (1/67) and the 2008-2010 unvaccinated group 8% (28/544) p=0.048.

HPV 11 prevalence. Data on HPV11 were not calculated due to too few cases.

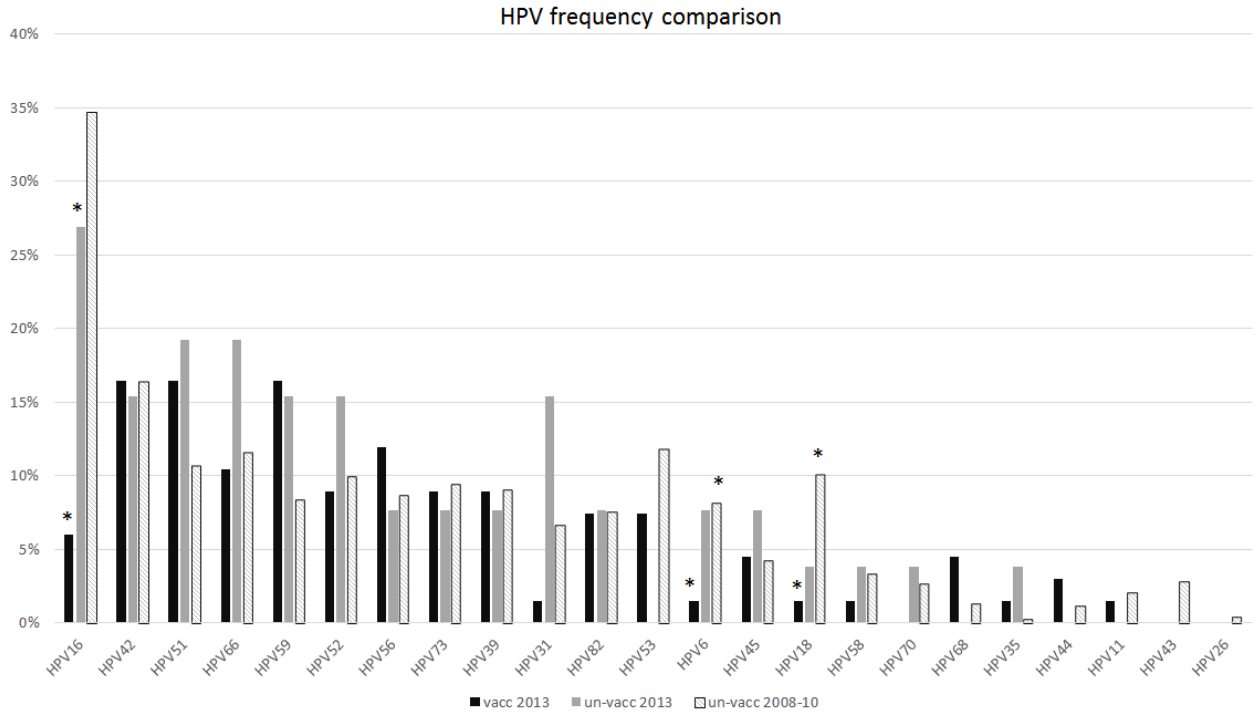

Fig.2 HPV prevalence rates indicated as percentage in the y-axis for vaccinated and non-vaccinated individuals and for different HPV-types indicated in the x-axis from 2013, as compared to the non-vaccinated cohort from 2008-2010.

Another HPV type outside the vaccine group showing a tendency to decrease was HPV31. It has been suggested that there is some cross-immunity with vaccine types, but this was not investigated in detail in this study.

Oral HPV prevalence during 2013 and in comparison to the 2009-2011 cohort

Of the oral samples collected during 2013, 2.3% (4/171) samples were HPV positive, and there was no significant difference in oral HPV prevalence between men and women (p=0.31).

However, all the HPV positive samples were obtained from young women. Three of the four young women were HPV vaccinated and one of four of the women was not HPV vaccinated.

In two cases there was an HPV type concordance between the oral and cervical sample.

There was one HPV16 positive oral sample and this was obtained from an HPV vaccinated young woman, who had an HPV33 positive cervical sample.

The second sample was positive for HPV52 and donated by an HPV vaccinated young woman, who also had an HPV18, 45, 51 and 52 positive cervical sample.

The third sample was HPV59 positive and obtained from an HPV vaccinated woman, who had a cervical sample that was HPV39 and 44 positive.

The fourth sample was positive for HPV51, and this sample was from a young woman, that was not vaccinated, but notably also HPV6, 16, 18, 31, 42, 51 and 60 positive in her cervical sample.

As the prevalence was so low, no statistically significant differences could be seen between the vaccinated and un-vaccinated groups.

Notably however, the overall HPV oral prevalence 2.5% in 2013 was significantly lower than that obtained previously in 2009-2011, with an oral HPV prevalence of 9.3% (45/483), p=0.002.

Urinary HPV prevalence in men during 2013

Urinary HPV prevalence was tested *for the first time* among the youth in this clinic.

Urine samples should not contain cells and therefore should not have cellular DNA. For similar types of viruses it has been possible to test urinary samples without extracting DNA from urinary samples.[33]

Prior to the test of urinary samples in this project, *a pilot experiment* was performed with urinary samples obtained from the Microbiology Clinic at the Karolinska University Hospital to test the method. Totally, 20 urinary samples that were used for testing of Chlamydia were obtained from young men. Among these samples, 4 were HPV16 positive (data not shown) indicating that it would be possible to test for the presence of HPV DNA in urinary samples.

At the youth clinic 2013, of the urine samples tested, 6% (3/47) were HPV positive, and all samples were from non-HPV-vaccinated young men. Only 2 HR-HPV types HPV 59 and 16 were detected in the urines from the males. More specifically, one sample was positive for HPV16, and two samples were positive for HPV 59. Thus there were very few samples detected in urinary samples of men and a comparison to those found in the cervix of young women was not done.

DISCUSSION

In this study, cervical and oral HPV prevalence was analyzed in HPV16, 18, 6 and 11 vaccinated and non-vaccinated young women and non-vaccinated men at a youth clinic in Stockholm and compared to base-line pre-vaccination data from two studies conducted 2008-2011 at the same youth clinic.[13,14] Furthermore, HPV prevalence in the urine of young men was also explored.

Shortly, the data showed that overall HPV cervical prevalence was similar in vaccinated and unvaccinated women in 2013 compared to that in unvaccinated women 2008-2011 and ranged between 60-74%. Notably, however, HPV16 cervical prevalence was significantly lower in the 2013 HPV vaccinated women compared to the unvaccinated women (7%) and (27%) respectively (p=0.033). Moreover, while cervical HPV18, 6 and 11 prevalence was not significantly different between vaccinated and unvaccinated women in the 2013 cohort, there was a statistically significant lower prevalence of HPV18 and 6 in the 2013 vaccinated group compared to the 2008-2011 unvaccinated group. Finally, oral HPV prevalence was significantly lower in 2013 (2.3%) compared to oral prevalence between 2009-2011 (9.1%). Male urinary prevalence was low with only 6% positive samples. Taken together the data suggest that the gradually initiated catch-up HPV vaccination program may be having an affect, but below specific points will be discussed in more detail.

The collecting of the samples during this project period (although the study in the end continued for 1.5 years), ranged for only three months and this could be suggested as an issue. However the sample sizes were not unreasonable for this type of study. The smallest subgroup, cervical samples for non-vaccinated women, was still above twenty-five in total. In addition, the base line studied contained a larger number of samples and had a very high HPV prevalence of different HPV types, so it was still possible to compare possible changes over time for some of the HPV types included in the vaccine. An issue with HPV epidemiological studies can be a low prevalence of HPV positive samples in the study in general, giving poor statistical power. [32] However for the two most important types HPV16 and 18, the HR-types affected by the vaccine, the larger comparison dataset is of great use. It consisted of >500 samples in total, and had 190 and 44 positive for HPV types 16 and 18 respectively, being more reliable in estimating the actual prevalence of HPV types.[13,14]

Using Biswas 2013 method for sample size, for HPV16 (with an HPV prevalence of 27% among HPV unvaccinated and 7% in HPV vaccinated individuals, only 90 samples would be needed (calculations not shown).[34] The significance of the data for HPV16 are thus reliable since the sample size of cervical samples was >90. For the other HPV types, with smaller differences with >500 samples required for a reliable result, the data are less reliable.

Notably, also of importance the 2008-2010 study also used the same extraction methods, lending more credibility as a comparison set to the 2013 study. There was nevertheless one difference in between the studies, in that in the 2008-2011, 24 HPV types were tested, for while in this study testing was performed for 27 HPV types, thus including HPV30, 67 and 69 with limited evidence or inadequate evidence of these three being HR risk types.

During the base line study in 2008-2010, the most common HR-HPV types were HPV16, 51, 18, 52, 73, 39, 56, and 59. In this study HPV 51, 59, 56, 39, 73 and 82 were still commonly reported, while HPV16 and 18 were not among the most common HR-types. The fact that HPV 51, 56, 59, 39 are commonly reported is in line with other pre-vaccination studies.[35-38] Typing for HPV73 and 82 is new and not usually performed in other studies and therefore comparisons could not be performed.

Proving differences in HPV prevalence **within** the 2013 cohort was somewhat more difficult due to these smaller sub-group sizes, 67 vaccinated and 26 unvaccinated women. For HPV16 with 5 and 7 HPV positive samples in vaccinated and unvaccinated groups respectively, the statistics were likely still reliable. For HPV18, on the other hand, with only one positive in either group, and for HPV6 and 11 as well more material would have been desired. This is likely due to the lower prevalence of these three HPV types in the 2008-2011 cohort as well as in the 2013 vaccinated and unvaccinated cohorts. Nevertheless, it was possible to compare HPV18 and HPV6 prevalence over time. Both HPV18 and HPV6 cervical prevalence were significantly lower in the vaccinated 2013 cohort females compared to that in the non-vaccinated 2008-2010 female cohort. This study suggested a trustworthy indication that HPV16, 18 and most likely also HPV6 had decreased in cervical prevalence in the HPV catch-up vaccinated group 2013 as compared to the unvaccinated group from 2008-2010.

In two later studies, in continuation to this one, the first that went on until 2014[39], and the last completed in 2015[40] (including 335 women and 122 men), it was shown that HPV16, and 6 had decreased in samples of catch-up HPV vaccinated as compared to un-vaccinated women within the same 2013-2015 cohort.[40] However, it was more difficult to show significant differences for HPV18 and 11 since the numbers of HPV-positive samples were much lower.[39,40] Nonetheless, there was a tendency for the vaccine HPV types HPV16, 18, 6 and to decrease when compared to the 2008-2010 cohort, but the calculation was only done for HPV16 and HPV31 (a subtype that is similar to HPV 16 and where HPV vaccination cross-protection has been suggested).[40]

An issue in this context that comes up is why HPV vaccination does not eliminate the different HPV types included in the vaccines. However, it is important to recall that so far the youth that attend the youth clinic have all been catch-up HPV vaccinated and many likely after their sexual debut and have not been vaccinated in the school-based program.

In this project there was no information on when the youth had had their sexual debut, but we assume that there are similarities in sexual behavior among youth in Sweden and in another study information on both year of HPV vaccination and year of sexual debut were obtained.[41]

In that study, in 2013, of third year high-school students, where 64% of the women were vaccinated most women were vaccinated at the age of 16 years, while their sex debut occurred at the mean age of 15 years.[41] This suggested that there was a risk of acquiring an HPV infection of the HPV-types included in the vaccines during the period they were yet not vaccinated. In that study only oral HPV prevalence was studied and there are no data on cervical HPV prevalence.[41] Therefore its not surprising that catch-upp vaccinated women in this study, could still have vaccine-type HPV infections.

The data obtained at youth clinic in Stockholm at least with regard to HPV vaccination, most likely reflect a similar situation as in the high school situation, at least with regard to HPV vaccination, where many women are catch-up HPV vaccinated but not necessarily before their sexual debut. As time progresses, and young girls HPV vaccinated within the school vaccination program attend the same youth clinic, most likely there will be a steep decrease of the HPV types included in the school-based HPV vaccine.

Oral HPV prevalence was lower than cervical HPV prevalence. This was not entirely unexpected.[14] The reason for this is that in the oral cavity 0.5-15 liters of saliva is produced daily and this may therefore dilute the amount of virus that is available for analysis.[14] However, also in that study it was shown that HPV16 was a very dominant HPV type in the oral cavity and can explain why oral HPV-prevalence decreased after HPV vaccination.[14]

Oral prevalence being low was therefore not subdivided between vaccinated and unvaccinated groups in the 2013 cohort. There was nevertheless a significant decrease in oral HPV prevalence in the 2013 cohort compared to the 2009-2011 cohort (2.3% and 9.1% respectively).[14] This was not completely unexpected either, since in a school based investigation of 3rd year high grade students, where around two thirds of the young women were HPV vaccinated (not necessarily before their sexual debut) oral HPV prevalence was 1.8%.[42] At the time it was speculated that the youth at school were a different type of cohort than those at the youth clinic and therefore had lower oral HPV prevalence, but with this study it was interesting to disclose that oral HPV prevalence was similar in the two groups of youth.[14,42]

Again, in the continued study ranging from 2013-2015 at the same youth clinic and including 457 oral samples oral HPV prevalence had dropped to 1.5% indicating indeed a decrease in overall HPV prevalence after the initiation of HPV vaccination.[39,40]

Oral HPV-prevalence in our samples was also similar to that observed in an Australian study conducted on 307 Australian university students (18-35 years), where 32% of the students had been vaccinated with at least one dose of Gardasil.[43] Here, participants reported anonymously about life style including, sexual behavior. Seven of 307 (2.3%) students tested positive for oral HPV infection, with the majority being males.[43]

In another type of cohort in Stockholm, Sweden among mainly HPV non-vaccinated patients aged 3-56 years of age undergoing tonsillectomy, oral HPV prevalence was 10.3% and showed dominance of HPV69 and the reason for this is not known.[44] At the youth clinic oral HPV prevalence was lower 2013, whether or not this is due to HPV vaccination, is really unknown, and the age of the individuals in the two studies differ.

Compared to the previous studies of 2008-2010[13] and 2009-2011[14], a novel research-question was HPV prevalence in urine samples of the male youth at the same clinic. First of all, the possibility to test urinary samples was tested and shown possible, however these samples were obtained from a group of samples where there was no background information of the patients. From samples obtained from the youth clinic, only three samples were HPV positive.

When this project was initiated, an approximate HPV-prevalence of almost 20% had been hoped for in order to make comparisons with HPV types in the cervix of women. This was clearly too optimistic. The presently low HPV prevalence in male urines was however not completely unexpected. Other studies have indicated that there are problems with sensitivity of the sample extraction method, and have had issues detecting HPV from urethral swabs and urine.[45] Other studies on urine samples in men have been able to detect HPV-DNA reliably with PCR amplification, so it is difficult to draw conclusions on the actual prevalence in the male cohort.[46,47] Furthermore, studies in male urine samples were not performed during the 2008-2011 study, so there are no data for comparison. [13,14]

A study in 1993, Forslund *et al.*, investigated 138 healthy military conscripts, and found 8% urethral positive samples, and 5% positive urine samples in Southern Sweden.[47] These are comparable to what was found in the 2013 cohort. However, when applying the urethral and urine method on 11 men visiting a youth-STD clinic, 7 patients had HPV positive urethral samples, and 5 of these also had HPV-positive urine samples.[48] Therefore both the cohort, the sampling method and the method of analysis may play important roles in the number of HPV-positive cases obtained in urinary samples of men. Because the samples used here were self-taken by the patient, ensuring proper and consistent genital scraping by male patients would have been to time-consuming and complicated for the youth-centre personnel.

To conclude, in this study there was a significant decrease of HPV16 between the vaccinated and the non-vaccinated cohorts of 2013, and a decrease in HPV18 and 6 between the vaccinated cohort of 2013 and the non-vaccinated cohort of 2008-2011, indicating that HPV catch-up vaccination has an effect. That oral HPV prevalence has decreased also in men suggests possible herd immunity. Urine HPV prevalence in men was low and not useful for studying prevalence of different HPV types in this cohort. Future studies on larger cohorts for change in HPV18, 6 and 11 prevalence and investigations on possible changes in male HPV prevalence could be of interest. Nonetheless, this study gives good support for the HPV catch-up vaccination program, and proves that at least HPV16 prevalence is going down.

ETHICAL ASPECTS AND IMPACT OF THE RESEARCH ON SOCIETY

This study was performed conferring to the obtained ethical permissions 2012/1756-31/2 and 2013/1427-3 from the regional ethical committee in Stockholm, at Karolinska Institutet.

The research that was conducted was not associated with any risk or physical pain for the participants, since taking cervical, oral and urine samples is pain-free and there is no risk for any side effects.

There were however other ethical issues.

For the youth: At the youth clinic, the youth may be stressed due to personal concerns and many are <18 years of age. They are there for birth control, or examined for sexually transmitted diseases (STDs). This means that one has to be considerate with the young individuals during the study.

The ethical committee accepted that the study could be performed without contacting the parents of the participants <18 years of age, since in many cases these individuals were visiting the youth clinic without the knowledge of their parents and the issues they wanted to discuss were sensitive. For the sensitivity issue, the study was performed anonymously and therefore no feedback could be given to the participants whether their samples were HR HPV positive or not. Importantly, however, having an HPV positive cervical infection is extremely usual between the ages of 15-30 years of age and most infections are cleared spontaneously within 2 years. HR-HPV is therefore not used for screening for 23-29 year olds for pre-stages of cervical cancer, but instead cytology is used. We concluded, therefore that the young women should not be followed up for their HPV infections within this study, but participate in the regular screening programs instead.

Finally, as always in a scientific study, all participation is voluntary and the visitors have the right to not participate and have the full right to obtain full treatment irrespective of whether they participate or not as also mentioned in the informed consent form.

For society: This study is of benefit for society in my opinion HPV vaccination will help lessen a substantial cause of human suffering. Here, we have followed the effects of HPV catch-up vaccination and discovered relatively modest means of prevention of HPV infection after catch-up vaccination and if this vaccine can prevent 500 000 cervical cancers per year globally it is of great use.

Nevertheless, there is concern in more sexually restricted societies against the vaccine since it could promote sexual activity, or that there are serious side effects. So far none of this has been shown.

FUTURE PERSPECTIVES

As mentioned above, this project was the initiation of a study in 2013 that lasted until 2015, so it already had some future perspectives. It followed youth a youth clinic, with women that had been HPV catch-up vaccinated, as well as women and men that had not been HPV vaccinated at all. Despite this it was possible to follow a significant decrease in HPV type 16 in cervical samples.

A very interesting project would be to continue and follow up cervical and oral prevalence at the same youth clinic, when young women attending the youth clinic have been vaccinated through the school vaccination program.

This would suggest that since the school based HPV vaccination program started in 2012 with 10-12 year old girls, it would take at least 3 years before these young girls at the age of 15 years of age would start attending the youth clinic.

This would mean that one could start a new study in 2017 and continue for at least three to five years. During this time period it could be possible that the vaccine HPV types HPV16, 18, 6 and 11 may almost disappear. However, then one must be sure that young girls continue to want to be vaccinated.

Theoretically, it would also be interesting to conduct a life style investigation, similar to that conducted at a high school setting, with a questionnaire regarding life style and sexual habits, but unfortunately, this would need extra personnel that are qualified for this type of work. Moreover, it could be difficult to conduct such a study in a setting a youth clinic, where the youth most likely are rather stressed.

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APPENDIX 1

A method used for detection of HPV DNA – the Magpix Luminex System – a theoretical background

To detect HPV in this study a bead based multiplex system was used and here a short theoretical background to the system is presented. The Magpix from Luminex Inc is an instrument for the analysis of up to 50 different molecules by bead-based multiplexing. The basic components of this system is an array of magnetic and flourescent beads, each with a unique colour and with different probes, for further details see the texts in Fig. 1-3:

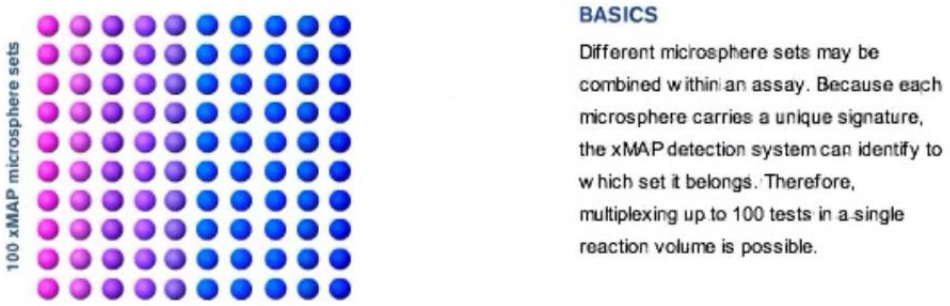

Fig 1. Magnetic beads with different colour identities. This separates samples from each other.

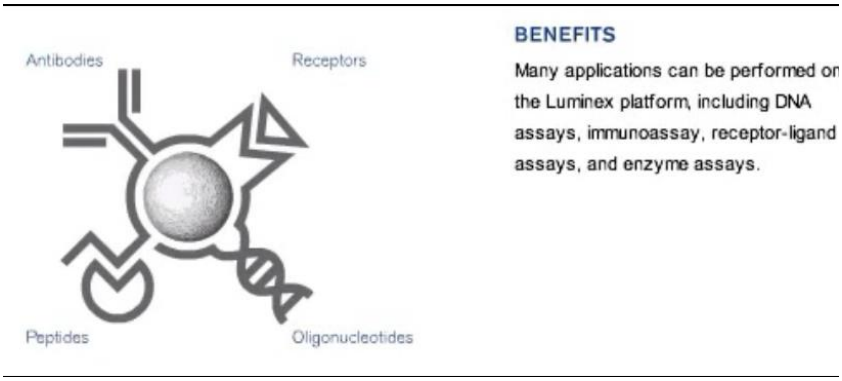

Fig. 2. Magnetic beads with specific probes binding to the molecules of interest in a sample, as well as a flourescent marker binding if molecule of interest is present.

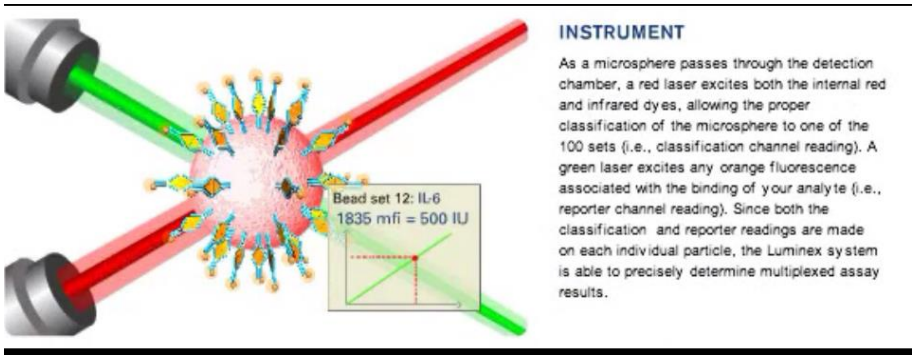

Fig. 3. After washing away of unbound beads

The basic principle behind an assay on a MagPix system is as follows:

Steps before the analysis:

1. Probes specific for a molecule of interest are coupled to the specific magnetic beads, covered in avidin.The molecule of interest can be a strand of DNA, RNA or a protein.

Preparation of samples for analysis on the MagPix system

2. The samples are incubated with the beads and the probes bind to the matching molecules of interest if these are present in the samples. When amplifying genetic material through PCR, this can be accomplished through incorporation of biotin into one of the primers. Biotin has little chemical effect, but binds strongly to avidin, the substance covering the beads.
   The biotin also enables the binding of a flourecent marker at a later stage in the analysis.
3. After washing away non-bound material from the beads, the detection molecule, flourescent streptavidin, binds to the biotin

Analysis of the samples on the MagPix system

4. The samples are inserted in the MagPix on a 96-well plate

5. The beads in each sample are attached to a magnetic plate.

6. The beads with the sample-biotin-detection molecule complex attached is luminated by a red light.The Bead give of a specific light depending on the bead type. Thus the instrument will know the identity of the bead. At least 50 beads of each included type are usually analyzed.

7. The bead is then luminated with a green light, that makes the detection molecule give of a green light, confirming if the bead-probe complex came into contact with their corresponding molecule while in the sample mix. The strength of the signal is related to the number of molecules attached.

8. As the lights are luminating the beads, a camera takes images of the well-plate.
   The beads are ID:d by their color, and the bioitin response shows the amount of expression for the target molecule.

The Luminex system has been used for a variety of applications; e.g. analysis of DNA, RNA, microRNA, proteins and antibodies from a number of organism e.g. humans, bacteria, viruses as well as different type of materials eg. tissue, blood and urine.

Benefits of analysis using suspension array compared to conventional PCR gels:

The benefits of a system for bead-based multiplexing compared with conventional PCR-gel techniques are more than tenfold. In a typical Agarose-Gel-PCR an amount of about 100 ng of DNA is used. In a PCR amplification for a Luminex evaluation only about 5 ng of DNA is required. For PCR amplification a new gel is required for each subtype of HPV, this is time-consuming, and the gel-making is in itself a process that can go wrong if agarose concentrations are off, heat wasn't optimal, bubbles were formed in the product or other misshaps. Also the time required for the analysis short. One 96-well plate is analysed in approximately 1 hour. When PCR products are analysed the PCR can be set up in the morning followed by analysis on the MagPix instrument in the afternoon. Thus one (or more) 96-well plate with samples can be analysed in one day.

Magpix Luminex:

The bead is a luminating device that gives a way of differentiating between different DNA types in a sample mechanically by way of light given off when exposed to a light source. The way this works is by attaching probes binding to different protein or DNA types, and to a specific variant of bead. The beads are then exposed to PCR products that bind to the probes. An additional "detection" molecule is also added, and binds to all of the DNA types. Before the beads are exposed to a light source, they are magnetically removed from the fluid PCR-product-bead-mix. As the beads are exposed to light, they shine, and the different light spectras are detected by a camera and recorded, showing that each type of bead is available for processing. The Magpix machine also has a separate light detecting the probes on the beads, which in turn confirms which types of DNA were available in the PCR products, e.g. which beads are "positive".