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RESEARCH

Aline Simen-Kapeu

Epidemiological Study of Tobacco Use and Human Papillomavirus

Implications for Public Health Prevention

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Epidemiological Study of Tobacco Use and Human Papillomavirus

Implications for Public Health Prevention

Academic dissertation

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To my beloved family – and my wonderful husband and daughters, Abida Danielle Kapeu and Olive Lanpeerbas Kapeu

"*The fear of the Lord is the beginning of wisdom.......................*" *(Proverbs 9:10).*

Abstract

Aline Simen-Kapeu. Epidemiological Study of Tobacco Use and Human Papillomavirus – Implications for Public Health Prevention. National Institute for Health and Welfare (THL), Research 20. 150 pages. Helsinki 2009.
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Cervical cancer (CC) is one of the most common forms of cancer in women. The sexually transmitted oncogenic human papillomavirus (HPV) types are the necessary etiological agents of CC. However, only a small fraction of HPV-infected women go on to develop cancer. Other avoidable co-factors that act in conjunction with HPV to promote cervical malignant lesions need to be verified and tackled. Tobacco exposure, a potential environmental cofactor of CC, has attracted increasing attention since the early 1980s.

My aim in this thesis was to assess the role of tobacco exposure in cervical precancerous lesions and cancer of the uterine cervix as well as in multiple HPV infections, and to evaluate the impact of tobacco smoking on the immune response to natural HPV infection as well as to HPV vaccination.

A population-based case-control study of CC was nested within a joint cohort of five Nordic serum banks from Finland, Iceland, Norway and Sweden. The samples of cases and controls were analyzed for cotinine (a biomarker of tobacco smoking) and antibodies to HPV types 16 and 18, herpes simplex virus type 2 (HSV-2), and *Chlamydia trachomatis* (C. *trachomatis*). Due to small sample size, the first study material (171 cases and 496 controls) (Paper I) had limited power to distinguish whether tobacco smoking was an independent cofactor in cervical carcinogenesis or whether its relative role was due to residual confounding by the oncogenic HPVs. In the second study material (Paper I), we assembled almost four times bigger independent material, including 588 cases and 2,861 controls. We identified smoking as an independent risk factor for CC. A highly significant 2- to 3-fold increased risk of invasive CC (Odds ratio (OR) = 2.1; 95% confidence interval (CI): 1.4–3.2) and squamous cell carcinoma (SCC) (OR = 2.7; 95% CI: 1.7–4.3), free of residual confounding bias, was found among HPV16/18-seropositive heavy smokers. In addition, the point estimates increased with increasing age at diagnosis (probably indicating longer exposure) and increasing cotinine level.

In a cross-sectional analysis (Paper II), we compared the association between tobacco smoking and chewing and the risk of multiple HPV infections and cervical squamous intraepithelial lesions (SILs) in two populations with different routes of tobacco exposure. We studied 2,162 women from Côte d'Ivoire, West Africa, and 419 women from Finland, Northern Europe, with baseline data on cervical screening, smoking and chewing habits, HPV DNA status, C. trachomatis status and human immunodeficiency virus (HIV) seropositivity. In both settings, tobacco

consumers (chewers or smokers) ≥30 years of age tended to have an increased risk of low-grade SIL (LSIL). Among tobacco chewers (Côte d'Ivoire), the risk of high grade SIL (HSIL) was five times higher in both young (OR = 5.5, 95% CI: 1.2–26) and older (OR = 5.5, 95% CI: 2.1–14) women compared to non-chewers. We found an increased, albeit not significant, risk of both LSIL and HSIL, in HPV-DNA positive women ≥30 years of age and actively exposed to tobacco through smoking or chewing. There was no increased risk of multiple HPV infections among tobacco consumers.

In a cohort study (Paper III), we evaluated the association between humoral immune response to HPV and smoking in 191 HPV infected women prospectively followed-up for 10 years by cytology and HPV DNA analyses. The baseline sample and the last follow-up sample were analysed for serum cotinine levels, Immunoglobulin (Ig) A and IgG antibodies to HPV16 and 18, and *C. trachomatis* using ELISA methods. Young women (<30 years of age) who smoked were less likely to either seroconvert or maintain detectable HPV16/18 antibodies over the follow-up time (up to 10 years) than non-smokers. This suggests that among young women with oncogenic HPV16/18 infections, smoking impairs the humoral immune response to high-risk HPV types.

A phase III double-blind, randomized controlled trial enrolled 4,808 16- to 17-year-old females in Finland to receive either the prophylactic HPV16/18 AS04-adjuvanted vaccine-like-particle (VLP) vaccine or hepatitis A vaccine (Havrix^{TM}) as a control (Paper IV). We conducted a pilot study among 216 participants to compare HPV16/18 antibody levels of nonsmokers and smokers 7 months post-vaccination (one month post the third vaccination dose). Baseline and month 7 serum samples were analysed for cotinine levels and IgG antibodies to HPV16 and 18. We found that women who smoked appeared to have comparable levels of anti-HPV16 and 18 antibodies to nonsmokers at month 7 post-vaccination. Our data suggest that smoking may not have an impact on the humoral antibody response following HPV vaccination.

Alongside the development and combination of HPV vaccination programmes and screening and early diagnosis of CC, our findings support public health initiatives intended to prevent tobacco smoking and chewing exposures, particularly among young women.

Keywords: human papillomavirus, seroepidemiology, tobacco smoking, tobacco chewing, immune response, fertile-aged women, HPV vaccine, cervical cancer prevention

Abstract in Finnish

Aline Simen-Kapeu. Epidemiological Study of Tobacco Use and Human Papillomavirus – Implications for Public Health Prevention [Epidemiologinen tutkimus tupakoinnista ja ihmisen papilloomaviruksesta – merkitys kansanterveystyölle]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 20. 150 sivua. Helsinki 2009. ISBN 978-952-245-126-2 (painettu), ISBN 978-952-245-127-9 (pdf)

Kohdunkaulan syöpä on yksi naisten yleisimmistä syöpämuodoista. Seksin yhteydessä tarttuvat syöpävaaralliset ihmisen papilloomavirukset (HPV) ovat kohdunkaulansyövän välttämättömiä syytekijöitä. Vain pieni osa HPV-infektioon sairastuneista naisista saa syövän. Muut vältettävissä olevat osatekijät, jotka saavat yhdessä HPV:n kanssa kohdunkaulansyövän esiasteet etenemään on identifioitava ja voitettava. Tupakointi, mahdollinen ympäristöperäinen kohdunkaulansyövän osatekijä, on herättänyt kasvavaa mielenkiintoa 1980-luvulta lähtien. Tarkoitukseni oli tässä työssä määrittää tupakoinnin rooli kohdunkaulansyövässä ja sen esiasteissa sekä HPV-infektioissa, ja arvioida tupakoinnin vaikutusta luonnollisen HPV-infektion ja toisaalta rokotuksen seurauksena syntyneelle immuunivasteelle.

Väestöpohjainen upotettu tapaus-verrokkitutkimus hyödynsi viittä pohjoismaista (Islanti, Norja, Ruotsi, Suomi) seerumipankkikohorttia. Tapausten ja verrokkien näytteistä tutkittiin kotiniini (tupakoinnin biomarkkeri) ja HPV, herpes simplex virus ja klamydia vasta-aineet. Pienen otoskoon vuoksi ensimmäisen tutkimusmateriaalin mahdollisuudet erottaa onko tupakointi kohdunkaulan syövän kehittymisen itsenäinen riskitekijä vai selittyykö osuus syöpävaarallisten HPV:n jäännössekoitusvaikutuksella (osajulkaisu I) ei ollut mahdollinen. Toiseen tutkimusmateriaaliin (osajulkaisu I) keräsimme neljä kertaa suuremman, erillisen, materiaalin, jossa oli 588 tapausta ja 2861 verrokkia. Osoitimme tupakoinnin olevan kohdunkaulansyövän itsenäinen riskitekijä. Löysimme erittäin merkitsevän 2–3-kertaisen kohdunkaulansyöpäriskin (vaarasuhde, OR = 2.1; 95 % luottamusväli (CI): 1.4–3.2) ja levyepiteelisyöpäriskin (OR = 2.7; 95% CI: 1.7–4.3) HPV16/18 vasta-ainepositiivisten aktiivisten tupakoitsijoiden joukossa, joka oli vapaa jäännössekoitus-vaikutuksesta. Lisäksi piste-estimaatit olivat sitä suurempia mitä korkeampi tutkittavien ikä oli diagnoosihetkellä (so. mitä pitempi altistuminen) ja mitä korkeampi heidän kotiniinitasonsa.

Tekemässämme poikkileikkaustutkimuksessa (osajulkaisu II) vertasimme tupakoitsijoiden ja purutupakan käyttäjien useiden HPV infektioiden ja kohdunkaulansyövän esiasteiden (SIL) riskiä kahdessa populaatiossa. Tutkimuksessa oli 2 162 naista Norsunluurannikolta Länsi-Afrikasta ja 419 naista Suomesta, Pohjoi-Euroopasta, joista oli käytettävissä tiedot kohdunkaulansyövän seulontatutkimuksesta, tupakoinnista ja purutupakan käytöstä, kohdunkaulan HPV DNA löydöksistä, klamydia ja HIV vasta-ainetiedot. Molemmissa tutkittavien ryhmissä tupakka-altistus (purutupakan käyttäjillä ja tupakoitsijoilla) liittyi kohonneeseen matala-asteisen

SIL-muutoksen riskiin sekä nuoremmilla (OR = 5.5, 95% CI: 1.2–26) että vanhemmilla (OR = 5.5, 95% CI: 2.1–14) tutkittavilla. Löysimme, ei-tilastollisesti merkitsevästi, kohonneen matala- ja korkea-asteisten SIL-muutosten riskin yli 30-vuotiailla tupakka-altistuneilla (purutupakan käyttäjät tai tupakoitsijat) tutkittavilla. Heidän useiden HPV-infektioiden riskinsä ei ollut kohonnut.

Kohorttitutkimuksessa (osajulkaisu III) arvioimme tupakoinnin liittymistä kiertäviin HPV vasta-aineisiin 191 HPV-infektioon sairastuneella naisella, joita seurattiin aina 10 vuotta kohdunkaulansyövän seulontanäytteillä joista määritettiin myös HPV-DNA.Tutkimuksen alku- ja loppuhetkillä otetut näytteet tutkittiin kotiniini, ja HPV16 ja HPV18 immunoglobuliini (Ig) G ja IgA vasta-aineiden suhteen, myös klamydia vasta-aineet määritettiin ELISA-menetelmällä. Nuoret tupakoitsevat naiset serokonvertoivat HPV16/18 viruksille tai säilyttivät nämä vasta-aineensa 10 seurantavuoden ajan vähemmän todennäköisesti kuin tupakoimattomat. Tämä viittaa siihen, että nuorilla naisilla, joilla on syöpävaarallisten HPV16/18 virusten aiheuttama infektio, tupakointi huonontaa vasta-aineresponssia korkean riskin HPV-virustyypeille.

Kaksoissokkoutettuun, satunnaistettuun faasi III tutkimukseen osallistui Suomessa 4808 16–17-vuotiasta naista, ja he saivat joko HPV16/18 AS04-adjuvantoitua viruksen kaltaisista partikkeleista (VLP) koostuvaa rokotetta tai kontrollina hepatiitti A rokotetta (HavrixTM) (osajulkaisu IV). Teimme pilottitutkimuksen 216 tutkittavan joukossa vertaamalla HPV16/18 vasta-aineita tupakoimattomilla ja tupakoitsijoilla 7 kuukautta rokotuksen aloittamisesta (kuukausi kolmannen rokotuskerran jälkeen). Alkuhetken ja 7 kuukauden seeruminäytteistä analysoitiin kotiniini ja HPV16 ja HPV18 IgG vasta-aineet. Naisilla, jotka tupakoivat ja jotka eivät tupakoineet HPV16 ja HPV18 vasta-aineet olivat samalla tasolla 7 kuukautta rokotuksen jälkeen. Tuloksemme viittaa siihen, että tupakoinnilla ei ole vaikutusta HPV rokotuksen seurauksena syntyvään vasta-aineresponssiin.

Tulostemme mukaan HPV-rokotus ja seulontaohjelmia, ja kohdunkaulansyövän varhaista diagnostiikkaa kehitettäessä myös kansanterveystyö tupakoinnin ja purutupakan käytön vähentämiseksi olisi perusteltua, erityisesti nuorilla naisilla.

Avainsanat: ihmisen papilloomavirus, seroepidemiologia, tupakointi, purutupakan käyttö, immuuniresponssi, hedelmällisessä iässä olevat naiset, HPV rokote, kohdunkaulansyövän ehkäisy

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Original publications

List of abbreviations

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cells
CC	Cervical cancer
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CO	Carbonmonoxide
COHb	Carboxyhemoglobin
CRPV	Cotton-tail rabbit papillomavirus
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ETS	Environmental tobacco smoke
FMC	Finnish Maternity Cohort
GYTS	Global Youth Tobacco Survey
hrHPV	high-risk human papilloma virus
HC	Hybrid capture
HIV	human immunodeficiency virus
HPV	human papilloma virus
HSIL	high-grade squamous intraepithelial lesion
HSV-2	herpes simplex virus type 2
IARC	International Agency for Research on Cancer
IFN	Interferon
Ig	Immunoglobulin
IMC	Icelandic Maternity Cohort
ISH	In situ hybridisation
LBC	Liquid-based cytology
LSIL	low-grade squamous intraepithelial lesion
MHC	major histocompatibility complex
MONICA	Monitoring trends and determinants in cardiovascular disease
NSMC	Northern Sweden Maternity Cohort
OR	Odds ratio
ORF	Open reading frame
PAP-smear	Papaniculaou-stained cytological cervico-vaginal smear
PATRICIA	Papilloma trial against cancer in young adults
PCR	Polymerase chain reaction
PIN	Personal identification number
RCT	Randomized control trial
RNA	Ribonucleic acid
RR	Relative risk
SCC	Squamous-cell carcinoma
SHS	Second-hand smoke

SIR	Standardized incidence ratio
STP	Smokeless tobacco products
UPR	Upstream regulatory region
VIA	Visual inspection with acetetic acid
VILI	Visual inspection with Lugol´s iodine
VIP	Västerbotten Intervention Program
VLP	Virus-like particle
WHO	World Health Organization

List of original publications

This dissertation is based on the following original articles referred to in the text by their Roman numerals:

I **Simen-Kapeu A**, Luostarinen T, Jellum E, Dillner J, Hakama M, Koskela P, Lenner P, Löve A, Mahlamaki E, Thoresen S, Tryggvadóttir L, Wadell G, Youngman L, Lehtinen M. Is smoking an independent risk factor for invasive cervical cancer? A nested case-control study within Nordic Biobanks. Am J Epidemiol 2009;169:480–488.

II **Simen-Kapeu A**, La Ruche G, Kataja V, Yliskoski M, Bergeron C, Horo A, Syrjänen K, Saarikoski S, Lehtinen M, Dabis F, Sasco AJ. Tobacco smoking and chewing as risk factors for multiple human papillomavirus infections and cervical squamous intraepithelial lesions in two countries (Côte d'Ivoire and Finland) with different tobacco exposure. Cancer Causes Control 2009;20:163–170.

III **Simen-Kapeu A**, Kataja V, Yliskoski M, Syrjänen K, Dillner J, Koskel P, Paavonen J, Lehtinen M. Smoking impairs human papillomavirus (HPV) type 16 and 18 capsids antibody response following natural HPV infection. Scan J Infect Dis 2008;40:745–751.

IV **Simen-Kapeu A,** Surcel H-M, Apter D, Paavonen J, Lehtinen M. Impact of smoking on humoral Immunoglobulin G antibody response to a human papillomavirus type 16 and 18 AS04-adjuvanted virus-like particle vaccine – A pilot study. Manuscript submitted.

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1 Introduction

Cervical cancer (CC) is the second most common cancer among women worldwide. The majority (83%) of cases occur in the developing world, where in most countries, CC is the leading cause of cancer mortality among females (World Health Organization (WHO) 2003, Ferlay et al. 2004). Cervical infection with oncogenic human papillomaviruses (HPVs) is the main cause of CC (Walboomers et al. 1999). Of the numerous oncogenic high-risk (hr) HPV types, 70% of CC is attributed to HPV16 and HPV18 (Bosch et al. 2002, Lehtinen et al. 2001). However, infection with HPV cannot be a sufficient cause of CC because of the high numbers of HPV-infected women who do not develop cancer (Walboomers et al. 1999). It is likely that environmental and host-related cofactors act in conjunction with HPV to promote malignant progression of squamous intraepithelial lesions (SIL), which is the clinical manifestation of hrHPV infection.

Tobacco exposure, an environmental factor in human cancers, is of interest in cervical carcinogenesis because of: 1) the correlation of increased incidence lung cancer and CC (Korhonen et al. 1999), 2) the consistent association of smoking with cervical intraepithelial neoplasia (CIN) grade 3 and CC (Plummer et al. 2003), 3) the comparable strength of the association between tobacco smoking and chewing and CC (Rajkumar et al. 2003) 4) biologic plausibility, including the observation of nicotine-derived carcinogens in cervical mucus after smoking (Holly et al. 1993) and 5) the potential for intervention through anti-smoking campaigns (Nandakumar et al. 2005).

For development, evaluation and effective implementation of prevention strategies and therapeutic agents against hrHPV infection and CC, a better understanding of the impact of tobacco exposure on hrHPV infection, associated natural and vaccine induced immune responses, and cervical carcinogenesis is required.

2 Review of the litterature

2.1 Tobacco use

2.1.1 History and epidemiology

The history of smoking starts among the Native Americans who used it for ceremonial purposes 5000 years BC. Christopher Columbus first brought tobacco to Europe from the West Indies in 1492. From the beginning it was used for medical purposes and in history it is mentioned when the Queen of France, Catherine of Medici, was cured from stomach pains by tobacco. She got the tobacco from Jean Nicot and named it "Nicotiana". Soldiers during the great European wars spread the use of tobacco, mostly used as snuff or smoked in pipes. It was not until the Crimean War, in the middle of 19th century, that cigarettes became more common.

When the first cigarette machine was constructed in 1870, cigarette smoking flourished. This was also the start for the big tobacco companies (Goodman 1995). Cigarette smoking was from the beginning a masculine habit and spread among soldiers during World War I and II. Women began to take up smoking during and after World War II, thus putting children in closer contact to environment tobacco smoke (ETS). The era of the well-educated and career-oriented women began; smoking became a sign of independence and was seen as part of women's liberation. Cigarette smoking then spread to other groups in society. While the well-educated women are now dropping the habits because of increased health awareness, it has now become most prevalent among underprivileged women with low education attainment (Torell 2002).

Worldwide, about 50 % of males and 10 % of females are smoking, but there are great variations among nations. In Finland, 26 % of 15 to 64-year-old males and 18 % of females smoked daily in 2005 (Helakorpi et al., 2005). Smoking prevalence among 13 to15-year-olds ranges from 1 % to 40 % in different countries (Global Youth Tobacco Survey (GYTS) Collaboration Group 2002). During the last decade smoking prevalence has decreased in the Western world but increased in the developing countries (Shafey et al. 2003). In the developing countries too, men started smoking first and women followed soon thereafter. Globally, tobacco consumption as well as production is growing and women in Asia and Africa are now the main target group for the tobacco companies (Mackay and Eriksen 2002).

Though smoking prevalence in the Western world is decreasing, smoking has kept an aura of tough and smart glamour, and around 100, 000 new young smokers are recruited daily (Mackay and Eriksen 2002). In total, about 1/3 of the adult population smokes and WHO has calculated that 1000 cigarettes are manufactured per year per person, including women and children (Mackay and Eriksen 2002). A

TABLE 2.1. Prevalence of tobacco use and number of smokers by WHO region and levels of development in 2000

	Prevalence (% of the population ≥15 years of age)			No. of tobacco users (thousand)		
	Men	Women	Total	Men	Women	Total
Who region						
African Region	29.4	7.4	18.4	51,967	13,420	65,387
Region of the Americas	32.0	20.9	26.3	94,035	64,072	158,107
Eastern Mediterranean Region	35.3	6.1	21.0	52,543	8,670	61,213
European Region	44.9	18.7	31.2	150,628	68,545	219,173
South East Asia Region	48.1	5.3	27.3	251,699	26,484	278,183
Western Pacific Region	61.2	5.7	33.8	390,632	35,784	426,146
Level of development						
Developed	33.9	21.2	27.4	114,783	75,891	190,673
Developing	49.8	7.2	28.9	809,725	114,718	924,443
Transitional	54.1	13.9	32.7	82,837	24,153	106,989
World	47.5	10.3	28.9	11,005,927	217,755	1,223,682

From Guindon and Boisclair 2003.

nearly two-fold difference in smoking rates is seen in men across different WHO regions, with the lowest level in the Eastern Mediterranean Region (34.2%) and the highest in the Western Pacific Region (62.3%). Based on these weighted prevalence estimates, there are over 1.2 billion smokers across the six WHO regions, women being in the minority in the developing countries (Table 2.1).

The prevalence of smokeless tobacco use is high among women. In Mumbai, 59% of women used smokeless tobacco (Gupta 1996). The prevalence was similar to that of other South-Asian female populations. Around 49% of UK-Bangladeshi females, 35.5% of Nigeran females and 59% of rural Malesian females use smokeless tobacco (Thomas et al. 2004, Gan 1995, Croucher et al. 2002). Women appear to have a higher prevalence of the chewing habit in many countries of the South due to the belief that tobacco has many magical and medicinal properties; keeping the mouth clean, getting rid of bad smell, curing toothache, controlling morning sickness, and minimizing labour pains (Muwonge et al. 2008).

Tobacco use prevalence can be decreased by a variety of tobacco prevention and control efforts. Reporting on the adverse health effects from smoking the anti-smoking debate was accelerated in the 1980's when it was shown that passive smoking was also a health hazard. During the 1990's numerous conventions, national as well as international, addressed the smoking issue. Educational, clinical, regulatory, economic, and comprehensive approaches are widely used and studied. WHO and European Union, have made up rules and recommendations for how the

"pandemic of smoking" can be defeated. Tobacco control is highly cost-effective (World Bank 1999). Many countries have passed laws on smoke free areas, rules for cigarette commerce and public health interventions to control tobacco use.

As an example in Finland, the Tobacco Control Act was passed as early as in 1976 (Puska et al. 1997). It prohibited smoking in most public places, restricted tobacco advertising, and set a 16-year age limit for tobacco purchases. Further amendements to the Act were made in 1995, when, for example, the age limit for tobacco purchases was raised to 18 years, and in 2000, when ETS was included in the national list of carcinogenic substances. Among Finnish adult males, smoking prevalence is nowadays one of the lowest in Europe (Shafey et al. 2003). In general, the smoking trends suggest that the impact of tobacco policy is decreasing smoking initiation in youth (Helakorpi et al. 2004); for example the legislation appears to have decreased purchases from commercial sources to minors (Rimpelä and Rainio 2004).

2.1.2 Global burden of tobacco use for public health

Tobacco is packed with harmful and addictive substances. Scientific evidence has shown conclusively that all forms of tobacco cause health problems throughout life, frequently resulting in death or disability. Smokers have markedly increased risks of multiple cancers, particularly lung cancer, and are at far greater risk of heart disease, stroke, emphysema and many other chronic diseases. The use of smokeless tobacco causes cancer in humans (International Agency for Research on Cancer (IARC) 2004). Smokeless tobacco contains carcinogens, which contribute to cancers of the oral cavity and the risk of other cancers. Smokeless tobacco use also causes a number of noncancerous oral conditions and can lead to nicotine addiction similar to that caused by cigarette smoking.

Women who smoke suffer additional health risks. Smoking in pregnancy is dangerous to the mothers as well as to the foetus, especially in countries where health facilities are inadequate. Smoking is not only harmful during pregnancy, but has long-term effects on the offspring. This is often compounded by exposure to passive smoking by the mother, father or other adult members (Fenercioglu et al. 2009).

Of today's global population, 650 million will eventually be killed by tobacco. Approximately half of smokers are killed by their habit – a quarter while still in middle age (35-69 years) (Peto et al. 1996). On average, smokers die ten years younger than non-smokers. Tobacco kills more than Acquired Immuno Deficiency Syndrome (AIDS), legal drugs, illegal drugs, road accident, murder and suicide combined (Doll et al. 2004). Tobacco already kills more men in developing countries than industrialized countries, and it is likely that death among women will soon be the same. Annual deaths due to tobacco in industrialized countries were 1.3 million in 1995 and reached 2.1 million in 2001 and will increase further to 3 million by

2025–30, whereas in developing countries this was 0.2 million in 1975, 2.1 million in 2001, and 7 million by 2025–30, which is a very rapid increase (WHO 2008). While 0.1 billion people died from tobacco use in the 20th century, ten times as many will die in the 21st century. The main diseases by which smoking kills people are substantially different in America (where vascular diseases and lung cancer predominate) (Peto et al. 2004), than in China (where obstructive pulmonary disease predominates, causing even more tobacco deaths than lung cancer) (Liu et al. 1998, Niu et al. 1998), or in India (where half of the world tuberculosis deaths take place, and the ability of smoking to increase the risk of dying from tuberculosis is of particular importance) (Gajalashmi et al. 2003). Around the world, it is estimated that there are currently 30 million individuals who start to smoke every year. With current smoking patterns, worldwide mortality from tobacco is likely to rise from 2–3 million deaths a year in 2001 to about 10 million a year around 2030 (WHO 2008). A survey conducted in 1990 in 44 industrialized countries showed that smoking caused an average of 24% of all male deaths – but 35% of these deaths occur in middle age (35–69 years). This proportion was about 12% in Chinese middle-aged men but is expected to rise to about 33% by 2030. Currently, smoking causes 7% of all female deaths overall.

2.1.3 Smoking forms of tobacco use

There is a variety of smoking tobacco products on the world market (Table 2.2).

Cigarette is any roll of tobacco wrapped in paper or other non-tobacco material; filter-tipped or untipped; approximately 8 mm in diameter, 70–120 mm in length.

A ***cigar*** is any roll of tobacco wrapped in leaf tobacco or in any other substance containing tobacco. There are four main types of cigars: little cigars, small cigars "cigarillos", regular cigars and premium cigars. Some little cigars are filter tipped and are shaped like cigarettes. Little cigars contain air-cured and fermented tobacco and are wrapped either in reconstituted tobacco or in a cigarette paper that contains tobacco and/or tobacco extract. Cigarillos are small, narrow cigars with no cigarette paper or acetate filter. Regular cigars are up to 17 mm in diameter, 110-150 mm in length. Premium cigars (hand-made from natural, long filter tobacco) vary in size, ranging from 12 to 23 mm in diameter and 127 to 214 mm in length (Stratton et al. 2001). The chemical composition of the tobacco leaf is determined by plants genetics, cultivation practices, weather conditions and curing methods (Tso 1991). Cigarettes and cigars use blended tobaccos and the type of tobacco used in these products has a decisive influence on the physicochemical nature of the smoke they produce.

Bidis are the most popular form of smoking of tobacco in India. They are also becoming increasingly popular among teenagers in the USA (Malson and Pickworth 2002). A bidi is made by rolling a rectangular piece of a dried temburni

leaf around approximately 0.2–0.3 g of sun-dried, oriental tobacco and securing the roll with a thread. These cigarettes are perceived by some as a better-testing, cheaper, safer or more natural alternative to conventional cigarettes (Malson et al. 2001, Stanfill et al. 2003).

Chuttas are coarsely prepared cheroots with 2–9 cm long, prepared by rolling local tobacco inside a sun-dried tobacco leaf. They are usually the products of cottage or small-scale industries. Nearly 9% of the tobacco produced in India is used for making *chuttas*. It is estimated that about 3000 million *chuttas* are made annually in India. The term "reverse smoking" is used to describe smoking while keeping the glowing end of tobacco product inside the mouth. Reverse *chutta* smoking is practised extensively by women in the rural areas of Visakhapatnam and the Srikakulam district of Andhra Pradesh (Van der Eb et al. 1993).

A ***cheroot*** is a roll made from tobacco leaves. *Cheroots* were commonly smoked by both Indian men and women in South India. *Dhumti* is a kind of conical cigar made by rolling tobacco leaf in the leaf of another plant. Unlike *bidis* and *chuttas,* ***dhumtis*** are not available from vendors but are prepared by the smokers themselves (Bhonsle et al. 1976).

Kreteks are types of small cigarettes that contain tobacco (approximately 60%), ground clove buds (40%) and cocoa, which gives a characteristic flavour and "honey" taste to the smoke (Stratton et al. 2001). *Kreteks* are indigenous to Indonesia, but are also available in the USA.

Pipe smoking is one of the oldest from of tobacco use. The different kinds of pipes used for smoking range from the small – stemmed European types made of wood to long-stemmed pipes made from metal or other material.

A *hookah* is an Indian white pipe in which the tobacco smoke passes through water before inhalation. It used to be more common among women, the reason being that it was inconvenient for men to carry a *hookah*, whereas women remain at home for most of the time.

Hooklis are clay pipes commonly used in western India. Once the pipe is lit, it is smoked intermittently. On average, 15 g of tobacco is smoked daily. Hookli smoking was common among men in the Bhavnagar district of Gujarat (Mehta et al. 1969).

A ***chillum*** is a straight conical pipe made of clay, 10–14 centimetres long, held vertically. It is exclusive and common among men and is confined to the northern states of India, predominantly rural areas (Wahi 1968).

2.1.4 Smokeless forms of tobacco use

Smokeless tobacco products have existed for thousands of years among populations in South America and Southeast Asia. Over time, these products have gained popularity throughout the world. Smokeless tobacco is consumed without burning

the product, and can be used orally or nasally. There are different types of smokeless tobacco products (STP) in use around the world. Oral smokeless tobacco products are placed in the mouth, cheek or lip and sucked (dipped) or chewed. Tobacco pastes or powders are used in a similar manner and placed on the gums or teeth. Fine tobacco powder mixtures are usually inhaled and absorbed in the nasal passages.

Smokeless tobacco comes in two main forms: ***chewing tobacco*** (loose leaf, in pouches of tobacco leaves, “plug” or “twist” form) and ***snuff*** (finely ground or cut tobacco leaves that can be dry or moist, loose or portions packed in sachets, and administered to the mouth, or the dry products to the nose or mouth). When administered orally, the tobacco can also be mixed with other psychoactive ingredients.

A list of the wide range of oral and nasal tobacco products used is presented below (Table 2.2).

TABLE 2.2 List of smokeless tobacco products by continent

Common name	Constituents	How used
EUROPE		
Moist snuff, Snus	Tobacco; water; sodium carbonate; sodium chloride; moisturizer; flavouring; nicotine	A pinch (called a dip) is usually placed in the upper gingivolabial sulcus. The average user keeps snus in their oral cavity for 11 to 14 hours per day.
Dry snuff	Tobacco	Inhaled up the nostrill
Nicotine gum (non-pharmaceutical)	Tobacco	Gum to be chewed
Gutkha	Tobacco	Chewed or smoked in pipe
Chewing tobacco	Tobacco	Chewed or smoked in pipe
NORTH AMERICA		
Dry snuff	Tobacco + aromatic oils, spices	Inhaled up the nostril
Loose leaf chew	Leaf tobacco; sweetener and/or liquorice	A piece of leaf is tucked between the gum and jaw, typically toward the back of the mouth. It is either chewed or held in place. Saliva spit or swallowed.
Moist plug	Enriched tobacco leaves; fine tobacco; sweetener and/or liquorice	Chewed or held between the cheek and lower lip. Saliva may be spit or swallowed.
Moist snuff	Tobacco	A pinch "dip" or held between the cheek/gum. Saliva may be swallowed.
Plug chew	Enriched tobacco leaves; fine tobacco; sweetener and/or liquorice	Chewed or held between the cheek and lower lip.1 Saliva may be spit or swallowed.
Twist roll (chew)	Tobacco; tobacco leaf Extract	Chewed or held between the cheek and lower lip. Saliva may be spit or swallowed.
Iq'mik	Tobacco, punk ash	Users pinch off a small piece and chew the iq'mik. Often, the user may premasticate the iq'mik and place it in a small box for later use by others, including children and sometimes teething babies.
SOUTH AMERICA		
Chimo	Tobacco resin; alkaline ash; Paullinia yoco; banana peel; sugar; avocado seed	A very small amount of the paste is placed under the tongue and absorbed there. Saliva is traditionally spat out. Chimo is popular as a replacement for cigarettes and provides a similar bolus of nicotine.
Dry snuff, Rapé	Dry tobacco powder with peppery smell	Sniffed through nostrils

Common name	Constituents	How used
INDIA SUBCONTINENT		
Gul	Tobacco powder, molasses, other ingredients	Often used for clearing teeth
Gutkha	Betel nut, catechu, tobacco, lime, saffron, flavouring, saccharine, mint	Held in the mouth and chewed. Saliva is generally spit out, but sometimes swallowed.
Khaini	tobacco; slaked lime paste; areca nut	Paste is placed in the mouth and chewed
Mawa	Tobacco; slaked lime; areca nut	Placed in the mouth and chewed fo 10 to 20 minutes
Tuibur, hidakphu	Tobacco water	Sipped and held in mouth 5–10 min and then spat out
Mishri (masheri)	Tobacco	Applied to the teeth and gums, often for the purpose of cleaning the teeth. Users then tend to hold it in their mouths (due to the nicotine addiction).
Nass (naswar, niswar)	Nass: tobacco, ash; cotton or sesame oil; water; sometimes gum. Naswar or niswar: tobacco, slaked lime; indigo; cardamom; oil; menthol; water	Held in the mouth for 10 to 15 minutes. Naswar is sometimes chewed slowly
Pan masala	Tobacco; areca nuts, slaked lime, betel leaf.	A quid is placed in the mouth (usually between the gum and cheek) and gently sucked and chewed. Pan masala is sometimes served in restaurants after the meal.
Zarda	Processed tobacco	Along with betel quid
Creamy snuff	Tobacco, clove oil, glycerine, menthol, spearmint, camphor	Often used to clean teeth. The manufacturer recommends letting the paste linger in mouth
Red tooth powder	Tobacco	
MIDDLE EAST		
Shammah	Tobacco; ash; slaked lime	
AFRICA		
Toombak	Tobacco; sodium bicarbonate	Product is rolled into a ball of about 10g called a saffa. The saffa is held between the gum and the lip or cheeks, or on the floor of the mouth. It is sucked slowly for 10 to 15 minutes. Male users periodically spit, while female users typically swallow the saliva generated.
Snuff	Tobacco	Sniffed through nostrils, portion bags introduced

From the European Scientific Committee on Emerging and Newly Identified Health Risks, 2007.

2.1.5 Assessment of tobacco exposure

The selection of a strategy to measure smoking exposure is affected by factors such as: (a) required accuracy of the smoking estimate, (b) need for immediate feedback to the smoker, (c) accurate disclosure by the target population, (d) concurrent use of nicotine replacement therapy, (e) need to distinguish between reduction and abstinence, (f) need for validating smoking status, and (g) availability of resources. The various approaches differ in accuracy, validity, objectivity, ease of measurement approach, acceptability and cost. Research evidence on the measurement strategy should guide the decision to use questionnaire data, a biomarker or both.

2.1.5.1 Self-reports

Self-administered questionnaires are a cheap method of assessing smoking status; they are easy to use with great feasibility (fit within setting) and accomplished by written or verbal communication. In this method, the participant responds to questions, either verbally or in writing, regarding smoking abstinence or cigarette consumption (number of cigarettes smoked, frequency, and duration). Questionnaires are noninvasive for the test subjects and the confidentiality of information reduces the refusal rate among participants. Self-reported information can be used to measure behavioural change, to evaluate the exposure risk or to study pathways to smoking cessation (Bauman et al. 1989, Wills and Cleary 1997). The validity of questionnaire data has been studied (Vartiainen et al. 2002). In special groups (adolescents, pregnant women), self-reported smoking is more likely to be under-reported (Owen et al. 2001, Britton et al. 2004, Burstyn et al. 2009).

2.1.5.2 Biochemical methods

The term biomarker means a measurement that reflects an interaction between a biological system and a chemical, physical, or biological environmental agent (Haufroid and Lison 1998). Biological quantification of tobacco use is based on some aspect of the composition of inhaled tobacco smoke. Tobacco smoke is composed of gaseous and particle components. The gaseous component is made up of room air, carbon monoxide, nicotine and volatilized hydrocarbons such as hydrogen cyanide. The primary particle component of tobacco smoke is tar, which carries nicotine. Substances such as nicotine, cotinine, thiocyanate, carbon monoxide and some minor alkaloids of nicotine have been identified and tested as biomarkers of both active cigarette smoking and second hand smoke exposure (SHS) (Woodward et al. 1991).

Nicotine

The major and most pharmacologically active alkaloid of tobacco is nicotine (Jacob et al. 1999). The amount of nicotine uptake is dependent on a smoker's inhalation behaviours (e.g. deep or long inhalation of smoke) and metabolism of nicotine (Benowitz 1999). Most nicotine is metabolized into cotinine and eventually excreted (see cotinine below). Nicotine may be extracted and measured from blood, saliva, and urine (Benowitz 1996). More recently, it has been measured from samples of hair and toenails (Al-Delaimy et al. 2002). Nicotine as a biomarker agent, however, is of limited use (Velicer et al. 1992). Any assay using nicotine must be very sensitive because of the small amount of nicotine present in body fluids. Furthermore, because of its short half-life (2 hrs) and individual variation in its rate of metabolism (Benowitz et al. 1996, Idle 1990), nicotine levels can be only approximated, and may give a biased estimate of tobacco use/exposure.

Thiocyanate

Tobacco smoke contains high concentrations of hydrogen cyanide gas, which is primarily metabolized into thiocyanate (SCN). Like cotinine, SCN can be measured in blood, urine and saliva (Velicer et al. 1992). The following issues affect the usefulness of SCN as a biomarker. Despite its long half-life (10–14 days), the sensitivity and specificity of the assay method are low. SCN levels are influenced by industrial exposure and dietary intake (almonds, bamboo shoots, sugar cane, cauliflower, broccoli, beer and ale (Benowitz 1999, Woodward et al. 1991). Because of these limitations, determination of SCN has not gained wider use (Scherer and Richter 1997, Velicer et al. 1992).

Carbon monoxide (CO)

Cigarette smoke contains a high concentration of CO in gaseous form. Regular cigarette smoking may produce carboxyhemoglobin (COHb) levels ranging from 5% (1 pack per day) to 9% (2–3 packs per day), whereas heavy cigar smoking can produce COHb levels up to 20%. CO has a half-life of 4–5 hrs in adults and can be measured in both exhaled alveolar air and blood (Stewart 1975). Although CO can be measured by analysis of hemoglobin for COHb using a carbon monoxideoximeter instrument, this approach is not favoured because the procedure to collect the specimen (blood) is invasive. Instead, a much simpler and direct measurement of CO can be accomplished using exhaled air and a simple handheld breath analyzer. This method does not require the samples, such as those of blood, saliva, or urine, to be collected and stored, and only minimal training is needed in using the device.

The immediately available measurement of CO level, which is shared with the smoker, can depict the detrimental effects of smoking. This may affect the smoker's subsequent smoking behavior (Secker-Walker et al. 1997). Thus, CO measurement

has been used as part of anti-smoking campaigns. Researchers have demonstrated high correlations among CO, self-reported smoking and urinary cotinine (Secker-Walker et al. 1997). Exhaled CO has been successfully used to corroborate self-report data, with concordance approaching 100% (Becona and Vazquez 1998).

Environmental sources of CO can result in CO levels indistinguishable from those produced by direct cigarette use, thereby confounding the measurement (Velicer et al. 1992, Becona and Vazquez 1998). Another disadvantage of CO measurement is the relatively short half-life of CO (4–5 hrs). In general population, false-negative rates of CO measurements have been found to range from 2% to 16% (Velicer et al. 1992). In addition, the sensitivity decreases with infrequent and irregular smoking patterns, causing those who are light or atypical smokers to appear indistinguishable from non-smokers (Jarvis et al. 1987, Lando et al. 1991).

Cotinine

Cotinine is a useful and popular biomarker of tobacco use. Most nicotine entering the body (70%–80%) is metabolized into cotinine. Cotinine is present in the blood serum, saliva, urine, amniotic fluid, cervical mucus and hair of both smokers and non-smokers exposed to tobacco smoke. It has been cited as the most useful marker for distinguishing tobacco users from non-users, for estimating the nicotine intake of tobacco users, and for specifying the exposure of nonsmokers to second hand smoke (Benowitz and Jacob 1994). Cotinine has an extended biological half-life (15–40 hrs). Its level in the body is directly related to the quantity of nicotine absorbed during the last few days (Wagenknecht et al. 1990). The presence of cotinine indicates exposure to nicotine, either from environmental exposure or direct consumption.

An advantage of cotinine as a biomarker is its high sensitivity. It can distinguish very low levels, such as from SHS in non-smokers, from levels associated with cigarette smoking. Small amounts of cotinine in the body can result from ingestion of foods rich in nicotine (such as cauliflower, eggplant, potatoes, tomatoes and black tea), but these levels are considered insignificant (Benowitz 1996). Measurement techniques have been developed. Cotinine can be quantified in blood, serum, saliva and urine. Various techniques are used for quantitative analysis including: (a) radio immunoassay, (b) high-performance liquid chromatography, (c) gas–liquid chromatography and (d) gas chromatography combined with mass spectrometry (SNRT Committee for Biochemical validation 2003, James et al. 1998). Woodward and colleagues (1991) compared cotinine levels with those from exhaled CO, self-reported tobacco exposure and thiocyanate. The results showed a high correlation among all the markers for the smoking group, but a lower correlation among the nonsmokers exposed to second hand smoke. The investigators concluded that cotinine is the most accurate discriminator between smokers and non-smokers (Woodward et al. 1991). In other studies, serum cotinine was demonstrated to be

a better measure of cigarette smoking than was questionnaire (Perez-Murray et al. 1993, Stable et al. 1995, Britton et al. 2004, Burstyn et al. 2009).

Exhaled carbon monoxide and cotinine (detected in blood, urine or saliva) are sufficiently sensitive, specific and feasible for general use, and are therefore frequently used as biomarkers of cigarette smoking.

New biomarkers and strategies to detect tobacco exposure

A number of new biomarkers are under development. One new strategy is to use hair specimens for analysis of cotinine in order to assess individuals' smoking history. Although there are limitations, such as confounders caused by hair color, hair dyes and other chemical treatments, a statistically significant correlation between hair cotinine and nicotine intake has been found (Eliopoulos et al. 1996). This strategy is promising because of the noninvasive nature of the test, the lack of stringent specimen storage requirements and the ability to detect a history of tobacco usage in patients who temporarily abstain from smoking prior to testing (Eliopoulos et al. 1996).

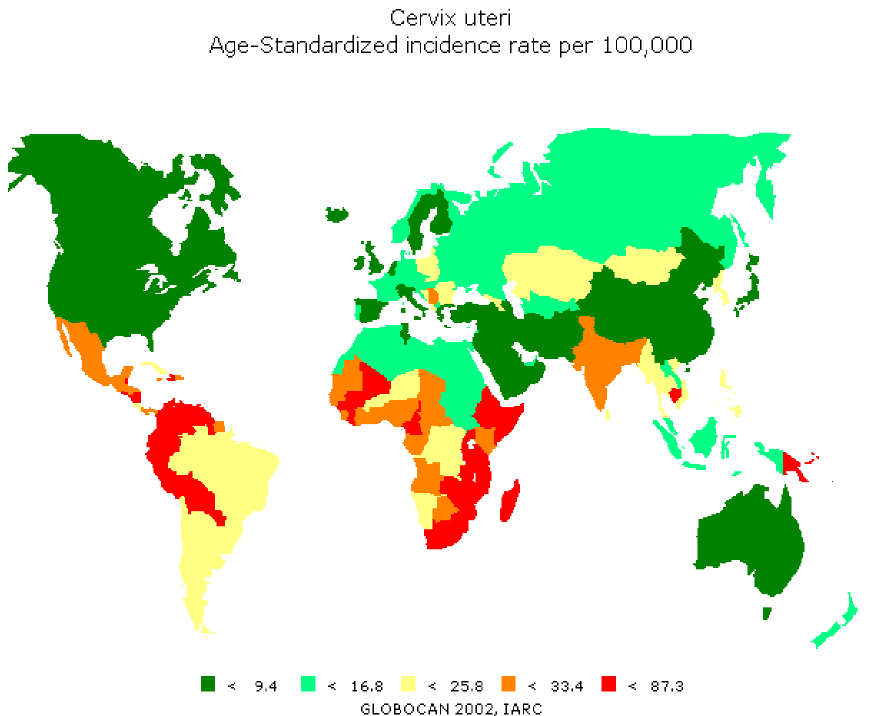
Biochemical validation is often considered to be a "gold standard" (= considered more accurate than self-reported habit) in validation studies. When used in combination with self-report, biomarkers provide information about the concurrent validity of self-report, and have been noted to increase the accuracy of self-reporting (Kathleen and Muñoz 2004, Wagenknecht et al. 1992, Clark et al. 1996, Becona and Vazquez 1998).

2.2 Cancer of the uterine cervix

2.2.1 Epidemiology

Cancer of the uterine cervix is the seventh most common cancer overall and the second most frequent cancer in women worldwide (Ferlay et al. 2004; Denny 2008). It is a major cause of morbidity, mortality and premature death among middle-aged women in developing countries, who account for 80% of the annual estimated 493,000 new cases and 274,000 deaths worldwide. In these low resourced countries, cervical cancer accounts for 15% of female new cancer cases, with an absolute cumulative risk of 1.5% before the age of 65 years. In developed countries, these proportions are smaller with only 3.6% of new cancers and a cumulative risk of 0.8%. If effective preventive interventions are not implemented, over 1 million women will suffer from cervical cancer annually by the year 2030 (Denny 2008), leading to a far greater in risk and disease burden in developing countries compared to developed countries, and increasing the social inequalities.

The highest incidence rates are observed in the developing countries in sub-Saharan Africa, Melanesia, Latin America and the Carribean, South-Central and Southeast Asia, with age-standardized (world population) incidence rates ranging from 18.7 to 42.7 per 100,000 (Maucort-Boulch et al. 2008). In more developed regions, these rates are generally lower than 14.5 per 100,000 (Parkin et al. 2005; Parkin 2008). These lower incidence rates have, however, materialized after the introduction of screening programmes in the developed countries in the 1960s. Earlier, the incidence in the developed countries was similar to that of developing countries today in most of Europe, North America and Japan (Gustafsson et al. 1997). Cervical cancer incidences were estimated to be 38.0 per 100,000 in the Second National Cancer Survey of the United States (Dorn et al. 1959), 37.8 per 100,000 in Hamburg, Germany in 1960–62, 28.3 per 100,000 in Denmark in 1953–57 and 22.1 per 100,000 in Miyagi, Japan in 1959–60 (Doll et al. 1966) The lowest rate of cervical cancer, 0.4 per 100,000, has been reported in Ardabil, northwest Iran (Sadjadi et al. 2003). Very low rates have also been observed in Israel (2.0 per 100,000), China (6.8 per 100,000), Western Asia (5.8 per 100,000) and Finland (4.0 per 100,000) (Parkin et al. 2005).



Mortality from cervical cancer is considerably lower than the incidence in both developing and developed countries but the mortality to incidence ratio is higher for the former (57%) than the latter (47%) (Ferlay et al. 2004). Survival rates vary between regions with good prognosis in regions with low incidence (survival obtained from case fatality ratio was 70% for USA, 66% for Western Europe and 65% for Japan in 2002) and fairly good prognosis even in some developing regions (55% in South America and 58% in Thailand) where many cases present at a relatively advanced stage (Parkin et al., 2005). However, poor prognosis is seen in sub-Saharan Africa (21%) (Parkin et al. 2008).

2.2.2 Natural history

Invasive CC is usually preceded by a long phase of pre-invasive, occult disease. This pre-invasive disease is microscopically assessed and characterized as a spectrum of progressive lesion with severity ranging from cellular atypia to various grades of dysplasia or cervical intraepithelial neoplasia (CIN) before progression to invasive carcinoma. Using different terminology systems (Table 2.3), cervical cancer precursor lesions are commonly classified into mild dysplasia or CIN I, moderate dysplasia or CIN II, and severe dysplasia or CIN III. However, the newer terminology of the precursor lesions classifies them as squamous intraepithelial lesions (SILs), which are graded as low (combines condylomatous (HPV) changes and CIN I) and high (encompasses more advanced CIN such as CIN II and III) (Sellors et al. 2003).

Infection of the cervical epithelium with specific hrHPV types plays a fundamental role in the development of cervical cancer through its precursor lesions (Zur Hausen 1999, Whiteside 2008). HPV DNA has been detected in virtually all cervical cancer specimens (Walboomers et al. 1999, Subramanya and Grivas 2008) with HPV16 having the dominating role followed to a lesser degree by HPV types 18, 31, 33 and 45 (IARC 2006). Most cervical abnormalities caused by HPV

TABLE 2.3 Terminology of cervical precancerous abnormalities

Dysplasia terminology	Cervical Intraepithelial Neoplasia (CIN) system	Bethesda system
Unspecified cellular changes	Cellular atypia	Atypical Squamous Cells of undetermined significance (ASCUS)
Mild dysplasia	CIN I	Low-grade squamous intraepithelial lesions (LSIL)
Moderate dysplasia Severe dysplasia/ Carcinoma in situ (CIS)	CIN II CIN III (includes CIS)	High-grade squamous intraepithelial lesions (HSIL)

infections do not progress to high-grade SILs (HSIL) or cervical cancer, but regress spontaneously. The long timeframe between initial infection and overt diseases indicates that other exogenous and endogenous cofactors, such as reproductive factors, other sexually transmitted diseases, smoking, nutritional deficiencies and genetic susceptibility, acting in conjunction with the hrHPVs probably participate in disease progression (Sellors et al. 2003, Stewart et al. 2003, Woodman et al. 2007, Castro 2008). Spontaneous regression of CIN suggests that a lot of women may not be exposed to these cofactors.

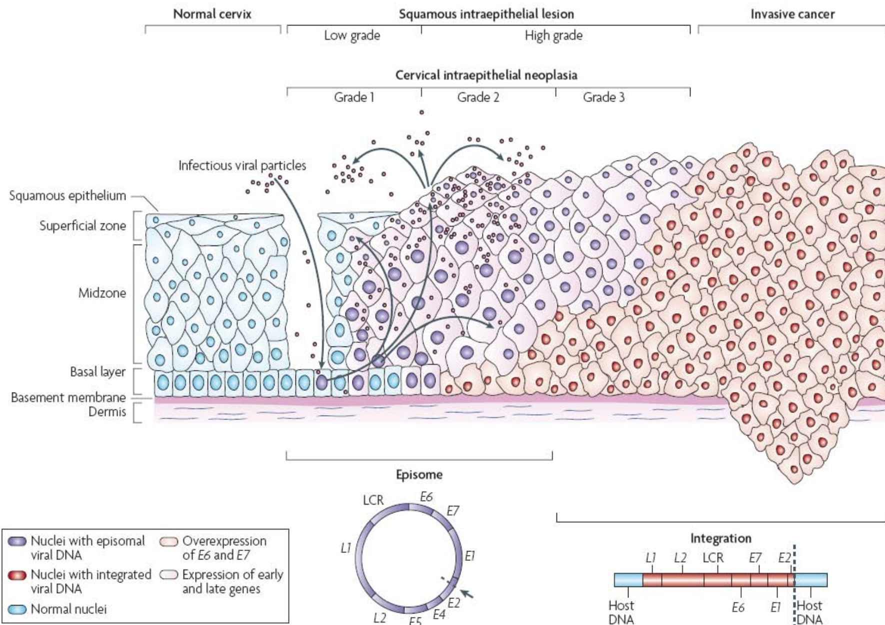


Figure 2.1 Natural history of HPV from Woodman et al. 2007 (Nature Reviews, Cancer)

Studies addressing the natural history of CIN, with particular emphasis on disease regression, persistence and progression, have demonstrated that most LSIL regress to normal within relatively short time-periods or do not progress to severe lesions or invasive disease (Mitchell et al. 1994, Melnikov et al. 1998, Holowaty et al. 1999, Schlecht et al. 2003, Bosch et al. 2008, Wheeler 2008). On the other hand, HSIL has a greater likelihood of progressing to invasive cancer, though a proportion of such lesions also regress or persist (Stanley 2007, Bosch et al. 2008, Wheeler 2008). The mean interval for progression from CIN to ICC ranges between 10 and 20 years.

2.2.3 Risk factors

2.2.3.1 Human Papillomavirus

Structure and classification

Human papillomaviruses belong to the *Papillomaviridae* family. They are small, non-developed, double-stranted DNA viruses with icosahedral symmetry. The virion has a diameter of 55–60 nm and the viral genome is approximately 7900 base pairs long (Chen et al. 1982). The protein coat is composed of 72 capsomers consisting of two structural proteins: one major protein (L1) representing 80% of the total capsid. L2 is the minor protein.

To date, at least 100 HPV types have been classified (de Villiers et al. 2004, IARC 2006). They are classified as genotypes and each type has a given number. The genotypes are based on the sequence homology of the L1 open reading frame (ORF) because this region is well conserved among all members of the papillomavirus family. If the DNA sequence of a new HPV type differs by more than 10% from the closest known papillomavirus type it will be recognized as a new type. A subtype is defined when there is 2–10% difference in the sequence homology. Less than 2% defines a variant (de Villiers et al. 2004).

HPVs are grouped according to the type of epithelia they infect. The majority of HPVs infect cutaneous epithelia or skin. Approximately 40 types infect mucosal epithelia and are called genital HPVs. These types are divided into high-risk types, including cell transformation and low-risk types, causing benign warts, and further divided by sequence homology into A9 (HPV16, 31, 33, 35, 52, 58, 67), A7 (HPV18, 39, 45, 59, 68, 70) (Centers for Disease Control 2008). It has been suggested that at least 14 types are high-risk types (Bosch et al. 1995, IARC 1996, IARC 2006). Main difference between the two major categories is represented by the exclusive capacity of the high-risk HPVs to integrate the host cell's chromosome (Ferenczy and Franco 2001).

Genomic organisation

The genome of HPV contains approximately eight ORFs, which are transcribed from a single DNA strand (Fehrmann and Laimins, 2003). In the upstream regulatory region (URR) which is a non-coding region, sequences for viral replication are found. The gene products can be divided into two classes: early (E) and late (L) proteins (Howley 2006) (Figure 2.2). The early genes are primarily responsible for viral DNA replication, transcription and transformation and the late genes express viral structural proteins that are responsible for maturation and assembly of the virus particle.

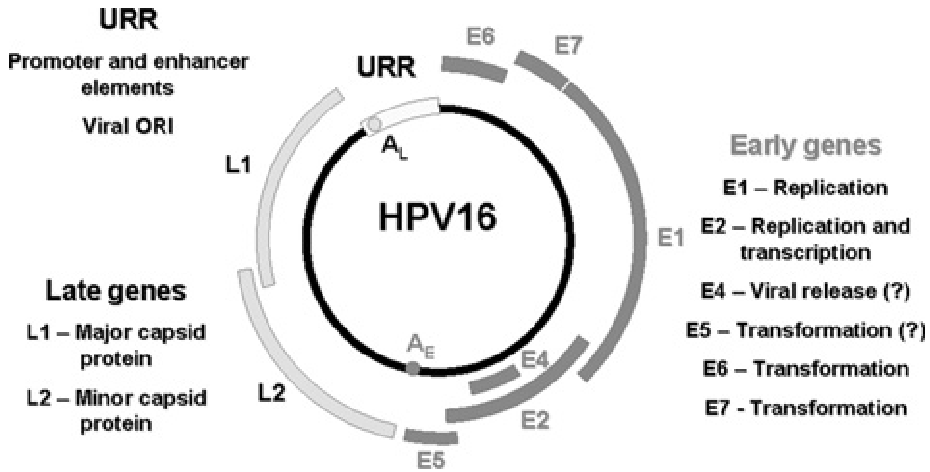


FIGURE 2.2 HPV 16 genome organisation

E1 is the largest ORF in the papillomavirus genome. It is the only papillomavirus protein with defined enzymatic activity (helicase and ATPase activity), which helps viral DNA replication to occur in an efficient manner (Wilson et al. 2002). E1 forms heterodimers with E2, which leads to the initiation of viral replication at the viral origin (Sverdrup and Khan 1995, Horner and DiMaio 2007).

The E2 protein has an important role in the life cycle of papillomavirus because it regulates viral transcription and replication (Lambert 1991). E2 has been shown to induce S-phase arrest, which allows sustained synthesis of viral DNA replication, something that is essential for completion of the viral life cycle (Hamid et al. 2009).

The ORF of E4 is found within the ORF of E2 but has a shorter reading frame. The protein is detected in productively infected cells. The E4 protein is translated from a spliced E1^E4 transcript to form a spliced E1^E4 fusion protein. The pattern of E4 distribution suggests that the E4 function may be required at all stages of the productive cycle (Knight et al. 2004, Doorbar 2006).

E5 is weakly oncogenic in tissue culture assays and improves the effectiveness of the transforming activity of E7 (Bouvard et al. 1994, Valle and Banks 1995). The HPV E5 protein is small, hydrophobic and located mainly at the endosomal membranes, Golgi apparatus and plasma membranes (Burkhardt et al. 1989, Conrad et al. 1993). The protein is probably expressed primarily during the late phase of the life cycle to modulate differentiation-induced functions like viral amplification and late gene expression (Fehrmann et al. 2003, Auvinen 2005).

The E6 and E7 proteins are encoded by all papillomaviruses and their ORFs are located in the 5' part of the early region. Together with E7 from high-risk HPVs, E6 can induce cellular immortalisation of keratinocytes (Hawley-Nelson et al. 1989, Munger et al. 1989). These genes are the main transforming proteins of the high-risk HPV types and act by modulating the activities of the cellular proteins that regulate the cell cycle. The E6 protein is one of the first genes expressed during

HPV infection. It is about 150 amino acids in size and contains two zinc-binding domains with the motif Cys-X-X-Cys. The zinc fingers are important for protein conformation and interaction with DNA. High-risk E6 proteins are found both in the nucleus and in the cytoplasm and have been reported to bind to more than 12 different proteins (zur Hausen 2002, Whiteside 2008). The E7 protein is rather shorter than E6, around 100 amino acids. E7 binds directly to the Rb gene and interferes with the ability of Rb to inhibit cell cycle arrest. This allows productive replication of HPV genes (Fehrmann and Laimins 2003).

L1 and L2 proteins that make up the capsid of the virus are synthesised in the late phase of the viral cycle. The role of the capsid is to protect the genome and to target cellular surface receptors involved in infection. The L1 protein can with or without the L2 protein self-assemble into virus like particles (VLP) when expressed in eukaryotic cells (Kirnbauer et al. 1992, Stanley et al. 2006). The VLPs are morphologically and immunologically comparable to HPV virions.

There is one part of the HPV genome that does not encode any known protein but still has an important function: the long control region (LCR). Its role is to regulate gene expression and replication.

Viral life-cycle

The viral replication cycle is one in which viral infection is targeted at basal keratinocytes but high level expression of viral proteins and viral assembly occur only in differentiating keratinocytes in the stratum spinosum and granulosum of squamous epithelium (Doobar 2007, Frazer 2009) . Although our knowledge is limited in some key areas of the immunubiology and pathogenesis of the viruses, particularly the immediate early events of viral replication, the sequence of events shown in Figure 2.3 (see below), in which viral genes are differentially expressed both temporally and spatially throughout the infectious cycle, is well accepted (Stanley 2006, Wang 2007).

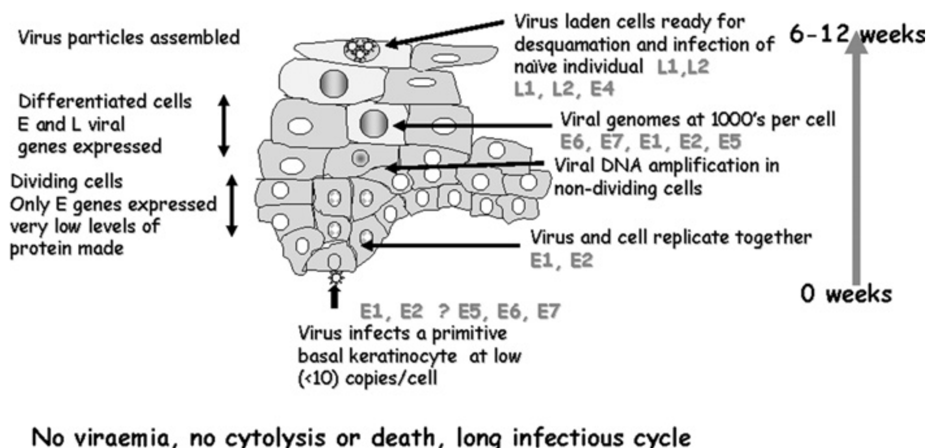


FIGURE 2.3 The HPV infectious cycle (From Stanley 2006)

The replication cycle takes a long time. Even in the best scenario, the time from infection to release of virus will take about 3 weeks since this is the time taken for the basal keratinocyte to undergo complete differentiation and desquamate. In humans the period between infection and the appearance of lesions is highly variable and can vary from weeks to months (Gillison and Shah 2003, Doobar 2007) indicating that the virus can effectively evade the immune system (Stanley 2006, Wang 2007).

The replication cycle is exclusively intra-epithelial, there is no detectable viraemia, viral gene expression is probably confined to keratinocytes and there is no cytolysis or cytopathic death as a consequence of viral replication, assembly and release (Stanley 2006). These key events for the virus occur in the differentiating keratinocyte, a cell destined for death and desquamation far from the sites of immune activity. HPV infection in consequence, is not accompanied by inflammation, there is no obvious 'danger signal' to alert the immune system—a viral strategy that results in persistent, chronic infections as the host remains ignorant of the pathogen for long periods.

HPV immunology

The immune system is complex and its responses can vary from person to person due to different gene disposition and environmental influences. The majority of HPV infected women clear the virus within a rather short period of time. The immune response probably has an important role in clearance of the virus. Understanding immunity to HPV is important in the development of both prophylactic and therapeutic measures against HPV infection and associated cancer.

Antigen presenting cells

Langershans cells (derived from dendritic cells) are the professional antigen presenting cells in the epithelium (Campaner et al. 2006). They present the endocytosed HPV antigens to T-helper cells by using the MHC class II molecules, and thus start up the immune response by secreting and inducing secretion of appropriate cytokines from the T-helper cells. Depending on the nature of the T-helper cells (Th1 or Th2 type cells) this may lead to enhancement of the cell mediated response or antibody response by pertinent cytokines (e.g. γ-interferon (IFN) or interleukin (IL)-4, respectively) (Bais et al. 2005, Nguyen et al. 2005). Due to viral or environmental factors the Langerhans cells can be depleted from the site of HPV infection, which may impair/delay the immune response. Some researchers have observed a decrease in the number and functions of Langerhans cells in the epithelium of the cervix in women who smoked, suggesting that the decrease in the antigen presentation could contribute to the development of CIN (Szarewski et al 2001, Arcavi 2004). Smoking is known to induce cytokines Th1

and Th2 and may thus have an adverse effect on the immune response (Cozen et al. 2004, Whetzel et al. 2007, Nakaruma et al. 2008).

Infected epithelial cells may act as (non-professional) antigen presenting cells (Stanley 2008). Mostly endogeneous antigens expressed by the genome of the infecting virus are presented by the MHC class I molecules to the T-cell receptor. The non-professional antigen presenting cells may lack pertinent cell surface molecules (e.g. B7) that are needed for cell-cell signalling in conjunction of the antigen presentation, and proper stimulation of the cytotoxic T-cells (Doorbar 2007). Co-factors of cervical cancer (e.g. *C. trachomatis*) may induce downregulation of these cell-surface molecules by perturbating the local cytokine responses, and thus "paralyze" immune response to HPV (Whiteside 2008, Simonetti et al. 2009).

Most if not all MHC (HLA) class II molecules responsible for antigen presentation are capable of presenting peptides derived from the L1 protein of HPVs (Zehbe et al. 2005). This is indicated by no genetic defects associated with susceptibility to HPV infections, no heterozygosity advance with regard to persistent HPV infections or cervical neoplasia, and the fact that 100% of individuals develop high titre antibody responses to vaccination after HPV L1 VLP vaccines. On the other hand, there exists a genetic predisposition to persistent HPV infection or associated cervical neoplasia (Castro et al. 2007). Most likely, however, this is due to differences in cytotoxic T-cell responses to the early viral proteins expressed in HPV transforme cells – the identified MHC class II associations may be explained by linkage disequilibrium with the MHC class I (Liu et al. 2007).

Antibody responses

The study of antibody responses to HPV is a useful tool in understanding the natural history of HPV infection, the cancer association of HPV and for vaccine development. Antibodies against L1-containing virus-like-particles (VLPs) are HPV type-specific and become detectable 2 to 12 months after infection (Carter et al. 1996, Schiller and Hidesheim 2000). Antibodies are not responsible for clearance of the virus but are involved in protection against the infection (Wang and Hildesheim 2003, Nguyen et al. 2005). The major isotypes of the antibody response against HPV are (Immunoglobulin) IgG1 and IgA. Studies on immune response to HPV infection showed that serum IgG antibodies persist longer than IgA antibodies (Wang et al. 2000). On the other hand, IgA antibodies appear early and are markers for recent or active HPV infection, whereas IgG antibodies are stable markers of lifetime cumulative HPV exposure (Dillner etal. 1996; af Geijersstam et al. 1998, Onda et al. 2003).

Serum IgG against HPV16 is detected in 50 to 60% of women who are positive for HPV16 DNA (Kirnbauer et al. 1994, Le Cann et al. 1995, Carter et al. 1996, Kjellberg et al. 1999). Most women who seroconvert will do so 6–12 months after infection but 10–20% convert during the time HPV DNA is detectable (af Geijersstam et al. 1998, Andersson-Ellstrom et al. 1996). When investigating

both serum and cervical IgA in women with incident HPV infection, the authors observed that within 18 months of the first detection of HPV16, 87.3% of the women had developed anti-HPV16 IgA in cervical secretions (Onda et al. 2003).

Women who cleared their infection revert to seronegativity faster than women with persistent HPV16 infection (Nardelli-Haefliger et al. 2003). HPV-seropositivity has in a few studies been associated with CIN persistence and severity of the lesion. Anti-VLP-L1 HPV16 antibodies were detected in approximately 30% of LSIL patients, and in 50% of women with HSIL (Sasagawa et al. 1996, Bontkes et al. 1999, Wideroff et al. 1999). Thus, it is natural that longitudinal seroepidemiological studies disclosed that HPV16-seropositive women have an increased risk for subsequent development of CC (Lehtinen et al. 1996, Dillner et al. 1997, Lehtinen et al. 2001).

Antibodies against non-structural HPV proteins have also been investigated. Anti-E2 IgG was detected in 67% of HPV16 DNA-positive women (Rosales et al. 2001). Anti-E6 and E7 antibodies can be found in CC patients but are not useful as indicators of CC prognosis (Silins et al. 2002). Lehtinen et al. evaluated HPV16 and 18 E6 and E7 responses in samples taken 1–20 years before time of diagnosis. Antibodies were detected in only 7% of women who subsequently developed cancer (Lehtinen et al. 2003). Although IgG antibodies to the HPV16 E2 were found to be inversely associated with progression of CIN, increased IgA and IgG antibody levels to the HPV 18 peptide indicated a significantly increased risk of adenocarcinoma (Lehtinen et al. 1992), suggesting that there may be differences in the humoral immune response to the major oncogenic HPV types.

Cell-mediated immune response

Cellular immunity plays a determining role in the early phase of HPV infection and is also involved in the immune response to CC (Frazer 2009). Immunohistological studies of genital warts and animal models have shown that non-regressing warts and lesions are accompanied with very low infiltrates of T cells (CD4+ and CD8+) (van der Burg et al. 2007, Kim et al. 2008), langerhans cells (Guess and McCance 2005, Caberg et al. 2008), proinflammatory cytokines (Ashrafi et al. 2006, Frazer 2009), and that there is a lack of systemic T cell responses to HPV proteins characteristic of an adequate regression (Suprynowicz et al. 2008, Fazer 2009). Evidence shows that HrHPVs downregulate IFN-inducible gene expression and its signalling pathways (Um et al. 2002, Stanley 2008). The Th1-Th2 model of immunoregulation may play a role in the natural history of HPV infection. A switch from Th1 to Th2 type cytokines has been suggested in cervical carcinogenesis (Frazer 2004, Bais et al. 2005). Keratinocytes infected by HPV can modify the immune response through the production of Th2 cytokines in squamous carcinomas, configuring an evasion mechanism against the T-cell mediated immune response (Frazer 2009).

Experimental studies show that cigarette smoking decreases the secretion of proinflammatory cytokines such as IL-1 and IL6 (Arcavi 2004) and suppresses IL2 and IFNγ production (Ouyang et al. 2000, Hussain et al. 2008), suggesting the possibility that cigarette smoking interacts to increase cervical carcinogenesis development.

HPV immune evasion

HPV infections are slow to induce measurable immune responses or clear, suggesting that HPV may have developed methods to evade host immune mechanisms. The primary mechanism of viral immune evasion for HPV infection is likely avoidance of antigen presentation (Doorbar 2007, Kanodia et al. 2007, Patel and Chiplunkar 2009). A brief description of different mechanisms involved is presented in Figure 2.4.

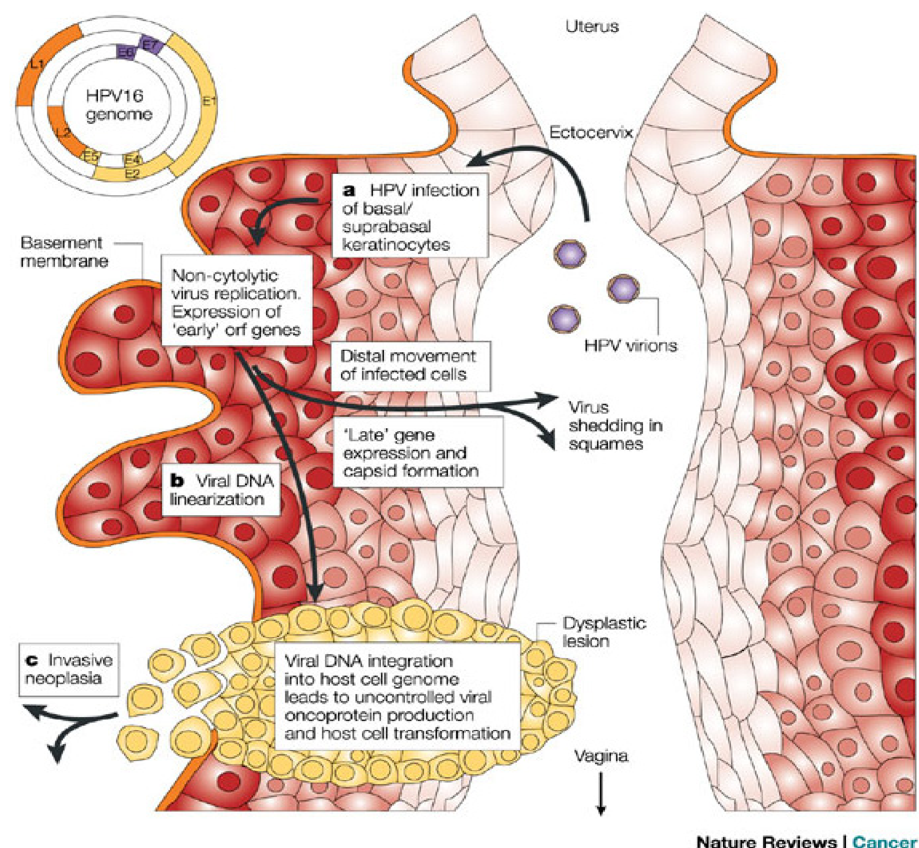


FIGURE 2.4 Human papillomavirus (HPV) immune evasion (Tindle 2002)

HPV infection and reproduction are totally dependent upon a complete programme of keratinocytes differentiation, expressing high levels of viral proteins only in the upper layers of the epithelia. Therefore HPV does not induce cytolysis or cytopathic death in its host cell but in the virus infected keratinocytes, which results in no alarm signal to the immune system and allows persistent and chronic infection (Tindle 2002, Kanodia et al. 2007).

Modes of transmission of HPV infection

HPV is essentially sexually transmitted (Svare et al. 1998; Centers for Disease Control and Prevention 2008). The mean incubation period is estimated to be 2 to 3 months, ranging from a few weeks to eight months (Handsfield et al. 1997). The HPV transmission probability per sexual partnership has been estimated to be 60% in mathematical modelling analyses (Barnabas et al. 2006). However, reports of high rates of incident, albeit transient, HPV infection in small children have raised the issue of alternative modes (mother to child) of acquiring the virus (Stevens-Simon et al. 2000, Sinclair et al. 2005, Cason and Mant 2005, Puranen et al. 1997, Rintala et al. 2005).

HPV detection

Until recently, the only means of diagnosis of HPV infection was direct or enhanced *Visual Inspection*, a specific but not particularly sensitive method (Denny et al. 2002, Sarian et al. 2005). The past decades have witnessed the introduction of increasingly effective molecular diagnostic techniques for HPV infection.

The *Southern Blot* method is the basis of all other specific detection methods. In this method, DNA is extracted and enzymatically digested. The resulting fragments are then separated by electrophoresis according to their size. The isolated fragments are denaturated and identified with the help of single-strand fragments of complementary DNA or RNA labelled with radioactive or calorimetric molecules. This requires relatively large quantities of DNA, is laborious and is impossible to automate (Cuzick et al. 1999).

The *Dot Blot* method (ViraPap, Viratype) was a simplified version of the Southern Blot method that avoids the enzymatic digestion and electrophoresis stages. It was partially automated but required large amounts of DNA and had a lower sensitivity and specificity (Cuzick et al. 1999).

The *filter in situ hybridisation* method was also derived from the Southern Blot but omitted the DNA extraction step. The target cells were applied directly to a solid support and treated to DNA denaturation and identification. While the method was simple, it did not perform adequately in clinical use (Demeter et al. 1987, Cuzick et al. 1999).

In situ hybridization (ISH) demonstrates nucleic acid sequences directly (in situ) in the cells. Cells or tissue sections are fixed on a slide and examined under a

light microscope enabling the assessment of DNA or RNA localization to be related to the morphologic aspects of the lesions. Identification of HPV18 in cervical adenoma tissue was confirmed with this methodology (Leminen et al. 1991). ISH with biotinylated HPV probes (Syrjänen et al. 1986) is a user friendly version of this method.

The *Hybrid Capture* Assay I (*HC-I)* was first introduced by Digene in 1995 (Clavel et al. 1999). HC-I was a relatively fast, liquid hybridization assay designed to detect 9 high-risk HPV genotypes (16, 18, 31, 33, 35, 45, 51, and 56) and one of 5 low-risk types (Cuzick et al. 1999). Initially, this was to be used to enhance the sensitivity of conventional Pap testing and to provide a meaningful negative predictive value for assessing cervical dysplasia. Since then, a second generation of Hybrid Capture assay (*HC II*) has been developed. The assay is a non-reactive, chemiluminescence that is easy to perform and can therefore be used in most clinical laboratories. The HC II is an effective test that requires less expertise than cytology (Terry et al. 2001). HC II detects more HPV genotypes and has high sensitivity and specificity (Hesselink et al. 2005, Brink et al. 2007, Kotianiemi-Talonen et al. 2008). The intra- and inter-laboratory agreement rates between these methods range from good to excellent, i.e. they are well reproducible (Schiffman et al. 2000, Gravitt et al. 1998, Castle et al. 2004, Kulmala et al. 2004, Carozzi et al. 2005).

The *Polymerase Chain Reaction* (PCR) is a selective target amplification assay capable of exponential and reproducible increase in the HPV sequences present in biological specimens (Brink et al. 2007). The amplification process can in theory produce one billion copies from a single double stranded DNA molecule after 30 cycles of amplification. The sensitivity and specificity of PCR-based methods do vary, depending on the DNA extraction procedures, site and type of clinical sample, sample transport and storage, primer sets, the size of the PCR product, reaction conditions and performance of the DNA polymerase used in the reaction, the spectrum of HPVDNA amplified and ability to detect multiple types (Brink et al. 2007). Most laboratories use PCR assays which utilize consensus primers including GP5+/6+, MY09/11, or SPF primer sets, directed to a conserved L1 gene, and hence able to detect all mucosal HPV types. Detection of the hybridized PCR product is done by a colorimetric reaction (Gravitt et al. 1998, Kleter et al. 1999) or by chemiluminescence (Van der Brule et al. 2002). Recently, a quantitative and high-throughput method was developed (Schmitt et al. 2006) based on Luminex suspension array technology. This method relies on detection of the consensus primer GP5+/6+ products with type-specific oligonucleotide probes coupled with fluorescence-labelled polystyrene beads, and allows detection of up to 100 different HPV types simultaneously. If one is interested in a particular HPV type, type-specific PCR can be applied. Confirmation of the specificity of type-specific PCRs, as with consensus primer PCRs, can be done by (regular or reverse) filter hybridization or by EIA, but also in real-time (Josefsson et al. 1999, Hesselink et al. 2005, Moberg et al. 2003). The possibility to sequence the amplicons has become more and more important in the identification of HPV subtypes (e.g. the African

vs. European subtypes of HPV16). Antibodies induced in natural infections by the different subtypes cross-react (Pastrana et al. 2004). A great advantage of real-time PCR assays is the possibility to quantify the HPV-DNA in the specimen. Several studies have shown that the amount of hrHPV present in a cervical smear (the "viral load") as measured by real-timePCR is predictive for the presence or development of high-grade cervical lesions (Josefsson et al. 2000, Ylitalo et al. 2000, Van Duin et al. 2002, Snijders et al. 2003, Snijders et al. 2006).

Detection of HPV-RNA will in the near future be applied to the distinction of transient HPV infections (or mere HPV DNA contamination) from clinically significant or persistent infections (Hafner et al. 2007, Khan and Singer, 2008).

HPV *serology* measures specific antibodies against different HPV types (Carter et al. 1996, Dillner et al. 1996). It is a way of detecting past infection and is very suitable for epidemiological studies because serum antibodies are stable over time (af Geijerstamm et al. 1998). Type-specific HPV serology has been important in the elucidation of the etiological role of HPV and CC (Lehtinen et al. 1996, Wang et al. 2000, Lehtinen et al. 2001). The most commonly used method is enzyme-linked-immunosorbent-assay (ELISA) using HPV virus-like particles (VLPs) as the antigen. The standard ELISA detects anti-HPV antibodies of the IgG or IgA isotopes. The sensitivity of serology for detecting anti-HPV16 antibodies is approximately 50% in best case scenarios, may be up to 65–75%, however, for HPV18 it is less than 50% (Kjellberg et al. 1999, Carter et al. 2000). As reported early by Kirnbauer and colleagues, IgG anti-HPV antibodies are found in only about 60% of women testing positive for cervical HPV DNA (Kinbauer et al. 1994). Anti-HPV antibody detection can therefore not be used as diagnostic tool on the individual level since all HPV infected women apparently do not seroconvert. HPV seropositivity is associated with the numbers of sexual partners and cytological lesions (Castle et al. 2002, Dillner et al. 1999).

HPV – a necessary cause of cervical cancer

The role of HPV in CC development has been studied in depth, culminating in the conclusion that the presence of certain HPV types is necessary for the development of CC (zur Hausen 2002, Roden and Wu 2006, Doorbar 2006, Walboomers et al. 1999, Bosch et al. 2002). Of the numerous HPV viruses, HPV16 is the type most commonly found in precancerous and cancerous lesions, followed by HPV18. In fact HPV16 and 18, along with 3 other hrHPV types (31, 33 and 45), are responsible for 85% of HPV infections that result in CC (Munoz et al. 2003). Other hrHPV types associated with CIN and CC are 35, 39, 45, 51, 52, 56, 58, 59 and 66 (with strong association) and 26, 68, 73 and 82 (with possible association) (zur Hausen 2006). Even though most sexually active women are exposed to HPV infection at least once in a lifetime and more than 50% of women worldwide will acquire a genital HPV infection (Gunell 2006), development of CC is, however, not common. Thus, HPV is not considered to be a sufficient cause of CC. A possible explanation is that

other cofactors are also necessary to trigger CC formation. This may be through alteration of the host (e.g. immune system), direct carcinogenic potential (creation of genotoxic adducts) or direct interaction with HPV in one way or another.

2.2.3.2 Other sexually transmitted diseases

Conflicting results on the role of herpes simplex virus type 2 (HSV-2) infection in cervical carcinoma and its precursors have been reported in several studies. HSV-2 may act in conjunction with amplified HPV infection to increase the risk of invasive cervical carcinoma (Hildesheim et al. 1991, Olsen et al. 1998, Smith et al. 2002). Evidence from longitudinal studies, however, indicates no role of HSV-2 in cervical carcinogenesis (Ferrera et al. 1997, Lehtinen et al. 2002).

Taking into account the central role of genital HPV infection, *C. trachomatis* is likely to be a risk factor of cervical squamous cell carcinoma (SCC) but its role has been difficult to prove (Koskela et al. 2000, Anttila et al. 2001, Wallin et al. 2002, Madeleine et al. 2007). Its effect may possibly modulate the host's immunity and/or precipitate chronic inflammation as persistence of oncogenic HPV infections is more likely among women with a previous *C. trachomatis* infection (Silins et al. 2005).

2.2.3.3 Age

Cancer of the cervix occurs most often in women over the age of 40. The rise in incidence of CC begins at ages 20–29, after which it increases rapidly reaching a peak between ages 45–49 in Europeans populations. In developing countries, the peak is often at a rather later (55–60) age (Curado et al. 2007, Stewart et al. 2003).

In Finland, the incidence of CC has increased especially in fertile-aged women during the first years, most likely due to an increase in HPV occurrence (Anttila et al. 1999, Laukkanen et al. 2003, Barnabas et al. 2006).

2.2.3.4 Tobacco

A positive correlation between the incidence of CC and other cancers known to be related to cigarette smoking across populations prompted the hypothesis that smoking may affect the risk of CC (Winkelstein 1977).

A review of some case-control (Stellman et al. 1980, Clarke et al. 1982, Brinton et al. 1986, Cuzick et al. 1996, Parazzini et al. 1998, Hirose et al. 1996) and cohort studies (Beral et al. 1988, Tverdal et al. 1993, Zondervan et al. 1996, Tulinius et al. 1997, McIntyre-Seltman et al. 2005, Tolstrup et al. 2006) shows that smokers have an increased risk of preinvasive neoplasms and invasive squamous-cell CC compared to non-smokers. In an earlier IARC evaluation of tobacco smoking, the

working group noted that the effect of smoking is confounded by sexual behaviour variables, but the data were not adequate to remove the confounding effect, and that a reasonable conclusion from the available studies of invasive CC is that the results, although they indicate a positive effect of smoking, are compatible with the residuals effects of variables that play a fundamental role in the aetiology of CC (IARC 1986). The extent to which the relationship between smoking and CC reflected a causal association independent of infection with HPV remained a cause of concern. It was believed that the association of smoking with CC may be causal, may even reflect causality via an effect of smoking on risk for HPV infection, or may reflect confounding or risk modification among women with HPV infection (IARC 1996).

In a few cohort studies (Deacon et al. 2000, Castle et al. 2002, McIntyre-Seltman et al. 2005, Mosciki et al. 2001) and case-control studies (Chichareon et al. 1998, Ngelangel et al. 1998, Munoz et al. 1993, Ho et al. 1998, Yoshikawa et al. 1999, Kjellberg et al. 2000, Gunnell et al. 2006) reporting on the association between smoking and CIN or ICC, the point estimates were adjusted for HPV DNA status. In these studies, the association between CC and smoking was not eliminated even though most studies additionally controlled for several well-established risk factors for CC. Women who smoked for a long period or who smoked heavily generally had the highest risk. In several of these studies, the relation was restricted to, or strongest among, recent or current smokers. The highest point estimates were observed among women who had started smoking early in life (La Vecchia et al. 1986, Daling et al. 1996, Plummer et al. 2003).

Several cohort studies (Deacon et al. 2000, McIntyre-Seltman et al. 2005, Tolstrup et al. 2006) and case-control studies (Bosch et al. 1992, Eluf-Neto et al. 1994, Chaouki et al. 1998, Olsen et al. 1998, Ho et al. 1998, Santos et al. 2001, Rolon et al. 2000, Castle et al. 2002, Shields et al. 2004, Rajeeran et al. 2005), including the IARC multicentre pooled analysis of 10 studies of invasive CC (Plummer et al. 2003), examined tobacco smoking as a co-factor to HPV infection by restricting the analysis to HPV DNA-positive study participants. The results from these analyses showed no significant alteration in risk whether or not the study participants were HPV DNA-positive. A nested case–control study of CIN 3 investigated the effect of smoking among HPV-seropositive cases and controls and found that the effect of smoking remained, and there was evidence of a dose-response relationship (Deacon et al. 2002).

No association between smoking and adenocarcinoma and adeno-squamous-cell carcinoma of the cervix has been observed (Brinton et al. 1986, Ursin et al. 1996, Chichareon et al. 1998, Ngelangel et al. 1998, Madeleine et al. 2001).

Tobacco chewing has rarely been studied as a risk factor of CC. To date, only one study has reported an association between tobacco chewing and increased risk of invasive CC in Southern India (OR = 4.0, 95% CI 1.2–13.3) (Rajkumar et al. 2007).

Smoking has been associated with persistent HPV infections (Szarewski et al. 2001) and decreased clearance of CIN lesions (Szarewski et al. 1996). Reduction in lesion size was highly correlated both with the reduction in reported amount of smoking and with the final-visit cotinine measurements (Szarewski et al. 1996).

Whether the link between smoking, HPV and CC is related to: 1) Genotoxic adducts of tobacco in the cervix epithelium (Ali et al. 1994, Phillips et al. 1994, Prokopczyk et al. 1997), 2) Effects on HPV transformation (Motoyama et al. 2004), or 3) Localised immunosuppression (Poppe et al. 1995), has been discussed. Tobacco-specific carcinogens and polycyclic aromatic hydrocarbons have been identified in the cervical mucus or epithelium of smokers (Melikian et al. 1999, Prokopczyk et al. 1997). These compounds can damage cellular DNA and may cooperate with HPV to produce malignant transformation (Simons et al. 1995). Finally there is also evidence that some studies have tried to show the effects of the harmful substances absorbed by the human body during the act of smoking in the immunological defence of the cervical epithelium. Current smokers have significantly lower Langherlans cell counts than nonsmokers in normal cervical epithelium and in CIN, HPV infection, or both (Poppe et al. 1995).

2.2.3.5 Immune compromization

Women infected with the human immunodeficiency virus (HIV), the virus that causes AIDS, or who have used medication that suppresses the immune system have an excess risk of developing CC. CC is one of the "AIDS-indicative diseases". Higher risk of HPV infection and lower HPV clearance have been observed in women infected with HIV than in uninfected women (Palefsky et al. 1999, Ho et al. 1998, Rowhani-Rahbar et al. 2007). Furthermore, the high prevalence, incidence and persistence/progression of SILs appear to be associated primarily with increased HPV persistence that may result from immunosuppression related to HIV infection (Hawes et al. 2006, Six et al. 1998).

Long-term immunosuppression seems to increase the risk of CC among other anogenital cancers: in a few population-based follow-up studies, the observed incidence of cervical cancer among renal transplant patients has been higher than the expected rate (IARC 2006). In a study conducted in Australia and New Zealand, the age-standardised incidence ratio in transplant recipients was 3.3 after a mean follow-up of 5.8 years, whereas, it was 0.74 for dialysis patiens (Fairley et al. 1994). In a register-based study from the Nordic countries, the standardised incidence ratio for cervical cancer in transplant recipients was increased after an average of 4.8 years of follow-up (Birkeland et al. 1995). The increase was most notable in fertile-aged women among whom impaired immune system probably allows persistent HPV infection.

2.2.3.6 Other risk factors

There is evidence that numerous other risk factors such as *irregular or total lack of screening* (Bosch et al. 1992, Leyden et al. 2005, Nygard et al. 2002), *number of sexual partners* (Biswas et al. 1997, Kjaer et al. 1992, Agarwal et al. 1993, Bosch et al. 1996, Castellague et al. 2002, Franceschi et al. 2003, Hammouda et al. 2005), *long term oral contraceptive use* (Appleby et al. 2007, Smith et al. 2003), m*ultiparity* (Autier et al. 1996, Franceschi et al. 2003, Hammouda et al. 2005, Munoz et al. 2002), *early age of sexual debut* (Biswas et al. 1997, Bosch et al. 1992, Ferrera et al. 2000), *low socio-economic status and ethnicity* (de Sanjose et al. 1997, Ries et al. 2008), *micronutrients deficiency* (Garcia-Closas et al. 2005, Potischman et al. 1996), g*enetic susceptibility* (Zelmanowicz et al. 2005, Magnusson et al. 1999) may increase the risk of CC. Highest risk estimates were observed among women with HPV infection.

2.2.4 Prevention

2.2.4.1 Screening and HPV testing

The objective of cervical screening is to prevent invasive CC by detecting and treating women with high-grade SIL i.e CIN 2 and 3 lesions.

Conventional cytology (Pap smear) screening entails collection of cervico-vaginal epithelial cells using a wooden spatula or a brush, preparation and fixation of the smear. Screening with conventional cytology has resulted in a marked decrease in cervical cancer incidence and mortality in a number of countries. However, in some countries the effect of screening has been virtually nonexistent (International Agency for Research on Cancer 2005). In addition to the lack of organisation, the blame has often been laid on the quality of conventional cytology. Worldwide, the variation in the accuracy of conventional cytology is wide: estimates for the sensitivity for CIN 2+ vary from about 30% to 90% and, respectively, for the specificity from 85% to nearly 100% (Soost et al. 1991, Nanda et al. 2000, IARC 2005, Arbyn et al. 2008c). Reproducibility of conventional cytology measured through intra and interobserver variability has been at best moderate to good (Branca et al. 1996, Cocchi et al. 1997, Branca et al. 1998, Woodhouse et al. 1999, Gupta et al. 2001, Chhieng et al. 2002).

Organised cytology screening with systematic call, recall, follow-up and surveillance systems has shown the greatest effect (e.g. Finland) (Nieminen et al. 2004; Kotaniemi-Talonen et al. 2007). Failure and difficulties in organizing Pap smear screening in low- and medium-resourced countries have prompted the search for and evaluation of alternative screening test such as visual screening after the application of acetic acid (VIA) or Lugol's iodine (VILI) and HPV DNA testing (IARC 2004, Sankaranarayanan et al. 2005a).

Liquid-based cytology (LBC) is a modification of conventional cytology that is widely used for primary CC screening. With this technology cervical samples are collected into liquid solution, which is then used for cytological slide preparation. The residual material may be used for additional testing, e.g. HPV DNA detection. Several LBC tests are commercially available, among which ThinPrep (by Cytyc Corporation) and the BD SurePath System (formerly AutoCyte PREP System, by BD Diagnostics, Diagnostic Systems – TriPath) are the best-known and most studied (Arbyn et al. 2008a).

Recent randomised studies and meta-analyses have shown that the performance of LBC in terms of CIN 2+ detection is comparable to conventional cytology (Davey et al. 2006, Ronco et al. 2007, Arbyn et al. 2008b). However, some studies have reported a smaller number of unsatisfactory smears with LBC than with conventional cytology, and the time needed for interpretation is possibly shorter with LBC (Colgan et al. 2004, Dowie et al. 2006, Doyle et al. 2006, Ronco et al. 2007).

The fact that cervical neoplasia are caused by persistent infection with oncogenic types of HPV has led to the evaluation of **HPV testing** as a primary screening and/or secondary test for cervical neoplasia. The optimal age for HPV testing is likely to be between 30-40 years i.e. before the risk of CC starts to increase. HPV testing is the most objective and reproducible of all currently available cervical screening tests. The sensitivity of HPV testing in detecting CIN 2 and 3 lesions varied from 66 to 100% and the specificity varied from 62 to 96% in several cross-sectional studies (Franco 2003, Koliopoulos et al. 2007, Sankaranarayanan et al. 2005). In randomized trials, the sensitivity of HPV test for the detection of CIN is greater than that of Pap testing (Kotaniemi-Talonen et al. 2005, Bulkmans et al. 2007, Mayrand et al. 2007, Naucler et al. 2007, Sankaranarayanan et. al. 2009). A pooled data from seven European perspective screening studies shows that the cumulative incidence of CIN3/cancer was 0.27% among women negative for HPV DNA, whereas the respective cumulative incidence among women with negative cytological test was significantly higher, 0.97% (Dillner et al. 2008).

Self-collected samples for testing of oncogenic HPV is a potentially viable screening option that holds promise for women in under-resourced areas or those who are reluctant to participate in screening programmes, save for the limited evidence supporting it (Stewart et al. 2007).

2.2.4.2 Vaccination

While early detection of asymptomatic precancerous lesions by screening and their effective treatment leads to the prevention of ICC and premature death, the fact that CC is caused by persistent infection by one or more of the 15 oncogenic HPV types, with HPV types 16 and 18 causing more than 70% of cervical cancers, provides the exciting opportunity for prevention through vaccination.

Vaccinations with virus VLP were first found to be safe and highly protective in animal models. Rabbits that were immunized with cotton-tail rabbit papillomavirus (CRPV) VLPs were protected upon CRPV challenge and neutralizing antibodies were detected in most of these studies (Breitburd et al. 1995, Jansen et al. 1995).

Currently, two vaccines against human papillomavirus infection are commercially available,bivalent Cervarix™ (by GlaxoSmithKline) and quadrivalent Gardasil™ (by Merck and Co. Incorporation). Both vaccines are made from L1 VLPs i.e. empty protein shells resembling the real virus, for which they are non-infectious. Both vaccines are targeted against HPV types 16 and 18 that are estimated to cause altogether 70% of cervical cancers worldwide (Muñoz et al. 2004). In addition, Gardasil additionally targets two low-risk HPV types, HPV6 and 11 that cause 90% of genital warts.

VLP vaccines to prevent papillomavirus infections in humans have been evaluated in several studies (phase I to phase III) (Harper et al. 2006, Koutsky et al. 2002, Koutsky and harper 2006, Villa et al. 2006, The Future II Study Group 2007, Paavonen et al. 2007). The results from these studies indicate that HPV VLP vaccines are highly immunogenic in early adolescents (aged 9–13) and young women. The antibody titres are 2-fold higher among prepubertant boys and girls than among young women aged 16–26 years (Villa et al. 2005, Pedersen et al. 2007, Reisinger et al. 2007, Block et al. 2007, Dawar et al. 2007, Petäjä et al. 2009). This is in concordance with high immuno-response of other vaccines in children (Reisinger et al. 2007). In phase II clinical trial, quadrivalent HPV6, 11, 16 and 18 L1 VLP-induced antibody titres reach their highest level 7 months following the first vaccine dose. The titres then decline, reaching a plateau 18–24 months later. This plateau is maintained for at least 5 years, with 5-year levels that are similar to the titres naturally induced by HPV types 6 and 18 and that are higher than the titres naturally induced by HPV types 11 and 16 (Villa et al. 2006). At 24-month follow-up, over 96% of participants in the HPV vaccine group were seropositive for HPV types 6, 11 and 16; however, only 68% of participants were seropositive for HPV type 18. The significance of this reduction remains unclear as immune memory is induced by the vaccine (The Future II Study Group 2007). This has not been noted with the AS04-adjuvanted bivalent HPV16/18 vaccine (Harper et al. 2006). Geometric titers (GMTs) for vaccine induced antibodies against HPV16 or HPV18 infections were substantially higher (≥11-fold) than those seen in natural HPV16 or HPV18 infections at all timepoints over 5.5 years (Harper et al. 2006). High seroconversion rates and strong vaccine-induced antibody response were also evident in the interim (14.8-month) results of the papilloma trial against cancer in young adults (PATRICIA) study, a large phase III trial of the AS04-adjuvanted HPV16/18 vaccine. At month 7, peak GMTs were 313-fold (HPV16) and 211 (HPV18) higher than corresponding values in women who had cleared natural HPV16/18 infections (Paavonen et al. 2007).

The results from vaccine efficacy (phase III) trials have shown high efficacy against HPV 16 and 18-related CIN 2/3 and adenocarcinoma *in situ* lesions among

adolescents and young women naïve for these HPV types and received at least one dose (of the three recommended) of the vaccine. The quadrivalent vaccine prevented 100% of HPV16 and HPV18-related cases of high grade (grade 2/3) vulvar or vaginal neoplasia, and 95% of HPV6, 11, 16, 18-related cases of CIN (grade 1, 2, 3) (The Future II Study Group 2007). The AS04-adjuvanted HPV16/18 vaccine demonstrated 90%–100% efficacy in preventing CIN 2+ and 89% efficacy in preventing CIN 1+ associated with HPV16/18 infections (Paavonen et al. 2007, Paavonen et al. 2009). Results from the phase II trial showed that the HPV16/18 vaccine efficacy was 100% in terms of preventing HPV16/18-associated CIN 1+ or CIN 2+ after at least 5.5 years of follow-up (Harper and Dubin 2007) .

HPV vaccination holds great promises for CC prevention, but there are still several issues that need to be resolved before it can be widely implemented in high-risk developing countries (Agosti et al. 2007). These include: current high costs of the vaccine, affordability, feasibility, acceptability, logistics of vaccine delivery (in view of the needs for three doses spread over 6 months, improved strategies and vaccine platforms to reach out to pre- or early-adolescent girls), long–term immunogenicity and efficacy in preventing cervical neoplasia, cross-protection against HPV types not targeted by the vaccine antigens and the efficacy of different, more logistically feasible dose regimes in including and maintaining immunogenicity and long term protection against cervical neoplasia. To initiate HPV vaccination in low- and medium-resource countries, vaccination costs should be dramatically reduced both by lowering the costs of the vaccine and of vaccine delivery.

A population-based phase III study in Finland will consider the long-term outcomes in HPV16/18-vaccinated adolescents versus those receiving a control vaccine or no HPV vaccine at all (Lehtinen et al. 2006). While prophylactic vaccination is likely to provide important future health gains if vaccination is offered to girls before onset of sexual activity, cervical screening should still be continued for women, as the risk of being already infected with the oncogenic HPV types remains. For the vaccinated birth cohorts the mode of screening most likely will change in years to come.

3 Aims of the study

The general purpose of this work is to contribute to understanding the role of smoking in the natural history of HPV infection and its progression to CC.

The specific aims were:

1. To ascertain whether the association between smoking and CC is due to an independent role of smoking or due to residual confounding by oncogenic HPV types.

2. To compare the association between tobacco chewing in Côte d'Ivoire, West Africa and tobacco smoking in Finland, Northern Europe, and the age-stratified risk of multiple HPV infections and cervical SIL.

3. To evaluate the role of smoking in the impairment of the immune response following natural HPV16 and HPV18 infection.

4. To evaluate the role of smoking in immune response following immunization with adjuvanted HPV16/18-AS04 VLP vaccine.

4 Materials and methods

4.1 Data sources and study participants

4.1.1 The Nordic population-based biobanks (Paper I)

4.1.1.1 Characteristics of the serum banks

Five population-based serum banks collaborated in this nested case-control study: the Finnish Maternity Cohort (FMC), the Iceland Maternity Cohort (IMC), the Northern Sweden Maternity Cohort (NSMC), the Northern Sweden Health and Disease Study (NSHDS) and the JANUS Project in Norway.

The FMC was established in 1983. By 2006 it comprised around 1,300,000 serum samples collected from 750,000 women, practically all pregnant women attending maternity clinics during the first trimester of pregnancy. The samples are stored at -25˚C at the National Institute for Health and Welfare laboratories in Oulu, Finland (Pukkala et al. 2007).

The nation-wide IMC contained 96,000 serum samples, by 2003, collected from 50,000 women at 12 to 14 weeks of pregnancy for rubella screening. The samples have been stored since 1980 at -20˚C in the Department of Medical Virology, Landspitali University Hospital, Reykjavik, Iceland (Pukkala et al. 2007).

The NSMC consists of sera collected since 1975 from pregnant women screened for rubella immunity during week 14 of pregnancy in Västerbotten county and especially in the 1980s for some of the adjacent counties in Northern Sweden. So far, almost 120, 000 samples from 86,000 women have been stored at -20˚C at the virus laboratory of Umeå University, Sweden (Pukkala et al. 2007).

The NSHDS contains the Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) cohort and the Västerbotten Intervention Program (VIP). The MONICA project contains material from population-based screenings for cardiovascular diseases carried out in 1986, 1990, 1994, 1999 and 2004. There are 14,000 sampling occasions of 9,000 individuals, 50% of whom are included in VIP. Established in 1985, the VIP is a long-term project intended for health promotion and including 74,000 individuals by 2004, of whom 70,000 had donated blood samples. The samples are collected annually from all residents aged 40, 50 and 60 years of age, in the Västerbotten county, northern Sweden, and stored as plasma at -80˚C at the University Hospital of Umeå, Sweden (Pukkala et al. 2007, Tedeschi et al. 2006).

The JANUS serum bank was established in Oslo, Norway in 1973 to identify early changes in chronic disease development. In 2003 the JANUS serum bank had 430,000 serum samples from 331,801 donors, 10% of them are Red Cross blood donors. About 145,000 female donors have been recruited during routine health

examinations in subsequent phases. Serum samples in 1974–1978 and in 1983–1991, are being stored at -25˚C (Pukkala et al. 2007).

4.1.1.2 Population-based cancer registries

The cancer registries of Finland, Iceland and Norway and the regional cancer registry in Umeå, Sweden are all population-based and country-wide (four northernmost Swedish counties for Umeå). Audits have shown that virtually all cases of cancer are being reported to the cancer registries by physicians and pathology/hematology laboratories, and 95% of them are histologically confirmed (Engeland et al. 1995). Following approval of the appropriate ethics committees, serum banks were linked to the national cancer registries using the unique personal identification number (PIN) available in the Nordic countries for each citizen.

4.1.1.3 Identification of cases and controls

To elaborate the association of smoking and CC, we performed two consecutive nested case-control studies (Studies I and II, based on 171 and 588 invasive CC cases matched respectively to 496 and 2861 unaffected controls). Study I had limited power to distinguish whether smoking was an independent co-factor in cervical carcinogenesis and in Study II, we assembled almost four times as large independent material. Most of cases in Study I were included in Study II (Table 4.1).

Cases were women diagnosed with invasive CC following their enrolment into one of the Nordic cohorts. Cases were identified by linking the serum bank data files with the national cancer registries. When serial blood samples were available, the first sample collected with the longest lag time to cancer diagnosis was chosen.

Controls were selected from the same serum bank as cases (Table 4.1). For each case, five female controls who were alive and free of cancer at the time of the case's diagnosis were randomly selected and matched for age at serum sampling (± 2 years), storage time (± 2 months) and for county in Norway and Sweden. If five controls could not be found, matching criteria of age at blood collection and length of frozen storage were widened.

The average lag times from serum sampling to cancer diagnosis was 4.7 years in Study I and 9.6 years in Study II. In Study I, the mean ages at diagnosis were as follows, the FMC: 34 years, the JANUS health examinations: 47 years, Norway (1974–1978): 46 years, Norway (1981–1992): 47 years and the NSHDS: 50 years. In Study II, the mean ages at diagnosis were as follows, the FMC: 38 years, the JANUS health examinations: 49 years, Norway (1974–1978): 56 years, Norway (1981–1992): 47 years, the JANUS Red Cross blood donors: 44 years, the NSMC: 37 years and the IMC: 34 years.

TABLE 4.1 Characteristics of the Nordic cohort for a nested case-control study of the cervical carcinoma risk associated with previous exposure to tobacco smoke.

Serum bank	Geographic location	Period of serum sampling	Female serum sample donors (no.) by the end of year 2003	Cases (no. in Study I/ Study II)	Controls (no. in Study I/Study II)	Age at diagnosis (years) in Study I/Study II
Janus Project	Three counties in Norway Several counties in Norway Red Cross blood donors in capital	1974–1978 1981–1992 1973–1991, 1997–2000	29,000 115,000 14,000	79/36 47/129 –/45	237/178 141/639 –/223	26–63/32–70 35–63/29–67 –/29–69
Northern Sweden Health and Disease Study (VIP & MONICA)	Two northernmost counties in Sweden	1985–present	38,000	4/–	12/–	39–60/–
Northern Sweden Maternity Cohort	Four northernmost counties in Sweden	1975–present	86,000	–/111	–/530	–/21–57
Finnish Maternity Cohort	Finland	1983– present	681,000	41/167	106/798	22–49/22–54
Icelandic Maternity Cohort	Iceland	1980– present	49,000	–/100	–/493	–/23–55
Total	Nordic countries	1973– present	1,008,000	171/588	496/2861	22–63/21–70

4.1.2 The DYSCER – Côte d'Ivoire study (Papers II)

The disease screening research study (DYSCER) was conducted from April 1995 to February 1996. The study took place in three outpatient gynaecology clinics of Abidjan, Côte d'Ivoire. Women attending the clinics were eligible for the study if they fulfilled the following inclusion criteria: age between 20 and 50 years, absence of history of lower genital tract neoplasia, and absence of active pregnancy. A questionnaire was administered to each participant to obtain information on tobacco use. The subjects underwent pelvic examination and the cervical Pap smears obtained were independently read twice, in Abidjan and in France, in order to minimize false negative/positive results. Colposcopy with biopsies was performed on women with HSIL. Cases included all women with cytological cervical SIL. Controls were chosen at random during the same time and in the same clinics among the women without cytological cervical lesions with a frequency matching for age (by decade) and center. A complete description of this material has been published previously (La Ruche et al. 1998).

4.1.3 The KUOPIO Cohort (Paper II, III)

A prospective follow-up study of 532 women (mean age 28.7 years, range 15 to 69 years) with cervical HPV infection was conducted at the Kuopio University Hospital, Kuopio, Finland between 1981 and 1992. The original aim was to establish prognostic factors associated with the clinical course of HPV infections identified in young women by cytology. The cohort participants were recruited among women whose routine Pap smear showed unequivocal changes of HPV infection, i.e., koilocytotic cells with or without dyskaryotic abnormalities. The participants were subjected to a thorough gynecological examination including colposcopy, repeat Pap smear, and directed punch biopsy (when clinically indicated). A questionnaire was also administered to each woman to obtain information on medical history and smoking habits. After the first visit, all the participants were scheduled to attend the gynecological outpatient clinic of the Kuopio University Hospital for follow-up visits at 6-month intervals. Cervical smears and serum samples were collected at each visit. A complete description of this material has been published previously (Kataja et al. 1992).

The comparative study (Paper II) included 2162 women (mean age 29.4 ± 7.1 years, range 20–50 years) from Côte d'Ivoire and 419 women (mean age 27.8 ± 7.9 years, range 15–49 years) from Finland with complete baseline data on HPV status, tobacco use, Chlamydia trachomatis infection and HIV status.

In Paper III, we collected data from the Kuopio Cohort. A total of 191 participants (mean age 27.5 ± 6.9 years, range 16-49 years) who had available HPV DNA biopsy data at the end of the follow-up, and for whom at least two serum samples (first and last samples of the follow-up) were available for serology and cotinine analyses, were included in the analyses.

These study protocols were each approved by the respective ethics committee in Côte d'Ivoire, France and Finland.

4.1.4 The PATRICIA Study (Paper IV)

Enrolment for the papilloma trial against cancer in young adults (PATRICIA) study took place from April 2004 to May 2005 in Finland. Healthy women aged 16–17 years were eligible to participate in the Finnish arm of this study with no exclusion criteria with regard to lifetime sexual partners before study enrolment. Individuals with intact cervix and agreeing to adequate contraception (barrier methods in combination with a spermicide or hormonal contraception) over the vaccination period were eligible for inclusion. Exclusion criteria were limited to women with a history of colposcopy, who were pregnant, or breastfeeding, or who had chronic or autoimmune disease or immunodeficiency.

Informed consent was obtained from each participant to the performance of any study procedures. All recruitment materials, informed consent/assent forms, protocols and amendments were approved by the Finnish National Ethical Review Committee. This study is registered with the European Clinical Trials Database.

This was a phase III double-blind, randomized controlled trial. In Finland, a total of 4,808 participants were randomised in a 1:1 fashion with an internet-based centralized randomisation system received either the AS04-adjuvanted HPV16/18 vaccine (GlaxoSmithKline Biologicals, Rixensart, Belgium) or, a control hepatitis A vaccine (GSK Biologicals), to provide a health benefit for all participants and ensure double-blinding. Allocation of treatment numbers was stratified by study site and by age. Because the study is gradually ending, a random sample of 216 study participants with individual vaccine allocation unblinded, wase selected from the three biggest Finnish study sites: the Finnish Family Federation, Helsinki, the University of Helsinki, Helsinki and the University of Tampere, Tampere for this study.

Each dose of HPV-16/18 L1 VLP AS04-adjuvanted candidate vaccine (Cervarix™) contained 20 mg each of HPV-16 and -18 L1 proteins self-assembled as VLP and adjuvanted with AS04 (50 mg 3-O-desacyl-40-monophosphoryl lipid A [MPL] and 500 mg aluminum hydroxide). The vaccine was produced using a Baculovirus Expression Vector System in which each type of VLP antigen was produced on a Hi-5 cell line derived from *Trichoplusia ni*. Each dose of the control hepatitis A vaccine contained 720 ELISA units (EU) of inactivated hepatitis A antigen and 0·5 mg aluminium hydroxide. The vaccines were supplied under double-blinding in identical 0·5 ml prefilled syringes and administered into the deltoid muscle on a 0, 1, and 6-month schedule. According to protocol the code was broken for the subset selected for this study in March 2009.

Blood samples were collected at baseline and at month 7 post-vaccination, and stored at the National Institute for Health and Welfare laboratories in Oulu,

Finland from each participant before the first vaccination and at month 7 to assess vaccine-induced immune responses to HPV16, HPV18 or related factors.

Participants completed and returned safety diary cards documenting symptoms experienced during the first 7 days after vaccination and symptoms within the first 30 days after vaccination as previously described (Paavonen et al. 2007).

4.2 Laboratory methods

For all the studies, the laboratory analyses were performed on coded specimens, with case-control status masked. The samples of a case and her matched controls were pipetted on the same microplate.

4.2.1 Cotinine detection (Papers I to IV)

Blood samples were measured for cotinine using a qualitative immunoassay method (OraSure Technologies, Bethlehem, Pennsylvania) that is carried out as a quantitative assay and is based on the competition between free cotinine in the sample and cotinine bound to horseradish peroxidase-labelled cotinine. Cotinine concentration was quantified by measuring the light absorbance of wavelengths of 450 nm and 630 nm and by comparing the cotinine concentration of each sample with the standard curve. This assay has a sensitivity of 95%–97% and a specificity of 99%–100% (Murray et al. 1993, Parish et al. 1995, Pérez-Stable et al. 1995). Careful testing of this method has revealed excellent correlations with established gas chromatography (Feyerabend et al. 1990) and radioimmunoassay (Knight et al. 1985). Regression dilution bias was assessed by measuring paired samples repeatedly with the same batch from the assay kit (Clarke et al. 1999).

4.2.2 HPV serology and detection (Papers I to IV)

The presence of IgA (Paper III) and G (Paper I, III, IV) antibodies specific for HPV types 16 and 18 was determined by means of a standard enzyme-linked immunosorbent assay (ELISA) as reported elsewhere (Dillner et al. 1996; Lehtinen et al. 2006). For the IgA assay, goat anti-human IgA HRP conjugate antibody (Novus Biologicals, Colorado, USA) was used for detection. IgG analyses were done applying VLPs kindly provided GSK Biologicals.

We employed baculovirus-expressed capsids comprising both the L1 and L2 proteins, with disrupted capsids of bovine papillomavirus used as the negative control. The specificity of HPV serology was found to be high, since no antibodies could be found in serum samples from virginal women and seropositivity had

a linear relation to lifetime number of sexual partners (Dillner et al. 1997). The sensitivity of HPV serology for detecting past HPV infection has been estimated to vary between 50% and 70% (Carter et al. 1996, Kjellberg et al. 1999). Seropositivity was defined as a titer greater than or equal to the assay threshold for HPV16 and HPV18. Dilutions 1/30, 1/300 and 1/3000 were used to identify the linear part of the absorbance reactions for expression of the results as OD values.

HPV DNA was detected using PCR (Côte d'Ivoire) (Paper II) and ISH (Finland) (Papers II and III) techniques. In Côte d'Ivoire, cervical specimen for HPV detection were collected with a plastic brush (Viba-Brush, CML, Nemours, France), placed in a transport medium (sterile RPMI 1640 medium supplemented with penicillin and fungizone), and frozen at -80˚C until testing. The presence of cervical HPV was tested in 547 participants including 224 women with cervical SIL (151 LSIL, 60 HSIL, and 13 cervical cancer cases) and 323 controls. HPV DNA was detected with a polymerase chain reaction (PCR)-based method using HPV L1 consensus primers (MY11 and MY09) affording the detection of a broad spectrum of genital HPV types as previously described (La Ruche et al. 1998).

For HPV DNA-positive samples, restriction fragment length polymorphism analysis of the amplimers produced during PCR was used to determine HPV types 6, 11, 13, 16, 18, 31, 33, 34, 35, 39, 40, 42, 44, 45, 52, 53, 56, 58, 59, 61, 62, 64, 66, 67, 68, 70, 71 (CP8061), 81 (CP8304), 82 (MM4 et IS39), 83 (MM7), 84 (MM8). Only samples that gave a strong enough signal to be detected clearly on gels after enzymatic digestion were considered for HPV typing. In Finland, formalin-fixed, paraffin-embedded biopsies from all patients were analysed using DNA-ISH technique with S- and biotinylated HPV DNA probes as described earlier (Syrjänen and Syrjänen 1986). Specific DNA probes for HPV types 6, 11, 16, 18, 31, and 33 were used. The presence of HPV DNA sequences in the lesions was indicated by dense condensations of black–silver grains, usually superimposed on the nuclei of the intermediate and superficial cells, in HPV lesions.

4.2.3 *Chlamydia trachomatis* serology (Papers I, II and III)

The presence of IgG antibodies specific for *C. trachomatis* was determined by the microimmunofluorescence (Hanna et al. 1980) (Paper I) and ELISA (Dillner et al. 1999) (Paper II, III) methods according to the manufacturer's instructions.

4.2.4 HSV-2 serology (Paper I)

The presence of IgG antibodies HSV-2 was also determined using a commercially available HSV-2 glycoprotein gG-2-based ELISA (Biokit SA, Barcelona, Spain) according to the manufacturer's recommendation (Lehtinen et al. 2002).

4.2.5 HIV (Paper II)

All sera from Ivorian women were screened in Abidjan by ELISA (Genelavia Mixte, Sanofi Diagnostics Pasteur, Paris, France). In Finland, there were no HIV-positive women diagnosed in the catchment area of Kuopio University Hospital between 1981 and 1986 (National Institute for Health and Welfare, Finland 2007).

4.3 Statistical methods

Paper I

The cotinine level that is accepted as defining an active smoker depends on the prevalence of smoking in the population (Murray et al. 1993, Cummings and Richards 1988). In this paper, the measured cotinine levels were categorized into 3 groups: less than 20 ng/mL for nonsmokers or persons passively exposed to tobacco smoke and 2 other categories of 20-<100 ng/mL and ≥100 ng/mL, corresponding approximately to average levels found among light and heavy smokers.

Relative risks, expressed as odds ratios (ORs), were estimated using conditional logistic regression for matched case-control sets of cervical cancer by means of GLIM4 (Numerical Algorithms Group, Oxford, United Kingdom) (Breslow and Day, 1980). The 95% confidence intervals (CIs) for the ORs were based on profile likelihood (Nelder, 1990). Heterogeneity in the point estimates was assessed with a likelihood ratio test. The nested models compared were one for overall effects of cotinine groups (20-<100ng/ml and ≥100 ng/ml) and the other one also including serum bank-specific effects of the cotinine groups. Unconditional logistic regression was applied to HPV16/18-seropositive cases and controls, including the matching variables (serum bank, subcohort, storage time and age at serum sampling) in the model.

Increasing tobacco smoke exposure was defined by blood cotinine levels. The cotinine levels for evaluating the relationship between cotinine dose and risk of invasive CC in Study II were quartiles among control women with cotinine level >20ng/ml and the cut-off level for heavy smoking which was close to the median, 102ng/ml. In the dose-response analyses the quartiles (68, 100, and 140ng/ml) of active smokers (>20ng/ml) were used. The dose-response relationship was tested by the likelihood ratio test by adding the numerical dose-response variable to the threshold model in which a variable indicated those, whose cotinine level was at least 20ng/ml. Likelihood ratio tests for linear trends in the logarithms of ORs were performed for increasing age at diagnosis among women with cotinine level 20-<100ng/ml and 100ng/ml or more with and without adjustment for HPV16/18, HSV-2 and *C. trachomatis*.

Paper II

In this study, different cut off levels were tested, and the optimum value, cotinine level of 20 ng/ml, was used as the cut-off. Descriptive statistics were used to determine the distribution of women according to cervical SIL, age and tobacco exposure, and HPV positivity. Frequency tables for tobacco use (smoking and/ or chewing) among women stratified by age were produced. The effect of tobacco smoking and chewing on the risk of LSIL, multiple HPV infections and HSIL was estimated by ORs with 95% CI, derived from the logistic regression analysis. Because very few Ivorian women reported smoking evaluation for this habit alone in the logistic regression analysis was restricted to Finnish women. We conducted stratified analyses according to age, and the ORs were adjusted for C. *trachomatis* and HIV seropositivity (for Ivorian women). The level of statistical significance was $p<0.05$. All the statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, USA).

Paper III

Descriptive statistics were used to analyse the distribution of HPV16/18 antibody isotypes at baseline. McNemar's test was used to determine the proportion of change over time in HPV serostatus (dichotomous outcome variable) to take into account the fact that the observed proportions are not independent. Seroconversion and persistence of HPV16/18 antibodies were assessed. Logistic regression models were fitted and the relative risks (estimated as OR and 95% CI) calculated using SPSS13.0 (SPSS Inc., Chicago, USA). The model estimated the odds of seroconversion and the odds of maintaining persistent antibody response for HPV16/18 smokers, and used individuals with cotinine level less than 20 ng/ml, as the reference category. The ORs were adjusted for chlamydia seropositivity, a marker of high-risk sexual behaviour. We also conducted stratified analyses according to age. The level of statistical significance was $p<0.05$.

Paper IV

Immunogenicity analyses were based on the according-to-protocol (ATP) cohort. The analyses included all women who met the eligibility criteria, received the three vaccination shots and complied with protocol procedures. Seropositivity rates for HPV16 and HPV18 were calculated. *P-values* were calculated with Mann-Whitney test to compare mean absorbance of anti-HPV16 and HPV18 antibodies between non-smokers and smokers at each timepoint in the vaccine and control groups. Mean absorbance level for each antigen was reported with standard deviation (±Sd). Statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, USA).

5 Results

5.1 Tobacco smoking as risk factor for invasive cervical cancer (Paper I)

Effect of increasing tobacco smoke exposure on cervical cancer risk

We found that the relative risk of CC increased with increasing serum cotinine levels. In both Study I cohort and Study II cohort serum cotinine level ≥100ng/ml, which indicated heavy smoking among females was associated with an increased risk of invasive CC (OR = 1.7, 95% CI = 1.2–2.5; OR = 2.5, 95% CI = 2.0–3.0 respectively). In the Study II cohort the HPV16 and 18 adjusted OR of SCC was markedly stronger in women with cotinine level ≥100ng/ml: 3.2 (95% CI = 2.6–4.0) compared to women with cotinine level 20-<100ng/ml: 2.2 (95% CI = 1.7–2.8) with only negligible overlap of the 95% CIs. Further adjustment for antibodies to HPV16/18, HSV-2 and *C. trachomatis* had no material effects on the point estimates. In both study cohorts, no serum cotinine levels indicative of smoking were associated with an excess risk of cervical adenocarcinoma.

Effect of increasing tobacco smoke exposure on cervical cancer risk among HPV16/18-seropositive and seronegative women

The large sample size of the Study II cohort allowed stratified analysis by HPV status among cases and controls. In HPV16/18-seropositives, significantly increased relative risk of invasive CC and of SCC were noted in women with cotinine level ≥100ng/ml (OR = 2.1, 95% CI = 1.4–3.2, OR = 2.7, 95% CI = 1.7–4.3 respectively). Adjustment for antibodies to HSV-2 and *C. trachomatis* had no effect on the point estimates. Among HPV16/18-seropositive women, cotinine level 20-<100 ng/ml was not associated with the risk of invasive CC (OR 1.1, 95% CI = 0.7–1.8). In HPV16/18-seronegatives, an increased relative risk of invasive CC and SCC was observed both in women with cotinine level 20-<100 ng/ml and ≥100 ng/ml (Table 5.1).

Table 5.1 Odds ratios (OR) for risk of cervical cancer in human papillomavirus (HPV) type 16 and/or 18 seropositive and seronegative non-smokers and smokers by increasing tobacco smoke exposure (defined as blood cotinine level), crude and adjusted for antibodies to herpes simplex virus type 2 (HSV-2) and *Chlamydia trachomatis* (Study II)

Cotinine (ng/ml)	Invasive cervical cancer		Squamous cell carcinoma	
	Crude OR 95% CI	**OR adj* 95% CI**	**Crude OR 95% CI**	**OR adj* 95% CI**
	HPV type 16 and/or 18 seropositives			
	253 cases 597 controls	247 cases 589 controls	202 cases 460 controls	197 cases 452 controls
<20	1	1	1	1
20-<100	1.1 (0.7, 1.8)	1.1 (0.7, 1.8)	1.2 (0.7, 2.0)	1.1 (0.6, 2.0)
100+	2.1 (1.4, 3.2)	2.1 (1.4, 3.2)	2.7 (1.7, 4.3)	2.7 (1.7, 4.4)
P value†	<0.001	<0.001	<0.001	<0.001
	HPV type 16 and/or 18 seronegatives			
	335 cases 2261 controls	330 cases 2234 controls	243 cases 1706 controls	240 cases 1686 controls
<20	1	1	1	1
20-<100	2.0 (1.6, 2.6)	1.8 (1.4, 2.3)	2.7 (2.0, 3.6)	2.2 (1.7, 3.1)
100+	2.6 (2.0, 3.4)	2.3 (1.8, 3.0)	3.4 (2.5, 4.5)	2.9 (2.2, 4.0)
P value†	0.04	0.05	0.06	0.06

* OR adjusted for herpes simplex virus type 2 (HSV-2) and *C. trachomatis*.
† Test for dose-response relationship (see statistical methods).

5.2 Tobacco smoking and chewing as risk factors for cervical SIL (Paper II)

The proportion of women exposed to tobacco was almost ten times higher in Finland (36.8%) than in Côte d'Ivoire (3.7%). In Côte d'Ivoire, tobacco chewing was more common than tobacco smoking (2.6% versus 1.4%), and concerned mostly women ≥30 years of age (4.8%).

Among tobacco chewers (Côte d'Ivoire), the risk of HSIL was five times higher in both young (OR = 5.5, 95% CI = 1.2–26) and older (OR = 5.5, 95% CI = 2.1–14) women compared to non-chewers (Table 5.2). Overall, the age-adjusted relative risk of cervical HSIL was also significantly increased among tobacco chewers (OR = 5.6, 95% CI = 2.5–12). In Finland, tobacco smokers (Finland) ≥30 years of age tended to have an increased risk of LSIL (OR = 2.5, 95% CI = 0.9–6.8) and HSIL (OR = 2.2, 95% CI = 0.5–8.7) (Table 5.2).

Table 5.2 Age-stratified tobacco smoking/chewing-associated OR with 95% CI of low-grade lesions and high-grade lesions in women from Côte d'Ivoire and Finland

Variables	Low-grade lesions		High-grade lesions	
	OR	(95% CI)	OR	(95% CI)
Côte d'Ivoire	*n = 165*		*n = 71*	
Age < 30 years				
Non-chewers	1.0		1.0	
Chewers	n.a		5.5	(1.2–26)
Age ≥ 30 years				
Non-chewers	1.0		1.0	
Chewers	2.0	(0.7–5.8)	5.5	(2.1–14)
Finland	*n = 60*		*n = 28*	
Age < 30 years				
Non-smokers	1.0		1.0	
Smokers	1.2	(0.6–2.5)	1.1	(0.4–3.1)
Age ≥ 30 years				
Non-smokers	1.0		1.0	
Smokers	2.5	(0.9–6.8)	2.2	(0.5–8.7)

Further, analyses were restricted to HPV DNA-positive women to assess independent tobacco smoking- and chewing-associated risk of cervical LSIL and HSIL. Despite the small numbers of women in both settings, we found an increased, albeit not significant, relative risk of both LSIL and HSIL, in HPV DNA-positive women ≥ 30 years of age and actively exposed to tobacco through smoking (OR = 2.2, 95% CI = 0.5–9.0 and OR = 1.8, 95% CI = 0.3–9.6 respectively) or chewing (OR = 2.3, 95% CI = 0.2–27 and OR = 7.3, 95% CI = 0.8–68, respectively).

5.3 Tobacco smoking and chewing as risk factors for multiple HPV infections (Paper II)

Being infected with two or more HPV types (multiple HPV infections) was common in HPV16 and/or 18-seropositive women (60.4% in Finland and 47.2% in Côte d'Ivoire). Antibodies to three HPV types or more were detected in 20 of 57 (35.1%) Ivorian and 50 of 129 (38.7%) Finnish women. The logistic regression analyses, however, did not show any increased risk of multiple infections among tobacco smokers/ chewers compared to non-smokers/chewers.

5.4 Effect of tobacco smoking on the immune response following genital HPV16/18 infection (Paper III)

Effect of smoking on the development of HPV16/18 antibody response

We assessed the effect of smoking on the development of HPV16/18 antibodies in initially HPV16/18 seronegative women as odds ratio. In women under 30 years of age, only three out of 29 (10%) smokers turned positive for at least one HPV16/18 antibody isotype (IgG/IgA) compared to 13 of 41 (32%) non-smokers (OR, 0.2; 95% CI 0.0–0.9) (Table 6). Smoking-associated relative risk of HPV16/18 seroconversion was not significantly decreased in the older age group (Table 5.3). There was no difference between HPV DNA positive or negative women with regard to the development of HPV antibodies.

TABLE 5.3 Smoking-associated relative risk (OR and 95% CI) of developing HPV16/18 antibodies in initially seronegative females with cytological atypia consistent with HPV infection

HPV16/18 seronegative at baseline	Change in serostatus n/N[d] (%)	OR (95%CI)	OR[a] (95%CI)
Age < 30 years			
IgA only			
Non-smokers[b]	7/47 (15)	1	1
Smokers[c]	1/32 (3)	0.1 (0.0–1.5)	0.2 (0.0–1.7)
IgG only			
Non-smokers	3/37 (8)	1	1
Smokers	2/20 (10)	1.2 (0.1–8.2)	1.1 (0.1–7.6)
IgA and IgG			
Non-smokers	13/41 (32)	1	1
Smokers	3/29 (10)	0.2 (0.0–0.9)*	0.2 (0.0–0.9)*
Age ≥ 30 years			
IgA only			
Non-smokers[b]	2/16 (12)	1	1
Smokers[c]	3/8 (37)	4.2 (0.5–32)	4.8 (0.3–59)
IgG only			
Non-smokers	2/26 (8)	1	1
Smokers	1/20 (5)	0.6 (0.1–7.5)	0.7 (0.1–8.4)
IgA and IgG			
Non-smokers	2/15 (13)	1	1
Smokers	2/7 (28)	2.6 (0.3–23)	3.2 (0.2–45)

[a] OR adjusted for Chlamydia trachomatis.
[b] Cotinine <20ng/mL; reference group.
[c] Cotinine ≥20ng/mL.
[d] n = number of females who changed their serostatus over time.
N = total number of non smoking and smoking females in each strata.

Effect of smoking on the stability of HPV16/18 antibody positivity

We assessed the smoking-associated relative risk of maintaining HPV16/18 antibody positivity in initially HPV16/18 seropositive women. We found a significantly decreased risk of constant IgG antibody positivity (OR, 0.1; 95% CI 0.0–0.8), based on one seroconversion case, among smokers. In women under 30 years of age, 13 of 21 (62%) smokers remained IgA or/and IgG positive until the end of follow-up compared to 20 out of 26 (77%) non smokers (Table 5.4). Smoking did not materially affect the risk of IgG antibody waning in women over 30 years of age (Table 5.4).

TABLE 5.4. Smoking-associated relative risk (OR and 95%CI) of persistence of HPV16/18 antibodies in initially seropositive females with cytological atypia consistent with HPV infection

HPV16/18 seropositive at baseline	**No change in serostatus n/N[d] (%)**	**OR (95%CI)**	**OR[a] (95%CI)**
Age < 30 years			
IgA only			
Non-smokers[b]	4/10 (40)	1	1
Smokers[c]	7/11 (64)	2.6 (0.4–15)	2.8 (0.5–17)
IgG only			
Non-smokers	8/12 (67)	1	1
Smokers	1/8 (12)	0.1 (0.0–0.8)*	0.1 (0.0–0.8)*
IgA and IgG			
Non-smokers	20/26 (77)	1	1
Smokers	13/21 (62)	0.4 (0.1–1.7)	0.4 (0.1–1.7)
Age ≥ 30 years			
IgA only			
Non-smokers[b]	7/18 (39)	1	1
Smokers[c]	2/6 (33)	0.2 (0.0–6.9)	0.2 (0.0–6.8)
IgG only			
Non-smokers	10/14 (71)	1	1
Smokers	3/4 (75)	1.2 (0.1–15)	1.2 (0.1–15)
IgA and IgG			
Non-smokers	22/36 (61)	1	1
Smokers	9/11 (82)	2.8 (0.5–15)	2.8 (0.5–15)

[a] OR adjusted for Chlamydia trachomatis.
[b] Cotinine <20ng/mL; reference group.
[c] Cotinine ≥20ng/mL.
[d] n = number of females who changed their serostatus over time.
N = total number of non smoking and smoking females in each strata.

5.5 Effect of smoking on the immune response following prophylactic HPV16/18 AS04-adjuvanted VLP vaccination

A total of 216 young women from the Finnish arm were included in this pilot study. Out of 103 participants from the HPV16/18 group, 46.6% (48) were smokers compared to 56.6% (64) smokers in the control group.

We evaluated differences in mean absorbance of anti-HPV16 and HPV18 antibodies levels from baseline to month 7 post-vaccination. The results showed that the mean absorbance of anti-HPV16 antibody levels was 1.97 (±0.78) among non-smokers compared to 1.88 (±0.73) among smokers. The mean absorbance of anti-HPV18 antibody levels was 1.44 (±0.85) among nonsmokers and 1.36 (±0.76) among smokers. The observed differences between the two groups were not statistically significant (Table 5.5).

TABLE 5.5 Comparison of mean absorbance level (with standard deviation (Sd)) of anti-HPV16 and HPV18 antibodies between. Non-smokers and smokers by vaccination group

	Vaccine (n = 103)			**Placebo (n = 113)**		
	Non-smokers (n = 55)* mean (±Sd)	**Smokers (n = 48)* mean (±Sd)**	***p-value*†**	**Non-smokers (n = 49)* mean (±Sd)**	**Smokers (n = 64)* mean (±Sd)**	***p-value*†**
HPV16						
Baseline	0.12 (± 0.08)	0.29 (± 0.52)	*0.2*	0.21 (± 0.43)	0.18 (± 0.29)	*0.9*
Month7	1.97 (± 0.78)	1.88 (± 0.73)	*0.4*	0.01 (± 0.04)	0.01 (± 0.02)	*0.7*
HPV18						
Baseline	0.07 (± 0.06)	0.18 (± 0.08)	*0.1*	0.10 (± 0.08)	0.10 (± 0.14)	*0.8*
Month7	1.44 (± 0.85)	1.36 (± 0.76)	*0.6*	0.01 (± 0.03)	0.01 (± 0.03)	*0.7*

* Nonsmokers (cotinine < 20ng/ml) and smokers (cotinine ≥ 20 ng/ml).
† *p-value* derived from Mann-Whitney test to evaluate mean absorbance difference between non-smokers and smokers.

6 Discussion

6.1 Tobacco exposure of fertile-aged women

There are an estimated 1.3 billion adult smokers (over 15 years old) among the world's six billion people. If the prevalence of tobacco use remains constant, the number of smokers will rise to 1.7 billion between 2020 and 2025 (Guindon and Boisclair 2003). Four-fifths of current smokers live in low-income or middle-income countries.

There are important gender-specific differences in tobacco use, with global prevalence among males (48%) about four times higher than among females (10%) (Guindon and Boisclair 2003). There may, however, be considerable female smoking that is underreported, or unreported, because of gender norms that stigmatize women who smoked. Male-female differences in use are the highest in the Western Pacific Region and the lowest in the Americas and Europe, where about one quarter of women smoke (Carrao et al. 2000).

Typically, the smoking epidemic starts in most populations among men and higher-income groups, and later affects women and low-income groups. However, global male rates have peaked and have stabilized or are in slow decline, while the prevalence of tobacco use among women is increasing (Mackay 2001, Molarius et al. 2001). In fact, the historical gender differences in uptake and prevalence are diminishing because of the increased prevalence of smoking among adolescent women. Recent findings of the GYTS, the largest global survey of adolescents (aged 13 to 15) and tobacco use, show that almost as many adolescent girls and adolescent boys are smoking in many parts of the world (GYTS Collaborating Group 2003). This is an indication of the increasing global smoking epidemic among women that will not peak until well into the 21st century. The prediction is that by 2025, 20% of the female population will be smokers, up from 12% in 2005.

Tobacco exposure is a potential environmental cofactor of CC. Therefore, the high frequency of tobacco smoking and chewing among women emphasizes the importance of assessing its impact on the development and prevention of cervical neoplasia.

6.2 Comparison of the study with findings from other studies

6.2.1 Tobacco smoking is an independent risk factor for cervical cancer

Smoking was associated with an increased risk of ICC/SCC after both adjustment and stratification for antibodies to oncogenic HPVs. While the former could be confounded by misclassification bias in the serological diagnosis of hrHPV infection, the latter provided estimates unconfounded by persistent hrHPV infection as determined by serology with up tp 75% sensitivity in cases and controls (Kjellberg et al. 1999). The Nordic joint study (Pukkala et al. 2007) is by far the largest nested case-control study on risk factors of cervical cancer. Study I and Study II showed significant increased risk of ICC/SCC among women with cotinine level >100mg/ml. We found that the point estimate was lower in Study I relative to Study II. Women were from the same population and the same design was applied in both studies. Chance could be one explanation of this observed difference. Another explanation could be the age difference since the Study II material included older women compared with the Study I material. The carcinogenic effect of smoking is likely to increase with inceased age at diagnosis (This study, Plummer et al. 2003) and is usually observed after a long period of exposure to tobacco smoke (Gunnell et al. 2006). The Study II material allowed stratified analyses to assess the independent role of smoking. We found a significantly increased two-fold excess risk of SCC, free of the non-differential residual confounding bias, among HPV16/18-seropositive women.

HPV VLP serology has been proven to be a specific albeit not sensitive marker of current and past exposure to HPV (Olsen et al. 1997). It permitted the delineation of HPV16/18-infected (seropositive) controls that could be compared with HPV16/18-infected (seropositive) cases. Our results are consistent with the few prospective studies that have controlled for HPV infection using high quality HPV assays (Plummer et al. 2003). In the UK, Deacon et al. analySed risk factors for progression to CIN 3 among HPV-positive women in a nested case-control study and reported a high risk of 2.2 (95% CI = 1.4–3.4) for ever smoking with a strong evidence of a dose-response effect.

Several case-control studies (Bosch et al. 1992, Eluf-Neto et al. 1994, Chaouki et al. 1998, Santos et al. 2001, Hildesheim et al. 2001, Olsen et al. 1998, Rolon et al. 2000), including the IARC multicentre pooled analysis of 10 studies of ICC (Plummer et al. 2003), examined tobacco smoking as a co-factor to HPV infection by restricting the analysis to HPV DNA positive study participants. The results from these analyses showed no significant alteration in risk of CIN or CIS whether or not the study participants were HPV-DNA-positive. Most of these studies, however, tested for HPV-DNA at only one timepoint, which favoured the detection

of amplified HPV DNA in the cases. Furthermore, controls testing HPV-positive just once most likely have a transient infection. This could bias the measure for lifetime exposure and impact of HPV infection. Such a problem does not exist for HPV-seropositives since the possibility that the HPV antibodies wane over time is highly unlikely (af Geijersstam et al. 1998).

The presence of tobacco carcinogens in cervical mucus has been described as a possible biological explanation for the epidemiological association (Simons et al. 1995). The mechanism could involve soluble carcinogens that may have a direct transforming effect on squamous cervical epithelium. Some other epithelial cancers, those of the nasal cavity and the oesophagus, show similar differences between SCC and adenocarcinoma with regard to smoking and HPV (IARC 2004, Björge et al. 1997, Mork et al. 2001). Cigarette smoking may also exacerbate the carcinogenic potential of HPV specifically via inhibition of IFN-gamma and/or tumour necrosis factor alpha, leading to a significant inhibition of the apoptosis, which may promote tumor growth (Suk et al. 2001, Hussain et al. 2008). Experimental studies show that cigarette smoking decreases the secretion of proinflammatory cytokines (IL1, IL6) and suppresses IL2 and IFNγ production (Ouyang et al. 2000, Arcavi 2004), suggesting the possibility that cigarette smoking interacts to increase cervical carcinogenesis development.

6.2.2 Tobacco use (smoking/chewing) and the risk of cervical SIL

The route of exposure to tobacco was different between Côte d'Ivoire and Finland probably due to socio-economic and cultural differences. In addition, screening for cervical cancer and laboratory analyses for HPV detection were performed in two different settings and at different times. The differences observed in the association of tobacco exposure and SIL by the route of tobacco exposure may, however, be valuable for future studies. Comparison of the separated results was feasible since the method used for the PAP smear test was comparable, and assessment and grading of cervical SIL was based on a comparable method. The lesions were classified into LSIL and HSIL according to the Bethesda System.

In the Côte d'Ivoire material, we identified a lack of statistically significant association between tobacco chewing and the risk of cervical LSIL in Ivorian women ≥30 years of age. Despite the low prevalence of tobacco use, our results showed that women who chewed tobacco had a significantly increased risk of cervical HSIL. To date, only one study has reported an association between tobacco chewing and increased risk of invasive CC in Southern India (Rajmakar et al. 2006). Smokeless tobacco products contain and deliver high quantities of nicotine comparable to those typically absorbed from cigarette smoking. Moreover, these products are carcinogenic to humans and may result in local and systemic health hazards depending of the way of administration and the content of various toxic

products, including nicotine and tobacco-specific nitrosamines (Hoffman and Djordjevic 1997, Cogliano et al. 2004).

There was an increased risk of LSIL and HSIL among Finnish women ≥ 30 years of age who smoked tobacco. This is in line with recent studies showing that tobacco smoking increases the risk of cervical SIL and CC (Kjellberg et al. 2000, McIntyre-Seltman et al. 2005, Tolstrup et al. 2006). The point estimates, however, did not reach statistical significance possibly due to the small number of cases.

The general degree of concordance between PCR and ISH is between 70 and 90%, the main difference yielding in the sensitivity of the methods particularly among patients with high-grade lesions (Takuma et al. 2005, Dabie et al. 2004, Biedremman et al. 2004). The failure of HPV DNA hybridization studies to find all cervical HPV infections has been known for a long time (Leminen et al. 1992). Possible recall bias may have led to under-reporting of smoking habits but it is unlikely to have affected the results as the accuracy of self-reported tobacco smoking habit is generally high in Finland (Vartiainen et al. 2002).

We further restricted the analyses to HPV DNA-positive women to assess the independent role of tobacco smoking and chewing in cervical SIL. Although Ivorian and Finnish women ≥30 years of age tended to have an increased risk of LSIL and HSIL, we could not draw any conclusion due to the small number of women. Syrjänen et al. did not report an increased risk of high-grade CIN among smokers (Syrjänen et al. 2007). It is possible that being exposed to tobacco through smoking or chewing is not an independent risk factor of cervical SIL as the increased risk could be mediated by acquisition of hrHPV infection (Syrjänen et al. 2007). When restricting the analyses to women who were HPV DNA-positive, it is possible that we restricted attention to women whose immune response to HPV was partially inadequate and, in whom, therefore, smoking and chewing status could not have had an additional effect (Rousseau et al. 2003).

6.2.3 Tobacco use (smoking/chewing) and the risk of multiple HPV infections

There was no association between tobacco use (smoking and chewing) and the risk of multiple HPV infections. Kataja et al. previously reported that current smoking was a strong risk factor for HPV infection among Finnish women, although the risk did not increase in parallel with the number of cigarettes and years smoked (Kataja et al. 1993). More HPV types were detected in Côte d'Ivoire than in the Finnish study, but similar negative findings were reported among tobacco chewers or smokers. In Canada also, Rousseau et al. did not find an association between tobacco smoking and multiple HPV infections among women (Rousseau et al. 2003). No data have previously been published on the association between smoking/chewing and the risk of multiple HPV infections among women from less industrialized countries.

6.2.4 Tobacco smoking impairs the immune response following genital HPV16/18 infections

To the best of our knowledge, this is the first follow-up study to relate smoking status based on cotinine measurements to the likelihood of developing or maintaining HPV16/18 antibody positivity. Young women who smoked were less likely to either seroconvert or maintain detectable HPV16/18 antibodies over time relative to non-smokers.

Smoking impairs protection against and/or immunosurveillance of HPV infections in the uterine cervix (Syrjänen et al. 2007, Wiley et al. 2006). In a cross-sectional study, HPV16 DNA-positive women aged 16 to 23 years who smoked were less likely to test positive for HPV16 IgG antibodies than non-smokers (OR, 0.5; 95% CI 0.3–0.9) (Wiley et al. 2006). The authors suggest that there was a dose-response relationship between smoking and anti-HPV16 antibody positivity. In our study the observed effect of smoking was possibly also due to the high tobacco exposure in young women, who tended to be heavy smokers. The follow-up time was not the same for all the women (range: 6–114 months) and this difference may have an effect on the point estimates. This is however unlikely, as there was no difference in the length of follow-up time between HPV-seronegative and HPV-seropositive women at last sampling (Chi squarre (χ^2) = 14.46, df = 12, p = 0.2). In addition, we did not observe a difference in the length of follow-up time between non-smokers and smokers (χ^2 = 7.11, df = 12, p = 0.8).

Seroconversions against the HPV16 and 18 capsids are seen within up to a year following primary HPV16 and 18 infections (Wang et al. 2000, af Geijeirsstam et al. 1998), and IgA antibodies appear in a maximum of 20 months (Onda et al. 2003). Our findings showed that tobacco exposure may significantly delay the development of HPV16/18 IgA or/and IgG antibodies in young women followed-up for up to 5 years. It is commonly known that smokers have 10 to 20% lower serum antibody (IgA, IgG and IgM) levels than non-smokers. The immunoglobulin levels are higher among past than current smokers, and increase with duration of smoking cessation (Anderson et al. 1982). HPV-specific antibody response is often delayed but our cohort study, with a long follow-up time, allowed us to make the distinction between females who, eventually, had responded and those who had failed to respond over time. Determination of HPV16/18 IgA and IgG antibodies was by standard ELISA (Pagliusi et al. 2007). Serum antibodies are not affected by differences in blood withdrawal or specimen handling (Lehtinen et al. 2006). Following natural HPV infection, IgG antibodies are detectable in up to 75% of women (Kjellberg et al. 1999). HPV16/18 IgG seropositive women <30 years who smoked, had a significantly decreased likelihood of being constantly IgG antibody positive suggesting that smoking may promote waning of IgG antibodies over time.

Interpreting the results was not straightforward due to the small sample size. Our old HPV DNA typing data probably distinguished HPV positive cases with high viral load only. This did not seem to have an effect as the results in DNA positive

and DNA negative women were comparable. However, having few strata with small numbers of individuals cannot exclude the possibility of change observations.

How tobacco exposure specifically influences HPV antibody response is not known. HPV-infected cells may also be more susceptible to DNA damage from specific tobacco carcinogens (Simons et al. 1995). In addition, the fact that some cigarette constituents have the ability to manipulate cytokine expression in a manner similar to hrHPV gene expression suggests that smoking/chewing may enhance the ability of HPV16/18 to escape from immune surveillance (Poppe et al. 1995, Arcavi et al. 2004, Hussain et al. 2008). Smoking has been associated with persistent HPV infections (Szarewski et al. 2001) and decreased clearance of CIN lesions (Szarewski et al. 1996). Reduction in lesion size was highly correlated both with the reduction in reported amount of smoking and with the final-visit cotinine measurements (Szarewski et al. 1996).

6.2.5 Tobacco smoking may not impair the immune response following bivalent HPV16/18 vaccination

This pilot study is the first to evaluate the impact of tobacco smoking, assessed via serum cotinine level, on the immunogenicity of bivalent HPV16/18 AS04-adjuvanted vaccine. Young women who smoked tended to have comparable absorbance levels of anti-HPV16 and HPV18 antibodies to non-smokers at 7 months-post-vaccination.

In contrast to the often inadequate immune response following natural infection, a prophylactic CC vaccine should induce long-term immune response with high and sustained local and systemic antibody levels (Stanley et al. 2006). The addition of an adjuvant to a vaccine significantly increases the humoral response to the vaccine antigens (Giannini et al. 2006). Geometric titers (GMTs) for vaccine induced antibodies against HPV16 or HPV18 infections were substantially higher (≥11-fold) than those seen in natural HPV16 or HPV18 infections at all timepoints for 5.5 years (Harper et al. 2006). High seroconversion rates and strong vaccine-induced antibody response were also evident in the interim (14.8-month) results of the PATRICIA study, a large phase III trial of the AS04-adjuvanted HPV16/18 vaccine (Paavonen et al. 2007).

Our study demonstrated no statistically significant differences in vaccine-induced humoral immune response to either HPV16 or HPV18 L1 VLP among young women who smoked compared to non-smokers after a 7-month follow-up period. In a recent study investigating the effect of self-report smoking on HPV16/18 L1 VLP vaccine among small numbers of young women with preexisting HPV infections, the authors found no vaccine efficacy for viral clearance in either smokers (VE: -51.9, 95% CI: -136.7 to 2.5) or non-smokers (VE: 6.2, 95% CI: -17.1 to 24.8) after 12 months of follow-up (Hildelsheim et al. 2007). A similar observation has also been described for the quadrivalent HPV6/11/16/18 vaccine (Giuliano et al. 2007).

Smoking is a risk factor for oncogenic HPV infections (Syrjanen et al. 2007) and cervical neoplasia (Plummer et al. 2003). Tobacco smoking prevalence has increased considerably among women during the last decade (Mackay 2001). Long-term immunogenicity and efficacy of HPV vaccine in preventing HPV infection and cervical neoplasia is unclear (Keam and Harper 2008). Although this pilot study was limited by the short follow-up period, the results showed that both the anti-HPV16 and anti HPV18 absorbance levels in HPV16/18-vaccinated smokers and non-smokers were comparable at month 7 post-vaccination. Comparison between the type-specific ELISAs determined antibody levels is, unfortunately, not possible. These observations, however, warrant further investigation on a larger number of vaccinated individuals.

6.3 Strengths and limitations of the study

Study design and bias

Case-control studies are susceptible to various forms of bias compared to cohort studies, the usefulness of the former in the process of causal inference is especially diminished. Our nested case-control design (Paper I) measured data on exposure and confounders before the diagnosis of the disease, thus reducing potential recall bias (pregnant women from FMC were a part of the material), the temporary ambiguity usually inherent in case-control studies. Potential recall bias may be a limitation in the second study (Paper II) as under-reporting of tobacco smoking habit among Finnish women may have distorted the true association between exposure and cervical HSIL. This is, however, unlikely because we found a good correlation between serum cotinine levels and the questionnaire data among Finnish women (data not shown).

Cases and controls were drawn from the same cohort, decreasing the likelihood of selection bias. In Papers II and III, we performed cross-sectional studies. Despite the fact that this prevents us from concluding the directionality of the observed associations, these cross-sectional analyses allowed hypothesis generation for future research.

Paper IV described the results from a random subset of a randomized prospective clinical intervention study (Paavonen et al. 2007). Computer-generated randomization was performed to avoid selection bias. Vaccine and Placebo groups were equally treated following a standardized written protocol in order to minimize bias in study management. In addition, the doubled-blinded design made it possible to avoid observer (ascertainment) bias.

Misclassification of the outcome variables (Papers I and II) was unlikely as the diagnoses for cervical SIL, SCC or carcinoma in-situ (CIS) were reported by experts in cytopathology and mostly based on results from histology. Samples from cases (Paper I) and their controls were batched together during the laboratory analyses

to avoid assay-drift bias. Moreover, great care was taken to avoid differential measurement errors in the determination of HPV antibodies positivity or in cotinine detection by blinding the laboratory personnel (Papers I, II, III and IV).

Confounding

We identified some potentiel confounders on the basis of earlier studies and biology. In the statistical analyses, we did not have questionnaire data on sexual habits, which may have resulted in slight over-estimation of the smoking-associated relative risks. This is however, unlikely as the point estimates were adjusted for antibodies to *C. trachomatis*, a sensitive surrogate of risk-taking sexual behaviour. Seropositivity to *C. trachomatis* shows a strong correlation with life-time number of sexual partners (Dillner et al. 1996).

Validity of the method used for tobacco exposure assessment

Complete questionnaire data on tobacco exposure history would have permitted us to assess the lifetime exposure to tobacco smoke, and to study the effects of smoking in relation to age at starting and stopping smoking. Self-report has proven to be a simple and powerful predictor of disease outcomes in epidemiological studies. Self-reporting, however, underestimates the true prevalence and is inaccurate as to the quantity of smoking exposure (Murray et al. 1993). When smoking is a daily habit, the act of smoking is unlikely to be closely self-monitored. Thus, in a questionnaire, the respondent may be imprecise about smoking behaviours, such as number of cigarettes smoked per day (Benowitz 1999). On the other hand, pregnant women (a considerable part of our study cohorts in Studies I and II materials) tend to belittle their smoking habit because of health conscience (Britton et al. 2004, Burstyn et al. 2009).

Biochemical assessment of tobacco exposure integrates different aspects of the true exposure, including tobacco composition, uptake, distribution and individual differences in metabolism. Cotinine is a good marker of nicotine, which is the important carcinogen in tobacco smoke. Although serum cotinine measures recent exposure to tobacco smoke near the time of specimen collection, it has a high sensitivity and specificity for tobacco exposure and the measurement error is negligible compared to questionnaires (Murray et al. 1993, Boffeta et al. 2006). Biochemistry of identifying tobacco exposure via serum cotinine favours the validity of our observations. This method addresses the issue of non-differential recall bias. In addition, it is the inhaled dose of tobacco smoke that is directly related to the development of tobacco-related diseases (Pérez-Stable et al. 1995). Cotinine levels remain stable in frozen samples (Boffeta et al. 2006) enabling the identification of dose-response effects.

Validity of the results

The validity of the study refers to the reliability of the results. From the first to the fourth study, great care was taken to ensure that the results do not reflect the presence of bias or confounding. In biobank-based research, the process from the decision to take a sample from an individual to the moment the sample is safely placed in the biobank consists of several phases including collection of samples, handling and storage of samples. Failure in one of these standardized procedures may have significant consequences as the quality of the material stored in the biobank as well as conclusions and recommendations based on the analysis of such material may be severely affected. Established quality control of serums banks and cancer registries (Paper I) increases the validity of the study (Pukkala et al. 2007, Sjöholm et al. 2007).

Sample size and generalisability of the results

Our Nordic joint study (Paper I) is by far the largest nested case-control study on risk factors for CC conducted to date. Updating the study material (Study I) eventually result in a sample size large enough to provide confidence in the estimates and therefore, ensure our ability to reveal associations. The population representativeness of the serum bank cohorts was recently assessed by Pukkala and colleagues (Pukkala et al. 2007) who found no significant difference in cancer incidence in the biobank cohorts involved in the present study (the Nordic maternity cohorts and the Janus biobank) as compared with respective national rates. Thus, our results can be generalized to all Nordic women, and there was no evidence that the odds ratios among serum banks were heterogeneous.

In Papers II and III, the cohort participants were recruited among women attending routine Papanicolaou (PAP) smear. The screening activities are integrated in the healthcare system in Finland. In the organised programme, the present coverage of invitations is 90% of the target age; the participation rate is higher than 70% (Anttila and Nieminen 2000). In our study, all women whose PAP smear showed unequivocal changes of HPV infection attended the gynecological outpatient clinic of the Kuopio University Hospital for follow-up visits at 6-month intervals. There was no loss to follow-up in the Finnish study. In Cote d'Ivoire (Paper II) cases were drawn from the three outpatient gynecology clinics in Abidjan and may not accurately reflect the target population.

Assuming interval validity (Papers II to IV) and despite small sample size (Papers II and III), our results should also be representative of the Finnish (Papers II to IV) and Ivorian (Paper II) targeted populations. The study groups had similar physiological, behavioral, racial and cultural characteristiques than their respective general population. Caution must be exercised, however, in extending these results to other countries citizens as numerous other differences exist between nations in relation to genetics and lifestyle factors.

7 Conclusions

Tobacco smoking was associated with an increased risk of CC/SCC after adjustment to oncogenic HPVs. Moreover, a statistically significant 2-fold excess risk of SCC was found among HPV16/18-seropositive women who smoked. The risk of CC associated to smoking was similar in seropositive and seronegative women.

Tobacco smokers tended to have an increased risk of HSIL in women ≥30 years of age. In less industrialized countries, which bear the heaviest burden of CC, exposure to tobacco is more common through chewing than smoking among women. Relative to non-chewers, women who chewed tobacco had a significantly 5-fold increased risk of cervical HSIL. Although being infected with two or more HPV types (multiple infections) was common in HPV16 and/or HPV18-infected women, we did not report an increased risk of multiple HPV infections among tobacco smokers/chewers compared to non-smokers/non-chewers.

We showed that following natural HPV infection, young women who smoked were less likely to either seroconvert or maintain detectable HPV16/18 antibodies over time than non-smokers.

We found that following AS04-adjuvanted HPV16/18 L1 VLP vaccination, young women who smoked or did not smoke had comparable short-term HPV16/18 serum antibody response at 7 months post vaccination.

We conclude that tobacco smoking is an independent risk factor for CC in women infected with oncogenic HPVs. In addition, the results suggest that smoking may induce impaired antibody response to oncogenic HPVs but not immediately after prophylactic HPV vaccination in young women. The evidence discussed in this dissertation supports comprehensive primary prevention initiatives against causes of CC, with a call for strengthening efforts to prevent tobacco smoking and chewing exposures among women.

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Aline Simen-Kapeu

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