Factors influencing the treatment outcome of Cervical Intraepithelial Neoplasia (CIN)

A Thesis Submitted To the University Of Manchester
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ABSTRACT

Carcinoma of the cervix is the second commonest female cancer in the world. Women with abnormal smears have an increase risk of developing cervical cancer. During the 8 years following conservative treatment of CIN the risk of invasive cervical cancer for these women is about five times greater than that of the general population of women. The occurrence of treatment failure of cervical intraepithelial neoplasia (CIN) may cause considerable management problems. It has been found to be associated with treatment related factors, however the wide variation in the reported failure rate suggests that other factors may be involved. Human papilloma virus (HPV) has been associated with the natural history of both CIN and cervical cancer. Follow up studies of women with mild CIN indicate that women with cervical lesions associated with HPV types 16 and/or 18 are more likely to progress to advanced disease. To date, there have been no published reports on the predictive value of HPV testing in the treatment outcome of CIN.

It has also been suggested that additional factors are required for neoplastic development since HPV has been detected in a wide range of asymptomatic controls and only a minority of persistent HPV infection progress to cancer. One of these factors may be exposure to chemical carcinogens such as those present in cigarette smoke.

CIN has been associated with smoking in the majority of the epidemiological studies addressing this relationship. The causal relation has not been proven yet.

The objectives of the study are, i) to determine whether detailed information regarding the smoking history, the sexual history and other known correlates of cervical neoplasia, could be used to identify those who were likely to have treatment failure of CIN, ii) to study any predictive value of pre- and/or post-treatment HPV testing, iii) to identify any particular post-treatment advice which might influence the treatment outcome, iv) to consider what if any changes in follow up protocols would benefit high risk patients, v) to analyse for known polymorphism the enzymes involved in carcinogenesis metabolism, vi) to quantitatively assess carcinogenic exposure.

A prospective, multi-centre, study was conducted on a cohort of women in the North West of England, attending for colposcopic examination after abnormal results of a cervical smear test between 1st of August 1995 and the end of July 1999. They were interviewed and asked to complete a questionnaire, which covered a detailed smoking history together with other risk factors associated with CIN. This cohort of women was used to undertake a nested case-control study. The cases were defined as those patients who had had treatment failure of CIN within the two years immediately following treatment, the diagnosis of which had to be confirmed histologically. The controls were selected from those patients with no treatment failure of CIN during the 24 months following treatment confirmed by two negative smears, the latest of which was taken at least 24 months after treatment. A pre-treatment biopsy as well as post-treatment cervical swab, 6 to 12 months following treatment, were taken and stored at $-70^{o}C$. After identification of the cases and matched controls, DNA was extracted from the relevant samples and analysed firstly for the presence of HPV using the PCR technique, secondly for genetic polymorphism of the metabolising enzymes using the PCR technique and

finally for the presence of the DNA adduct for N7-methyl guanine using the immunoslotblot technique. The life style data obtained from the cases and controls was also analysed.

The effect of pre-treatment and post-treatment HPV test results was assessed independently both in the absence and presence of the significant lifestyle risk factors.

Nine hundred and fifty-eight patients were recruited into the study; 77 cases were identified (8%) and 154 controls were selected based on the matching criteria. Cases and controls were similar in terms of their distributions of age at first sex, prevalence of genital warts. Post-treatment positive HPV testing was found to be significantly associated with treatment failure of CIN ($p = 0.001$). Combination of cervical cytology & HPV test led to detection of 73% of the treatment failure cases in the 1st follow up visit. Smoking status was found to be the most significant life style factor associated with the treatment failure of CIN ($p= 0.0013$). Current smokers have a three fold increased risk of treatment failure of CIN as compared to non-smokers, this risk increased to almost 19-fold (95% CI 1.65 to 212.2) after adjustment for the follow-up HPV status. Ex-smokers showed less risk than current smokers but still higher than non-smokers. A significant effect of the level of N7-methylguanine on the risk of treatment failure was detected ($p=0.030$).The combination of GSTM1 null genotype and of CYP2D6 genotype EM appeared to be associated with treatment failure

These findings may make it possible to conclude that positive post-treatment HPV, smoking, high level of DNA adduct and combination of GSTM1 null and CYP2D6 EM genotypes are expected to be associated with increase risk of treatment failure of CIN. The combination of HPV status and cytological on the first follow up visit, can predict treatment failure in 72% of the cases.

DECLARATION

I declare that no portion of this work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning. The role I performed in this work concerned designing the study, applying for funds to support the study, the recruitment of patients from the Royal Bolton Hospital and identifying the cases and controls. In addition, I was responsible for explaining the study, the method to be employed and the means of patient follow up to the other doctors who assisted me in the recruitment.

The laboratory work was mainly carried out by laboratory technicians, although I was available for two sessions every week to help with this work.

Nabil Nathan Acladious

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ABBREVIATIONS

Bp	Base Pair
BCIP	5Bromo- 4 Chloro 3Indolyl Phosphate
BSS	Balanced Salt Solution
0C	Degree Centigrade
CaCx	Cancer Cervix
CI	Confidence Interval
CIN	Cervical Intraepithelial Neoplasia
CT-DNA	Calf Thymus DNA
CYP	Cytochromoxidase
ddH2O	Double Distilled Water
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dUTP	Deoxyuracil Triphosphate
EM	Extensive Metabolisers
EDTA	Ethylenediaminetetra-acetic Acid
G	Guanine
GapDH	Glyceraldehyde-3 Phosphate De-Hydrogenase

GST	Glutathione S-transferases
H	Hour
HEM	Heterozygous Extensive Metabolisers
HPV	Human Papilloma Virus
IARC	International Agency for Research on Cancer
IQR	Inter-Quartile Range
Kb	Kilobase
LiCl	Lithium Chloride
Mg Cl2	Magnesium Chloride
Min	Minute
MNU	N-nitroso-N-methylurea
(NH4)2 SO4	Ammonium Sulphate
NaCl	Sodium Chloride
NaHCO3	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NBT	Nitroblue Tetrazolium
N7-meG	N7-methylguanine
ORF	Opening Reading Frame
PAH	Poly Aromatic Hydro-carbons
PCR	Polymerase Chain Reaction
Pk	Proteinase K
PM	Poor Metabolisers
Pmol	Picomole

SD	Standard Deviation
SDS	Sodium Dodecylsulphate
SIL	Squamous Intraepithelial lesions
TAE	Tris acetate EDTA
Taq	Thermus Aquaticus
TRIS-HCl	Tris(hydroxymethyl)-aminomethane Hydrochloride
T.Z	Transformation Zone
WHO	World Health Organisation
Umol	Micromole

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DEDICATION

To my family, my Wife, my Mother and my little angels,
Peter and Monica:
Thanks for your support.

Chapter 1 Review of literature

1.1 Introduction

Carcinoma of the cervix is the second commonest female cancer in the world with only breast cancer occurring more frequently (Shafi and Luesley, 1995). The implementation of the cervical screening programme has had a significant impact on the incidence of cervical cancer because of the early detection of pre-invasive disease and its effective treatment. The incidence of invasive carcinoma of the cervix in England and Wales fell from 16.1 new cases per 105 women in 1986 to 11.2 per 105 in 1993 (Duncan, 1997). However, patients with abnormal smears have an increase risk of developing cervical cancer. Within the 8 years following conservative treatment of CIN, the risk of invasive cervical cancer among these women is about five times greater than that among the general population of women during this period (Soutter et al., 1997). In addition, the occurrence of treatment failure of CIN may cause considerable management problems as colposcopic assessment is less reliable and concerns regarding the risk of invasive malignancy are heightened (Hammond, 1999).

Study of the relationship between Human Papilloma Virus (HPV) and Cervical Intraepithelial Neoplasia (CIN) has suggested that HPV 16 and 18 were associated with invasive cervical cancer, whereas types 6 and 11 were associated with condylomas and low grade CIN (Reeves et al., 1987). Follow up studies of women with mild CIN indicate

that women with cervical lesions associated with HPV types 16 and/or 18 are more likely to progress to advanced disease than are women with lesions associated with HPV types 6 and / or 11 (Reeves et al., 1987; Campion et al., 1986; Reeves et al., 1989). It has also been suggested that additional factors are required for neoplastic development since HPV have been detected in a wide range of asymptomatic controls and only a minority of persistent HPV infection progresses to cancer (Arends et al., 1998). One of these factors may be exposure to chemical carcinogens such as those present in cigarette smoke. Molecular epidemiological research, using PCR-based approaches, suggests that cigarette smoking may be an independent risk factor for cervical cancer (Kjaer et al., 1996; Olsen et al., 1998). Such an interaction between exposure to a virus and a chemical carcinogen is not unknown. Men exposed to both aflatoxins and hepatitis B Virus are at much greater risk of hepatocellular carcinoma. (Qian et al., 1994)

1.2 Historical Background

Even in antiquity man was aware of the toxic effects of animal venoms and plant poisons. Over the centuries, people increasingly realised that there is a relationship between the exposure to certain chemicals and the occurrence of toxic effect. Paracelsus (1493-1541) was one of the first to focus on the 'toxicon' i.e., the toxic agent as chemical entity. In 1775, Percival Pott, who linked the increased incidence of scrotal cancer observed in the English chimneysweepers to exposure to soot, was one of the first to explain an occupational disease in terms of exposure to chemicals. Since those days, many efforts have been made to characterise the biological and biochemical processes that may lead to

toxic effects (Doull and Bruce, 1986). A breakthrough in this regard took place in 1842 when Ure and Keller demonstrated for the first time a biotransformation of benzoic acid into hippuric acid in the cow. Another breakthrough took place in the late 1940s, when it was discovered that biotransformation or metabolism of exogenous compounds (xenobiotics), which was generally thought to serve as a detoxification mechanism by facilitating the excretion of compounds, was also involved in the toxicity induced by compounds like polycyclic aromatic hydrocarbons and aromatic amines. Only over the last few decades, however, it has become clear that many of the parent chemicals themselves are not toxic, but instead require metabolic transformation to toxic reactive intermediates. This process is commonly referred to as metabolic activation, bioactivation or toxication (Miller and Guengerich, 1982). In recent years, a new subdiscipline called molecular or biochemical toxicology emerged within toxicology. In this new subdiscipline, the processes occurring from the time an organ is exposed to a chemical to the expression of the toxic effect are generally distinguished in several phases.

In 1941, George Papanicolaou suggested that exfoliated cells in the vaginal pool could be used for the early detection of uterine cancer. In 1947, Ayre devised a wooden spatula for obtaining cervical smears and by the 1960s cervical cytology screening had become widespread, before any randomised controlled trials had been carried out. In 1976, a World Health Organisation group of experts suggested that it was necessary to conduct a randomised trial of cervical screening (WHO study group, 1976).

1.3 CERVICAL INTRAEPITHELIAL NEOPLASIA

1.3.1 Definition

CIN is recognised by disturbances of the cellular maturation and stratification and cytological atypia of the metaplasic squamous epithelium of the transformation zone of the cervix, of various degrees of severity, without invasion of the basement membrane. (Anderson et al., 1991)

1.3.2 Normal Cervix, Pathology and Pathogenesis of Cervical Intraepithelial Neoplasia.

The uterine cervix, together with the vagina and vulva form the lower female genital tract. The uterine cervix can be divided into two sections, the portio vaginalis, which is covered by stratified squamous epithelium and the endocervix, covered by mucus-secreting columnar epithelium. The squamous epithelium of the cervix is not usually keratinized. Nevertheless in uterine prolapse and certain pathological states the cervico-vaginal epithelium may be keratinized. In the past, five zones or layers of cells in this epithelium were originally described by Dierks in 1927: stratum basale, stratum spinosum profundum (parabasal layer) and spinosum superficial, stratum granulosum and stratum superficiale. It is now usual to divided the ectocervical epithelium into three zones (basal, mid and superficial zone).

The basal zone is one or two layers of cubical cells, approximately 10 mm in diameter, with large, dark staining nuclei and nuclei orientated perpendicular to the underlying basal lamina (basement membrane), and scanty cytoplasm with high nucleo-cytoplasmic ratio. The cells of this layer are actively dividing. The lower mid zone, also known as the parabasal layer, consists of a varying number of layers of polyhedral cells with large dark-staining nuclei. Normally mitotic figures may sometimes be seen in this layer as well as in the basal layer. In the upper mid zone, also known as the intermediate cell zone, the cells tend to be flattened, non-dividing, glycogen rich, the cytoplasm may be vacuolated and the nuclei stain less darkly then in deeper layers and are vesicular. The cells of the superficial zone (outmost layer) are flat with over all cell diameter of approximately 50 um. The nuclei are small and pyknotic and the cytoplasm glycogen-rich and eosinophilic (Rollason, 1998). (FIGURE 1.1)

FIGURE 1.1 Structure of the cervical squamous epithelium

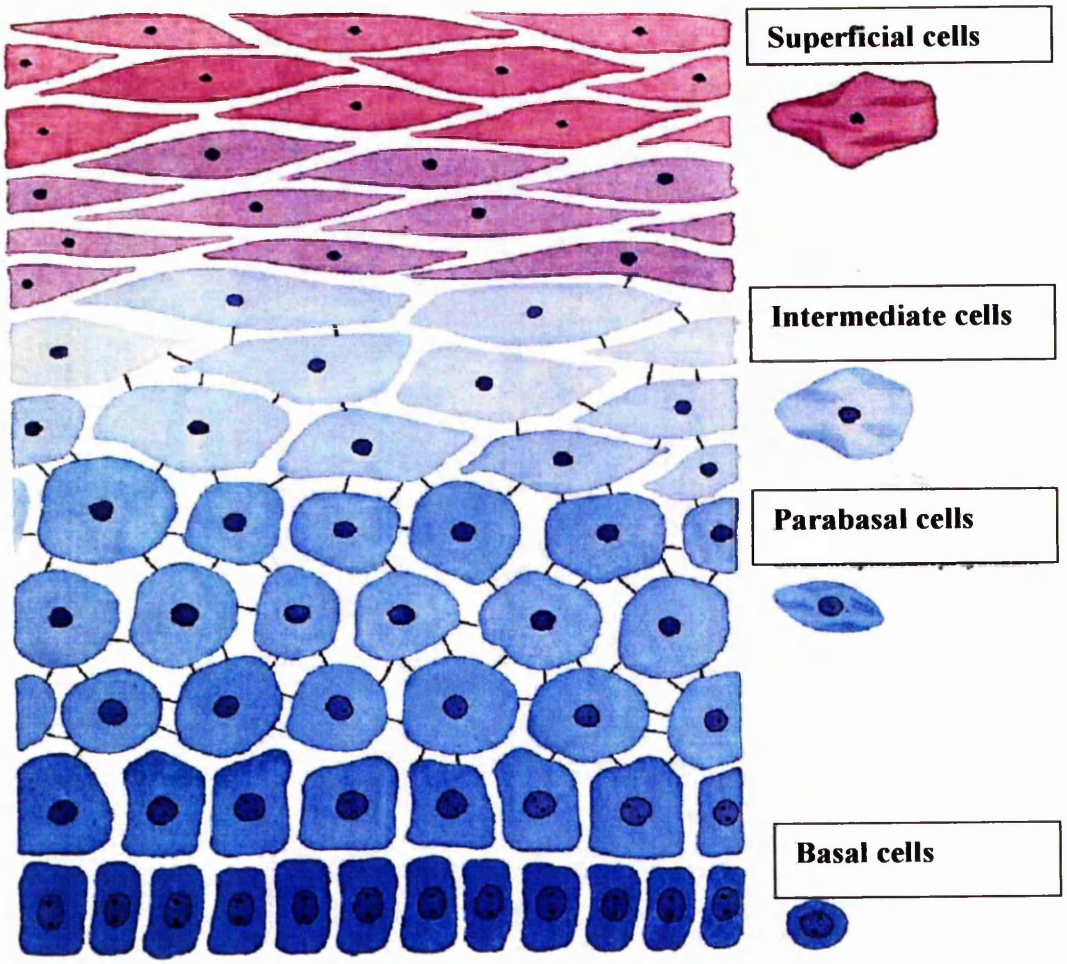
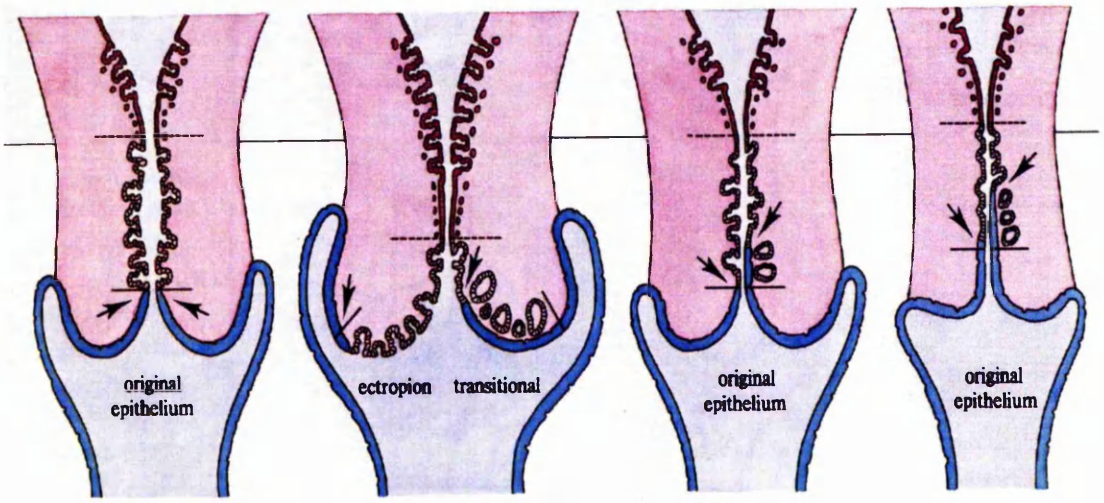


FIGURE 1.2 Position of the squamo-columnar junction at different stages of life



(1) **(2)** **(3)** **(4)**

1-Puberty

2-Childbearing

3-Perimenopausal

4-Postmenopausal

The columnar epithelium is thrown into folds and crypts as it lines the endocervical canal. The squamo-columnar junction defines the lower end of the endocervical canal anatomically and by the external os histologically. These two points do not necessarily coincide although they generally lie fairly close together. Before puberty and after the menopause the squamo-columnar junction may lie above the external os, whereas in the newborn and in the childbearing period it may lie outside the external os. At puberty, during pregnancy, and in some steroid contraceptive users, when the volume of the cervical wall increases, the lips of the cervix protrude carrying with them the endocervical mucosa on to the portio vaginalis, whilst at the same time the upper limit of the endocervical epithelium moves down, thus exposing the tissues previously found in the lower endocervical canal, to the vagina. This newly formed area has been termed endocervical ectopy (Buckley, 1994). (FIGURE 1.2)

The cervical ectopy zone is where the majority of intraepithelial and invasive neoplasms develop. The surface of an ectopy has a papillary structure; the crypts between the papillae open freely on the surface. The ectopic epithelium undergoes metaplastic transformation to squamous epithelium. Metaplasia starts at the periphery of the ectopy, and is detected by the appearance of cells that gradually become stratified beneath the layer of columnar cells, and assume the morphological features of squamous epithelia. Finally columnar cells are lost, and a mature stratified squamous epithelium, in which the vascular pattern of the ectopy is preserved, replaces the columnar epithelium. The area of the metaplastic squamous epithelium forms what is described as the transformation zone (T.Z).

Within this area a large number of dynamic, physiological as well as pathological processes occurs (Hoskins et al., 1992).

CIN is believed to occur in pre-existing metaplastic squamous epithelium of the transformation zone, which has become atypical. It is graded according to the degree of morphological and structural abnormalities. Grade I corresponds to the formerly called mild dysplasia, grade II to moderate dysplasia and grade III to severe dysplasia and carcinoma in situ. In addition, a cytological classification, called the Bethesda System. has been introduced. It combines clinically similar intraepithelial lesions into broad categories. Thereby, cytological diagnosis of HPV infection and CIN I are combined as low grade squamous intraepithelial lesions (SIL); while CIN II, CIN III, and carcinoma in situ are gathered together as high grade SIL (Kurman et al., 1991; Kiviat et al., 1992).

Cytological assessment of the grade of the lesion has its basis in the fact that a cervical scrape smear contains cells from the outer few layers of the epithelium. The degree of differentiation of the neoplastic focus may be deduced from the type of the predominant abnormal cell. The term dyskaryosis was coined by Papanicolaou to denote a cell with a malignant nucleus and a rather normal cytoplasm (dys: bad, abnormal; karyon: kernel). Intermediate dyskaryotic cells are suggestive of CIN I. Conversely, predominance of dyskaryotic parabasal or basal cells would indicate a minimally differentiated focus of CIN III. Moderately mature dyskaryotic cells would be suggestive of CIN II.

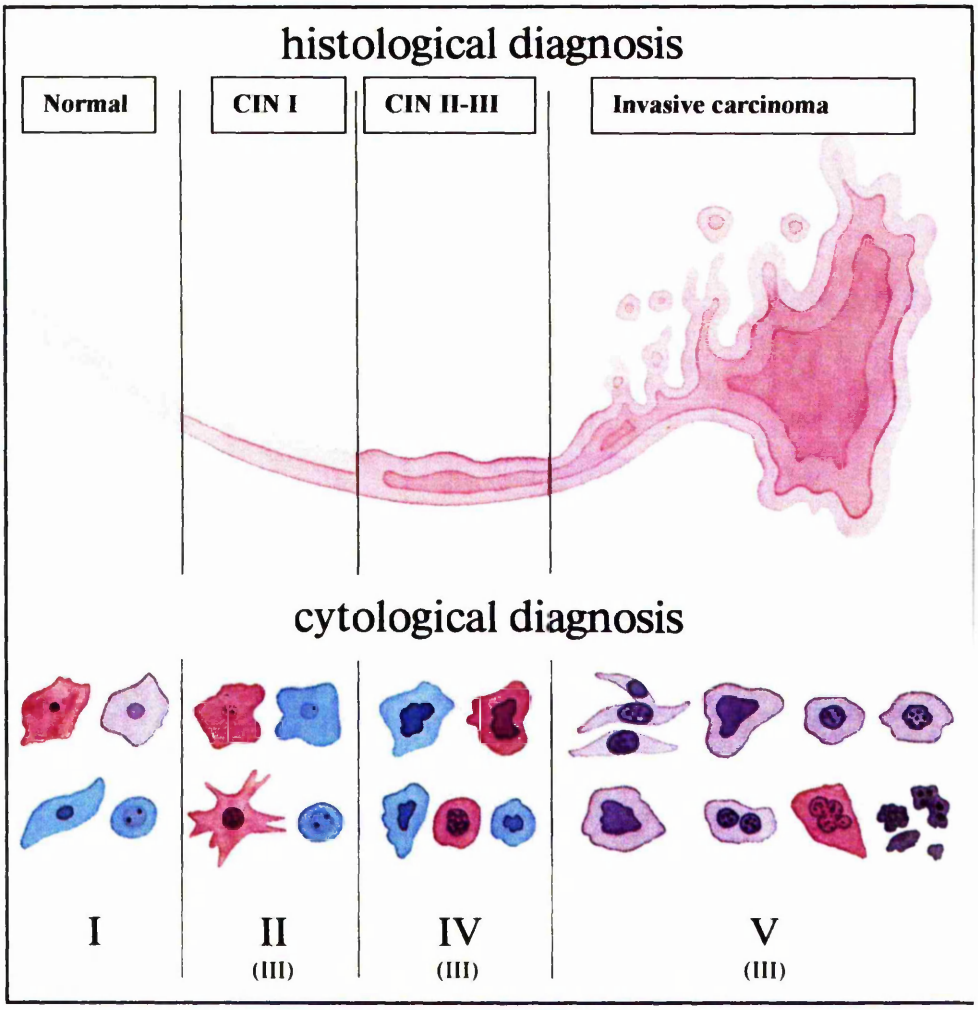
Histological examination of a biopsy specimen should be regarded as mandatory to define precisely the nature of a neoplastic lesion and for confirmation of the presumptive cytological diagnosis (Luesley and Barrasso, 1998).

Histological evidence of CIN is seen primarily in the nuclei, which are abnormal in all grades of CIN. The difference between the various grades lies mainly in the degree of squamous maturation. CIN 1 displays a considerable degree of squamous differentiation. The neoplastic cells retain their basaloid character for a third of the thickness of the epithelium and then mature and become stratified. The cells of the outermost zone attain the size and shape of intermediate squamous cells (FIGURE 1.3).

In CIN II, maturation does not progress beyond the inner intermediate or outer parabasal levels, the lower two thirds of the epithelium remain immature (FIGURE 1.3).

CIN III is characterised by little or no maturation, and the full thickness of the epithelium is made up of poorly differentiated cells, which form a single multi-layered stratum. In some cases a little enlargement or some flattening of the surface cells may be evident (FIGURE1.3).

FIGURE 1.3 Different grade of CIN and its cytological correspondent



Carcinoma in situ is thought to be the preinvasive stage of cervical cancer. It shows the usually accepted histological criteria of malignancy, including loss of stratification, loss of polarity, cellular pleomorphism with increased mitotic activity throughout the epithelium rather than only in the basal one-third. The main feature that distinguishes carcinoma in situ is that it involves surface and gland lumens of the cervix but it does not invade the basement membrane (Buckley, 1994). It is worth pointing that due to the similarities found between carcinoma in situ and CIN III, the former term is not in common use nowadays; instead, both lesions are referred to as CIN III. Figure 1.4 shows the histology appearance of CIN III.

1.3.3. Natural History of CIN

The natural history of CIN indicates that approximate likelihood of regression of CIN I is 60%, persistence 30%, progression to CIN III 10% and progression to invasion 1%. The corresponding approximations for CIN II are 40%, 40%, 20% and 5%, respectively. The likelihood of CIN III regressing is about 33% and progressing to invasion greater than 12%. Thus even the higher degree of atypia may regress. The main histological features identified in CIN III associated with microinvasive carcinoma or predictive of subsequent microinvasion are extensive involvement of the surface epithelium, lumenal necrosis, frequent mitosis, pericryptal inflammatory infiltrates and distinct nucleoli.(review by Arends et al., 1998)

FIGURE 1.4 CIN III histology

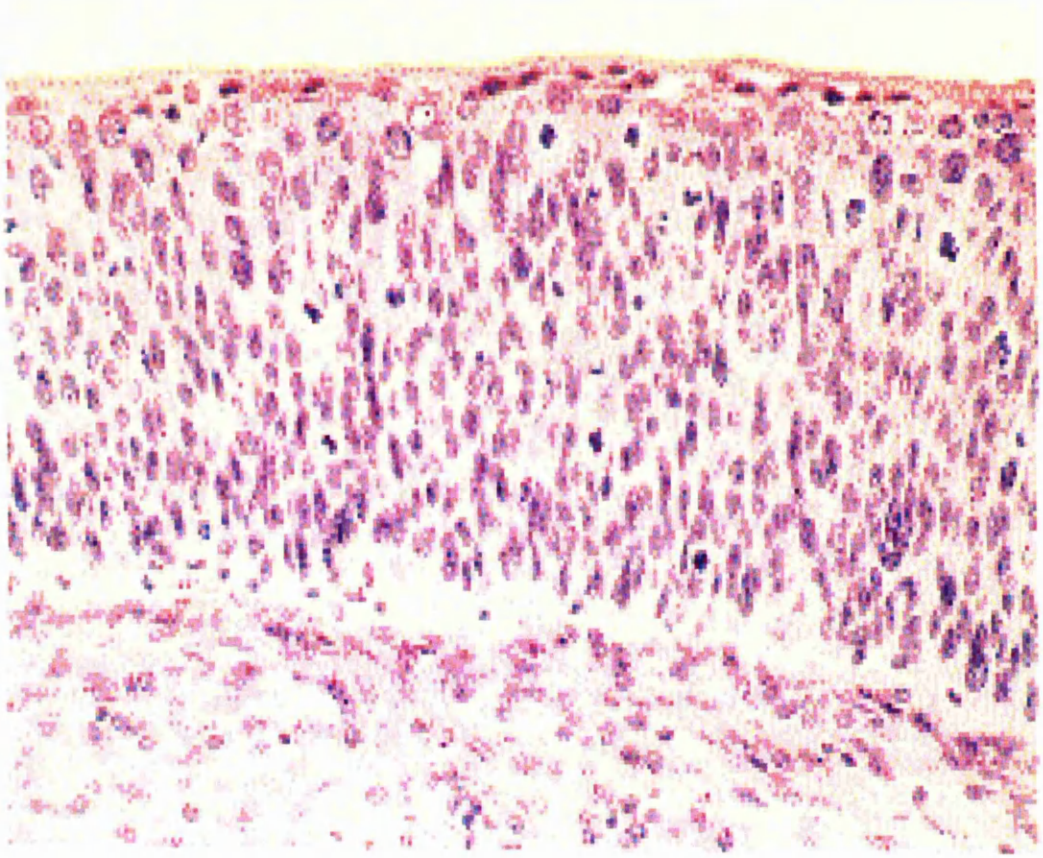
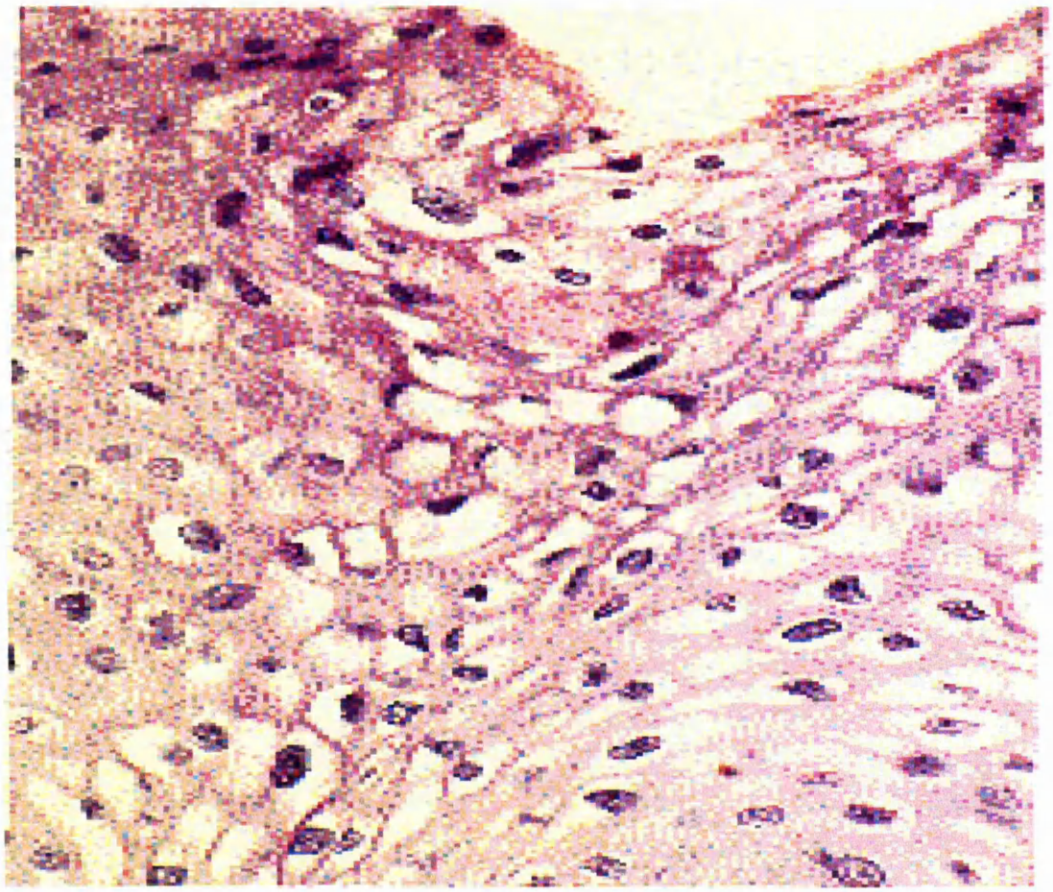


FIGURE 1.5 HPV infection associates with koilocytosis



HPV infection has been associated with the occurrence of CIN (Blomfield et al., 1998). HPV 16 and 18 were associated with invasive cervical disease while type 6 and 11 were associated with low grade CIN. Most HPV infections disappear within months to a few years of diagnosis (Hildesheim et al., 1994). Low grade cervical lesions also tend to regress to normality, however they can also progress to high grade lesions with an absolute risk of about 15-25% over 2-4 years (reviewed by Schiffman and Brinton, 1995). In most cases, progression of a benign HPV-induced lesion to invasive cancer does not occur abruptly, but through the development of cervical intraepithelial neoplasia (CIN) which are divided in three stages (CIN I, II and III) according to the severity of the lesion. (FIGURE 1.3)

The most important factor related to the progression from low grade CIN to high grade CIN is the presence of certain types of HPV. Among women suffering from CIN, the presence of cancer-associated types of HPV (high risk types especially type 16 and 18) predicts a higher risk of progression than either the low-risk types or HPV negativity (Campion et al., 1986; Kataja et al., 1990; Cruickshank et al., 1999). Also persistence of HPV infection (high-risk types) has been correlated with the progress of the disease from low to high-grade disease. (Ho et al., 1998b) In one study in the USA, the risk of progression from cytologically normal to biopsy confirmed CINII-III was increased by over 10 - fold among HPV DNA carriers as compared to HPV DNA negatives. Progression was largely attributable to HPV 16 (Koutsky et al., 1992). Wallin et al (1999) concluded that a single positive finding of HPV DNA in a pap smear confers an increased

risk of future invasive cervical cancer.

Using sensitive PCR-based techniques HPV DNA can be identified in more than 80% of women with CIN depending on the grade of CIN. Approximately, 50% of CIN 1 are associated with HPV-16 (Lehn et al., 1988; Hording et al., 1995). In addition, infection with more than one type of HPV is commonly found in CIN1 (Lungu et al., 1992). High grade neoplasia (CIN2 and 3) is frequently associated with HPV-16, HPV-18, HPV31, HPV33 and HPV 35 (reviewed by Park et al., 1995). The most prevalent HPV type related to CIN 2 and CIN 3 is HPV-16, which has been detected in 30-77% of this kind of lesions (Lungu et al., 1992).

Smoking appears to be the most important risk factor which can affect the progress of CIN after the HPV infection (Kjellberg et al., 2000).

Other host factors involved with the progression from low to high CIN grades are probably immunological (reviewed by Schiffman and Brinton, 1995). Another factor to be considered is parity, which might influence immunity, hormonal and nutritional status, as well as traumatic mechanisms (Munoz et al., 1993).

It is not clear if all cases of cervical cancer develop by passing through each stage of the CIN. It has been shown that some patients seem to develop high-grade cervical lesions from primary HPV infections, without showing any evidence of intermediate CIN lesions (Koutsky et al., 1992). However, this observation might be the result of a rapid

development of the lesions, whose detection was missed between the examination periods (the patients were followed every four months).

1.3.4 Epidemiology of cervical intraepithelial neoplasia.

1.3.4.1 Incidence

Enough data has accumulated to suggest a dramatic increase over time in the incidence and prevalence of CIN. The average age of women with CIN has been reported to be decreasing, which probably reflects earlier age of acquisition of HPV (Cox, 1995) .

The risk of development an abnormal smear in the United Kingdom is 45 / 1000 women screened of all age groups (Dept of Health, Statistical Bulletin, 1997), and numerous other risk factors for the development of CIN have been recognised, many of them relating to HPV infection, sexual behaviour and life style factors e.g. smoking (reviewed by Arends et al., 1998; Blomfield et al., 1998). The malignant potential of CIN grade III is about 12%.

1.3.4.2 Risk factors for CIN other than HPV and smoking

a) Sociodemographic factors

The risk of development of CIN increases with age, peaks at 25-30 years of age, then declines. Moreno et al (1995) in a study carried out in Spain and Colombia concluded that both CIN III and invasive cancer had similar Sociodemographic risk profiles. There is a ten fold increase in the risk of cervical cancer between those countries such as Israel, Spain or Finland, in which the incidence of the disease is low and Colombia and Paraguay in which it is high. There is also a significant difference in the incidence of cervical cancer in different ethnic groups within the USA. As the incidence of invasive cervical cancer rises to a plateau around 35/39 years in white women in the USA but continues to rise at later age in black women (Bosch et al., 1998).

Another valid observation is that cervical neoplasia affects more women in lower social classes (Fasal et al., 1981; Brinton et al., 1987), a fact, confirmed by the increased prevalence of HPV DNA in women of lower educational and income levels (Hildesheim et al., 1993). The reasons for the association between risk of CIN and these sociodemographic factors might be related to the lack of accessibility to good medical care in some poor communities, or even the underuse of available medical services (e.g. smear programs) due to lack of educational and social encouragement (Suarez et al., 1994) or may be due to the different genetic polymorphism of the metabolising enzymes of

environmental carcinogens. Anderson et al (1993) found that it is not useful to use social criteria to identify women who are at increase risk of high-grade disease.

b) Marital and sexual factors

An analysis stratified by HPV status in the Colombia and Spain study (Munoz et al., 1992) showed that in the HPV-negative cases , the disease frequency was still related to sexual behaviour. The key risk factor was the number of sexual partners. Amongst women who were HPV positive, the effect of the number of sexual partners does not seem to be abolished. This finding suggests that the number of partners is a strong surrogate measure of HPV infectivity among women who are HPV positive.

It is largely accepted that the risk of developing CIN is strongly influenced by sexual behaviour. Women having sexual relations at early ages (before the age of 16) are at higher risk than either virgins or women whose sexual experiences began later in life (after the age of 20) and the risk is also influenced by the lifetime number of sexual partners, with relative risk of about three or more for women reporting more than five partners compared with those reporting only one. When HPV infection is taken into account, the effect of lifetime number of partners is greatly weakened but often remains apparent especially in HPV negative women (reviewed by Schiffman and Brinton, 1995).

Some studies have shown that pregnancy at an early age (Bosch et al., 1992) and multiparty (Parazzini et al., 1989; Kjaer et al., 1990; Schiffman et al., 1993a) make additional contributions to the risk of CIN. It has also been suggested that pregnancy may

affect cell growth either directly or indirectly through immunological or hormonal influences on HPV (Pater et al., 1990).

All the factors described above may increase the risk of CIN by allowing HPV infection by causing cervical trauma, at an age when the cervix is undergoing important developmental changes.

Male factor has also been considered among the risk factors for CIN (Brinton et al., 1989a; Agarwal et al., 1993). History of sexually transmitted disease, including common genital warts (HPV-6 and HPV-11 related), was a risk factor in the male partners.

Finally, there is little evidence to indicate that the age at menarche, or menopause or the characteristics of the menstrual cycle are risk factors for CIN (Brinton et al., 1987).

c) Contraceptives.

Data reported about the effects of contraceptives in the development of cervical neoplasia are inconsistent (Piper, 1985; Brinton et al., 1986a; Beral et al., 1988; Berry et al., 1993). However, it appears that a prolonged use of combined oral contraceptive pill increases significantly the risk of cervical cancer (Beral et al., 1988; Berry et al., 1993) and dyskaryotic cytology (Blomfield et al., 1998), including the new generation progestogen combined oral contraceptives (Bagshaw, 1995).

Special interest has been centred on the possible interactive effects of oral contraceptives and HPV, since it has been suggested that sex steroid hormone receptors in HPV-induced cervical lesions may act as cofactors in promoting HPV-related cervical neoplasia (Monsonego et al., 1991). However Ho et al (1995) showed that there was no association between the persistence of HPV and cervical dysplasia and the use of combined oral contraceptive pills.

Additional evidence suggests that oestrogen increases transcription of HPV-16 proteins (Mitrani-Rosenbaum et al., 1989) and progesterone induces oncogenic transformation in the presence of HPV-16 DNA and ras oncogene in a rat model (Pater et al., 1990). This data suggests that oral contraceptives may promote HPV activity once infection has occurred.

Use of barrier methods of contraception (diaphragm and condom) were found to have a low risk of cervical dysplasia. The apparent protective effect is small and information is limited. It is plausible that the diaphragm may protect the cervix from HPV infection and that spermicides have antiviral properties (reviewed by Schiffman and Brinton, 1995). However, Hermonat et al (1992) showed that the spermicide nonoxynol 9 does not inactivate HPV .

d) Dietary Factors.

Dietary factors may play an important role in the chemoprevention of CIN and Cx Ca., both in their role in the repair of DNA damage and in their effects on local cellular immunity. Micronutrients and medications are assumed to affect the carcinogenic process at the cellular level.

Many investigators have considered the influence of diet on the development of cervical neoplasia. To summarise, three study designs used in the study of this relationship: the food record, the food frequency questionnaire and serum nutrient levels. Potischman (1993) has reviewed the studies using these three designs, acknowledging the inconsistencies in results and concluded that vitamin C is associated with decreased risks, particularly among smokers. In this regard, some micronutrients in particular have received more attention. Women from an American-Indian settlement, with low intake of vitamin C, folic acid and vitamin E, were found to be at an increased risk of having cervical dysplasia (Buckley et al., 1992). On the other hand, of all nutrients studied so far, the most consistent relationship was found between beta-carotene deficiency and CIN and CaCx (La Vecchia et al., 1984; Wylie-Rosett et al., 1984; Orr et al., 1985; Harris et al., 1986; Brock et al., 1988; Herrero et al., 1991).

Beta-carotene is a remarkably potent source of vitamin A (retinoic acid) (Reviewed by Mitchell et al., 1995). It has been demonstrated that expression of HPV messenger RNA decreases in the presence of retinoic acid (Bartsch et al., 1992). Retinoic acid has also

been shown to increase the secretion of the beta-transforming growth factor (BTGF) in cells immortalised by HPV. BTGF can suppress the expression of E6 and E7 oncoproteins in cervical epithelial cells (Woodworth et al., 1990; Batova et al., 1992). In addition, Meyskens et al. (1994), have shown that topically applied all-Trans-retinoic acid, can enhance the regression of early premalignant cervical lesions (CIN), but has no effect in more advanced dysplasia. The accumulated evidence suggests that some components of fruits and vegetables may protect against CIN. However, Mackerras et al (1999) conducted a double-blind end trial of beta-carotene and vitamin C in women with minor cervical abnormality and they concluded that these compounds have no effect on the progression or regression of CIN.

e) Infectious Agents other than HPV

The clinical and epidemiological evidence of the association of genital cancer with HPV and sexual behaviour, indicates the possible involvement of other sexually transmitted agents, such as Chlamydia trachomatis, Human Cytomegalovirus (HCMV), and Herpes Simplex Virus (HSV-2), in the development of CIN.

The results of studies associating Chlamydia trachomatis seropositivity with the risk of cervical dysplasia, have been inconsistent after adjusting for HPV infection (De San Jose et al., 1994). Using PCR-based techniques no direct association of human cytomegalo virus with CaCx, or interaction with HPV was found (Thompson et al., 1994; Koffa et al.,

1995). Various authors have reported an increased seropositivity of antibodies to HSV-2 in-patients with CIN, when compared to normal control subjects (Hildesheim et al., 1991; Jha et al., 1993). However, when CaCx populations are stratified according to HPV status, the association with HSV-2 is only found in HPV DNA-negative cases, suggesting that HSV-2 might have a separate etiological significance in the development of CaCx (De San Jose et al., 1994; Koffa et al., 1995).

f) Immunosuppression

It has been shown that immunosuppressed women, e.g. renal transplant recipients have an increased risk of HPV infection. (Porreco et al., 1975; Sillman et al., 1984)

Patients suffering from immunosuppression induced by the Human Immunodeficiency Virus (HIV) also demonstrate a very high prevalence of HPV DNA infection (Maiman et al., 1991; Vermund et al., 1991; Ho et al., 1994; Schafer et al., 1991). They also found that the risk of CIN closely correlated to the degree of immunosuppression. Compared to women in general, those who are HIV positive are characteristically vulnerable to pre-malignant cervical lesions. Maiman et al (1993) reported 12 and 24 months treatment failure rates of 47% and 63% respectively.

1.4 Human papillomavirus

1.4.1 The virus

Papillomaviruses are non-enveloped, DNA viruses, approximately 52-55 nm in diameter, that replicate in the nucleus of the squamous epithelial cell. The papillomaviruses are widespread in nature and are associated with naturally occurring cancers in a number of animal species (Schiffman, 1992). Some 82 types of Human Papillomavirus have been identified (Kino et al., 2000) and on the basis of the site of infection, can be classified into two main groups: cutaneous (found in cutaneous warts and epidermodysplasia verruciformis), and mucosal (predominantly found in anogenital lesions) (Herrington, 1994). Lesions associated with different types of HPV are summarised in table(1.1). Mucosal HPV types commonly found in benign lesions are considered " low risk viruses" and include HPV types 6 and 11, while those frequently detected in premalignant and malignant lesions (e.g. CIN & Ca Cx) are recognised as "high risk viruses" (types 16, 18, 31, 33, 52, 58).

According to the International Committee on Taxonomy of Viruses, papillomaviruses belong to a genus of the papovaviridae family, which also includes simian vacuolating 40 virus (SV4O virus) and polyoma virus. All of them are physically similar with icosahedral virion capsids and circular double stranded DNA's (Herrington, 1994). However, they are sufficiently dissimilar from the other members of the group, both in terms of virion size and genomic organisation, to be regarded as a separate group (Van Ranst et al., 1992).

Papillomaviruses infect humans and animals (e.g., bovine, rabbit, deer papillomaviruses have been identified). Although human and animal papillomaviruses share a similar genomic organisation they are highly species specific and do not cross-infect other species. Due to the fact that the capsid proteins of papillomavirus are antigenically similar, these viruses are not subdivided into serotypes based on structural antigenic features, but instead are subdivided into genotypes and subtypes based on their extent of DNA relatedness. Formerly, to be classified as a distinct HPV type a new isolate had to have less than 50% homology with the genome of other known HPV types (Chow et al., 1987). More recently, it has been proposed that, in order to define a new HPV type, the candidate virus should have been completely cloned and show less than 90% homology in its E6, L1 and URR regions with other HPV. Consequently, a subtype would be defined as sharing 90-98% homology in these regions, and a variant more than 98% (Van Ranst et al., 1992).

It is generally accepted that infection with high-risk HPV types is the primary risk factor for the development of CIN (Shafi and Luesley, 1995). The persistence of HPV infection has been correlated with the progress of the cervical lesions (Ho et al., 1995). However, the reported prevalence of HPV in the normal population and in premalignant lesions, varies according to the DNA detection method.

1.4.1.1 Detection of the virus DNA

Many methods are used for detection of HPV e.g. cytology, colposcopy, histology, but they are neither sensitive nor specific. The details of these methods are behind the scope of this study.

The methods of detection of HPV DNA can be roughly classified in two categories: (a) direct identification of the viral nucleic acids (southern blot hybridisation, dot blot hybridisation and hybrid capture), and (b) amplification of viral DNA and detection of amplified products (Polymerase Chain Reaction (PCR)-based techniques). The former type of analysis is now known to have the limitation of low sensitivity, giving attenuated results regarding the true association between HPV infection (Schiffman and Schatzkin, 1994).

The development of PCR technology has provided a highly sensitive and specific method for detecting HPV DNA, even in very small samples. However, PCR has the disadvantage of susceptibility to contamination, which has occasionally led to an over-estimation of the prevalence of HPV in the normal population (Tidy et al., 1989; Young et al., 1989). To solve this problem a number of precautions have to be taken when performing PCR assays. These include the use of different rooms or containers for the preparation of samples, electrophoresis and PCR solution preparation. Using PCR-based techniques, cross-sectional studies of cytologically normal women (Ley et al., 1991; Bauer et al., 1993; Melkert et al., 1993) have suggested that 20-40% of sexually active young women

have detectable HPV infection, and that the prevalence decreases with age. Byrne et al (1988) showed that the majority of HPV cervical infection disappears after treatment of CIN. Table 1.2 summarised the results of selected case control studies investigating HPV and CIN, in which hybridisation assays with amplification techniques were used (reviewed by Bosh et al., 1998).

TABLE 1.1 HPV types in benign and malignant tumours

Tumours	**HPV types**	
A) Benign and pre malignant	**Frequent**	**less frequent**
Skin warts		
• Plantar warts	1	2, 4, 63
• Common or mosaic warts	2, 27	1, 4, 7, 26, 28, 29,57, 60, 65
• Flat warts	3, 10	2, 26, 27, 28, 29, 41, 49
• Skin warts or RTR		1-6, 8, 10, 12, 15, 16, 17, 25, 27, 28, 29, 41, 49, 57, unclassified types
Anogenital lesions		
• Condylomata acuminata	6, 11	2, 16, 30, 40, 41, 42, 44, 45, 54, 55, 61
• CIN, VAIN, VIN and PIN lesions	6, 11, 16, 18, 31	30, 34, 33, 35, 39, 40, 42-45, 51, 52, 56-59, 61, 62, 64, 66, 67, 69
head and neck tumours		
• Oral papillomas and leukoplakias	2, 6, 11, 16	7
• Laryngeal papillomas (RRP)	6, 11	
• Conjunctival papillomas	6, 11	
• Nasal papillomas		6, 11, 57
B) Malignancies		
• Malignant skin tumours of RTR		1-6, 8, 10, 11, 14-16, 18-20, 23-25, 27, 29, 36, 38, 41, 47, 48, unclassified types
• Bowen's disease of the skin		2, 16, 34
• Cervical cancer	16, 18, 31, 45	6, 10, 11, 26, 33, 35, 39, 51, 52, 55, 56, 58, 59, 66, 68, unclassified types
• Non-cervical anogenital cancer	6, 16, 18	11, 31, 33
• Laryngeal cancer		6, 11, 16, 18, 35
• Oral cancer		3, 6, 11, 16, 18, 57
• Tonsillar/pharyngeal cancer		16, 18, 33
• Oesophageal cancer		6, 11, 16, 18
• Nasal cancer		16, 57

Abbreviations: RTR = Renal transplant recipients, CIN = Cervical intraepithelial neoplasia, VAIN = Vaginal intraepithelial neoplasia, VIN = Vulval intraepithelial neoplasia, PIN = Penile intraepithelial neoplasia, SCC = Squamous cell carcinoma, RRP = Recurrent respiratory papillomatosis.

TABLE 1.2 The results of case control studies investigating HPV and CIN, in which hybridisation assays with amplification techniques (Review by Bosh et al 1998)

Reference	Cases / Control (type, number)	HPV	HPV prevalence (%) Cases	Control	OR
1.Bosh et al 1993					
Spain	CIN III , 157/193	hpv 16	70.7 49	4.7 0.5	56.9 295.5
Cali, Colombia	CIN III 125 / 181	HPV 16	63.2 32.8	10.5 3.3	15.5 27.1
2.Schiffman et al 1993	CIN II-III 50/433	HPV 16/18	90 62	17.7 2.9	42 180
3.Van den Brule et al 1991	Pap III-VI 177/1762	HPV 18/16	76.3 47.5	6.1 2.2	49.7 39.9
4.Becker et al1994	CIN II-III 176/311	HPV 16	93.8 52.4	42.1 8.6	20.8 9.8
5.Kjaer et al 1996	CINIII 79/1000	HPV 16,18,31,33	77.2 62	15.4 8.2	13.6 32.9
6.Olsen et al1995	CINII-III 103/234	HPV 16	91	15	72.8 182.4

OR= odds ratio

TABLE 1.3 Smoking and cervical neoplasia: case- control studies CIN Grade III (Szarewski, 1998)

First author /year	No.cases /controls	current versus never smoke		heavy exposed		exposure variable	Dose response
		ad	unad	ad	unad		
Harris 1980	146/422	3.3	-2.2	4	2.1	>20 cig / day	yes
Lyon 1983	217/243	6.6	3	3.9	2.4	>20years smoked	no data
Marshall 1983	513/490	1.6		1.5		>1/2 pack years	no
Trevathan 1983	180/288		3.7	11.6		.12 pack year	yes
Zunzunegui 1986	39/39	0.6		3		>10 cig/day	inadequate dat
Brock 1989	116/193	5.3	4.5	7	5.1	>30day	yes
Cuzick 1990	284/833	2.4	1.7	4.5	2.4	>8 pack years	yes
Jones 1990	293/799	2.6	1.9	3.7	2.3	>30 cigs day	yes
Coker 1992	63/268	9.4	4.6	6.3	3.5	>10 years	yes
Munoz 1993	525/512						
Spain	249/242	1.8	1.3	3.1	-3.2	>5 pack years	yes
Colombia	276/270	2	2	2.1	-1.7	>5 pack year	no

ad=adjusted

unad=unadjusted

Cig= Cigarette

1.4.2 Genital HPV Infections

Genital HPV infections are usually transmitted by sexual acts (Ley et al., 1991; Hildesheim et al., 1993). This fact is supported by the observations that cervical HPV infections are very rare among virgins (Fairley et al., 1992), and cervical HPV prevalence peaks between the ages of 16-25, suggesting that the transmission of HPV infection to the cervix usually occurs soon after the initiation of sexual activity (Schiffman, 1992).

Although, sexual transmission is the most important route of genital HPV infection, vertical transmission has also been demonstrated and is the most likely explanation for the detection of HPV infections in new-born and older children (Roman and Fife, 1986; Cason et al., 1995). Additionally, fomite transmission of HPV is possible, as suggested by the presence of HPV DNA on underclothes, gynaecological equipment, fingers, pubic and perianal hair from patients with genital HPV (Ferenczy et al., 1989; Ferenczy et al., 1990; Boxman et al., 1999; Sonnex et al., 1999).

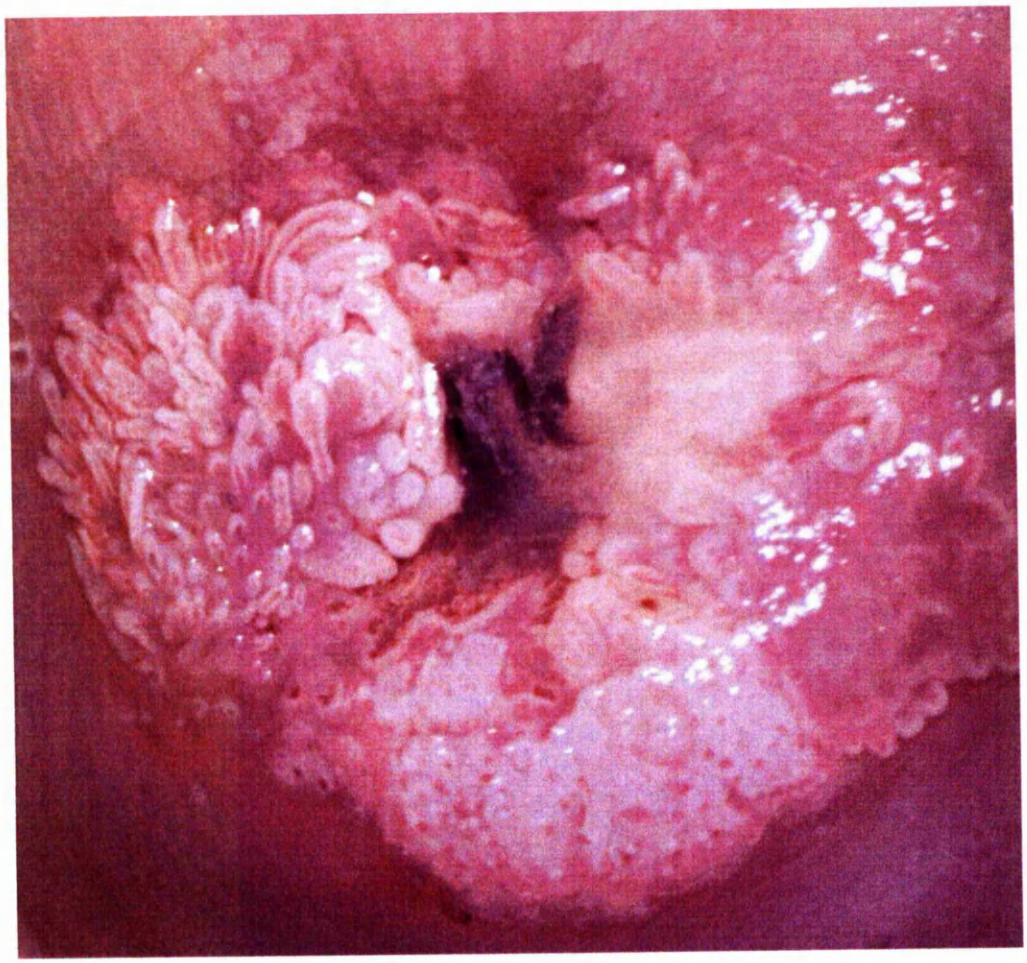
1.4.2.1 Pathology of genital HPV

Sexually transmitted HPV infect the epithelium of the lower genital tract producing papillomas and warts. The changes induced by an HPV infection are seen in intermediate squamous cells and consist of a large perinuclear vacuole in the cytoplasm hence the affected cell is referred to as a koilocyte (Gross and Barrasso, 1998). In the koilocyte the nucleus is enlarged, irregular and hyperchromic, binucleation is also a common feature.

Additional changes include individual cell keratinization, dyskeratosis and degenerate giant nuclei. (Figure 1.5)

The most common morphological manifestation of HPV infection in the lower genital tract is genital warts, or condylomata acuminata. In the female genital warts are commonly multiple and occur most frequently on the vulva, but spread onto the portio vaginalis and the cervix, producing pedunculated filiform lesions which may coalesce producing tumour-like masses (Figure 1.6).

FIGURE 1.6 Genital warts in the cervix



1.4.2.2 Incidence and epidemiology of genital HPV

The true incidence and prevalence of HPV are equally difficult to estimate because it is not a notifiable disease The peak prevalence of anogenital HPV infection is between 16 and 25 years of age, while younger and older women are less likely to test positive for HPV (Schiffman, 1992). Most HPV infections disappear within months or a few years of diagnosis (Schiffman and Brinton, 1995). It is estimated that the prevalence of non-expressed (latent) HPV infection and disease which is only identifiable colposcopically (i.e. subclinical) is at least three times the prevalence of disease which is clinically apparent without biological DNA or magnification (review in Schneider and Koutsky, 1992). Ho et al (1998b) found that the median duration of HPV infection in their cohort was 8 months and by 12 months after which 70% of the women were no longer infected.

In case series worldwide, most cervical cancers have been found to contain HPV of the same 10-15 types (Bosch et al., 1995). Additionally, HPV has also been detected in metastases of Ca Cx patients and corresponds to the type observed in the primary tumours (Lancaster et al., 1986). Bosch and Collaborators in 1995 in a multinational project using a PCR protocol based on consensus primers flanking the HPV L1 gene, showed that 93% of more than 1,000 cervical tumours from patients from 22 countries were positive for the presence of HPV DNA. In general, HPV 16 was the most common type found with an overall incidence of 50%, followed by HPV 18 with an incidence of 14%, HPV 45 (8%) and HPV 31 (5%). Distribution of the major HPV types was observed to vary

geographically (Bosch et al., 1995). HPV distribution worldwide is shown in figure 1.7.

It is clear that HPV plays a key etiologic role in cervical neoplasia. Nevertheless, the frequency of HPV infection in asymptomatic women, and the existence of non-HPV related cervical tumours, shows that HPV infection alone is not sufficient for the development of cervical malignancies. A number of other factors e.g. smoking, parity have been recognised as important in cervical carcinogenesis (Schneider, 1993), but HPV plays an essential role manifested by persistence of the viral genome during the entire natural life history of cervical cancer.(Hu et al., 1999)

1.4.3 HPV Biology.

The papillomavirus life cycle is intimately associated with the growth and differentiation of the host epithelial cells. Observation of a cross-section of infected epithelium shows that viral transcription starts in the lower, basal part, and overall expression increases from basal cell up to the top (McCance, 1994). (FIGURE1.8)

FIGURE 1.7 HPV distribution worldwide

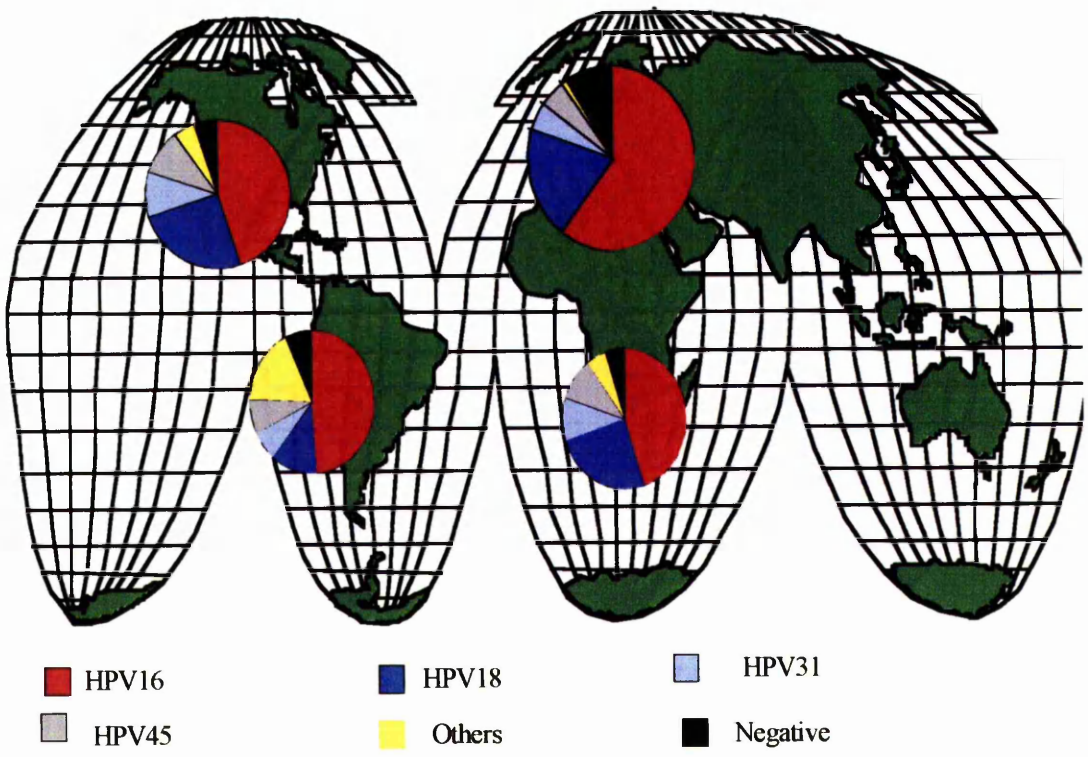
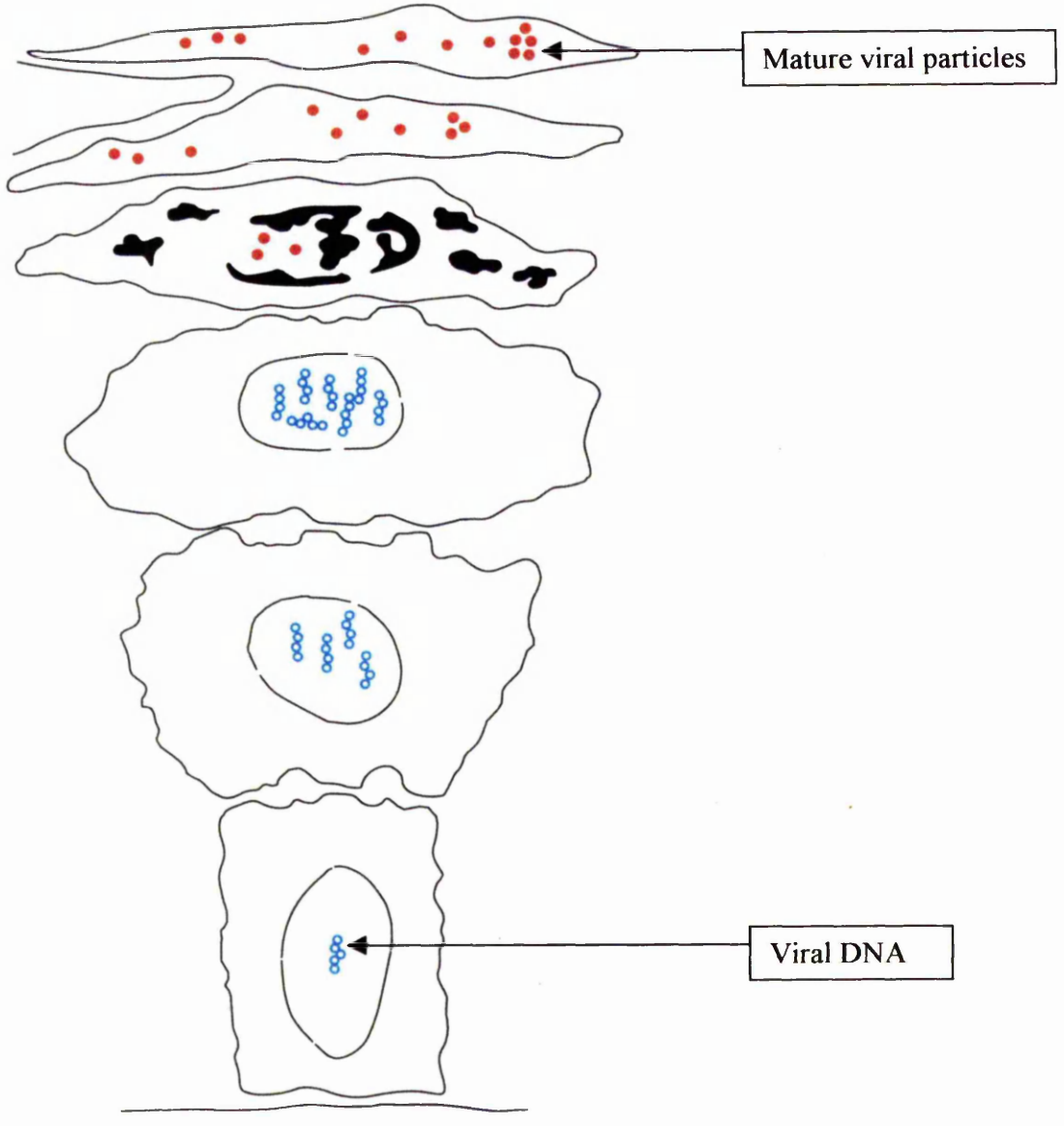


FIGURE 1.8 Replication of HPV in the epidermis



The means by which the virus gains access to basal epithelial cells is not known and, although abrasion and microwounds of the epithelium are postulated explanations, evidence for such a hypothesis is lacking (Schneider and Koutsky, 1992).

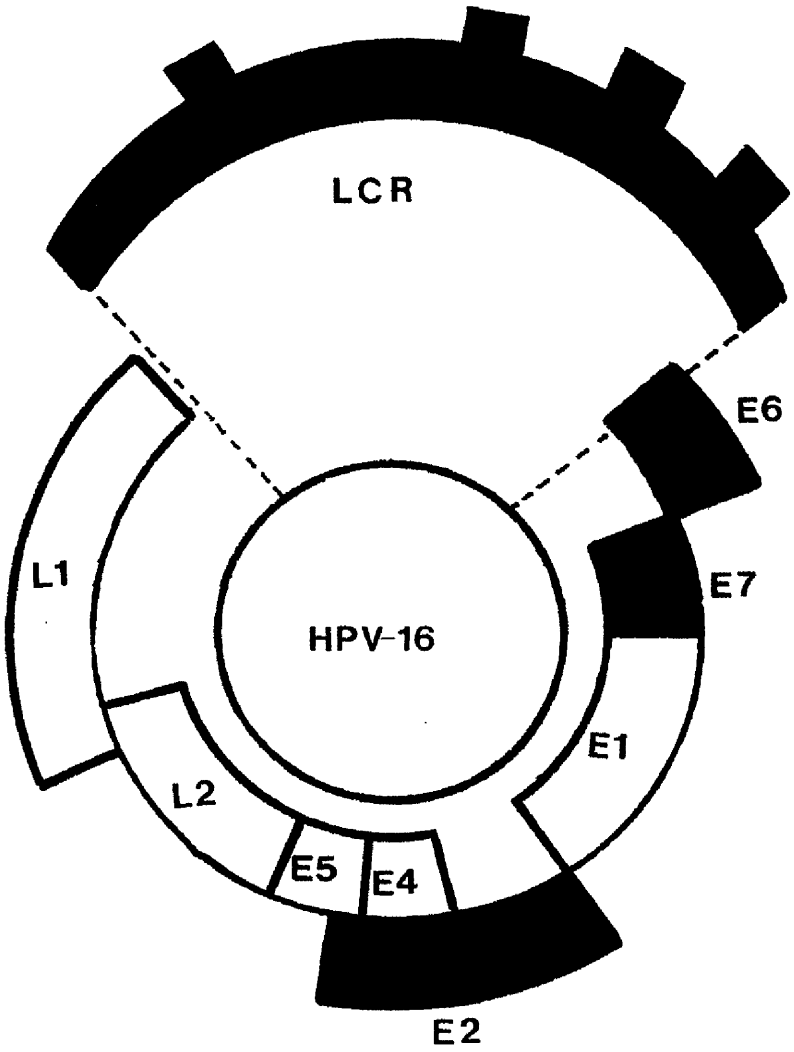
Following infection, HPV genomes are established in basal cells. Viral DNA remains in an episomal state (Durst et al., 1985) at a level of 20 to 100 copies per cell, and replicates coordinately with cellular replication (Laimins, 1993). As infected cells differentiate and migrate up from the basal to the middle, episomal DNA replication is stimulated markedly, producing an accumulation of a high viral copy number (up to thousands of copies per cell) in the cell nucleus (Lambert, 1991). Analysis of viral messenger RNA production using in situ hybridization has shown that early gene expression occurs throughout the epithelium, but late gene expression is only seen in terminally differentiated keratinocytes forming the superficial layers, where it is accompanied by capsid protein production and formation of complete virus occurs (Herrington, 1994; DiPaolo et al., 1993; Laimins, 1993). In vivo virion production occurs in lesions of the cervix (condyloma and CIN I) which exhibit only slightly altered patterns of differentiation in keratinocytes (Koss, 1987).

1.4.3.1 Genomic organisation and transcription of papillomavirus

The analysis of mRNAs and the nucleotide sequence of human and animal papillomavirus genomes has revealed overall structural similarities in their genetic organisation (Mansur and Androphy, 1993). It consists of at least 8 potential protein-coding sequences or open

reading frames (ORFs), all on the same strand of DNA (Figure 1.9). This is consistent with the observation that the same strand encodes all viral mRNA transcripts that have been detected. Papillomavirus genetic information is transcribed and translated in one direction from only one strand of the DNA (Amtmann and Sauer, 1982; Heilman et al., 1982; Engel et al., 1983). The other DNA strand contains only small ORFs and is assumed to be non-coding. This arrangement contrasts with the genetic organisation of other members of the papovavirus family, such as polyoma virus and SV40, where early and late functions are encoded in different DNA strands (Tooze, 1980).

FIGURE 1.9 HPV 16 open reading frames (ORFs)



Papillomavirus genomes can be functionally divided into two coding regions, and a non-coding segment of 0.4-1.0 KB. The early (E)-coding region contains the early genes El to E8, including information necessary for viral replication and cellular transformation. The late (L)-coding region contains the late genes L1 and L2, which are expressed only in differentiated keratinocytes and which code for the structural proteins of the virus capsid. The non-coding region is located between the end of the L1 ORF and the start of the E6 ORF. This region exhibits characteristic features including AT-rich regions, transcriptional enhancers, as well as binding sites for cellular transcription factors. The non-coding region is commonly termed the long control region (LCR) or upstream regulatory region (URR) (Laimins, 1993).

1.4.3.1.1 The upstream regulatory region

The URR is an approximately 400 base-pair DNA segment including the origin of replication. The URR, contains a complex array of overlapping binding sites for many different transcriptional activators, including virally derived transcriptional factors encoded by the early region (Turek, 1994). The URR regulates transcription from early and late regions and controls the production of viral proteins and infectious particles. Because of its potential for binding a wide variety of specific transcriptional factors, the URR may play a critical role in determining the host range of specific types of HPV (Turek, 1994).

Viral transcripts of the oncogenic E6 and E7 genes are controlled by these enhancers,

upstream of the E6 gene at a promoter referred to by nucleotide number as p97 in HPV 16 and HPV 31 (Baker et al., 1987) or p105 in HPV 18 (Schwartz et al., 1985; Schneider-Gaedicke and Schwartz, 1986).

1.4.3.1.2 Early region

The early (E) ORF's encode proteins involved in the DNA replication, transcription and cellular formation of papillomaviruses and are located downstream of the LCR. In bovine papillomavirus (BPV) 8 early ORFs were identified, designated El to E8, while HPV have only 6 early ORF (E1, E2, E4, E5, E6 and E7). It is believed that BPV E3 ORF does not encode any protein (Hermonat and Howley, 1987). The product of the BPV E8 ORF seems to be involved in replication; however, there is not an E8 homologue in HPV (DiMaio and Neary, 1990).

a) E1

The El ORF is absolutely required for virus DNA replication. Two replication functions have been ascribed to E1, the first being a modulator (E1-M) function encoded by the first one third of the ORF, whilst the second is a positive replication function (E1-R). (Chiang et al., 1991). The El protein is able to form complexes with E2 to regulate the extrachromosomal DNA replication and completion of viral cycle (Frattini and Laimins, 1994). HPV 16 E1 may have a role in repression of immortalization. (Romanczuk and Howley, 1992)

b) E2

The E2 ORF has been studied principally in the BPV system. The E2 of the BPVs is a nuclear protein with a predicted molecular weight of 45 to 48 KDa, which binds DNA as a dimer (Androphy et al., 1987). The DNA-binding and dimerization domain is located in the COOH-terminal part of the protein, whereas the NH2-terminal part is involved in transcriptional activation (Haugen et al., 1988; Gin and Yaniv, 1988). These two domains are connected by a flexible 'hinge' region. The sequence of the amino-terminal domain is more conserved than the carboxyl-terminal part, while the hinge is very little conserved among different viral types (Gin and Yaniv, 1988).

In HPV, the E2 is required for DNA replication in conjunction with E1. The transactivator E2 of HPV-11 (Chiang et al., 1992) can associate with the El polypeptide, and together are necessary for viral DNA replication. E2 clearly has an effect on the process of immortalization / transformation but the precise mechanism is still uncertain. The E2 is a nuclear protein; the nuclear localisation signal has been demonstrated to reside in the carboxyl-terminal 89 amino acids, for the case of the HPV 16-E2 protein (Sanders et al., 1995).

In HPV, E2 repressor forms have not yet been identified. In 1987, Cripe and co-workers suggested that the 5' part of the HPV 16-E2 ORF may encode a transcriptional repressor, but did not demonstrate the existence of such a repressor in HPV 16 infected-cells (Cripe et al., 1987). mRNA species with the potential to encode N-terminal truncated E2 forms have been observed in both, CIN and CaCx lesions (Durst et al., 1992).

Modulation of expression of the oncogenes E6 and E7 by E2 has important implications for malignant progression. In approximately 5% of advanced CIN (CIN 2 and 3) (Cullen et al., 1991; Daniel et al., 1995), as well as in over 80% of cervical carcinoma tumour biopsies (Durst et al., 1985; Cullen et al., 1991; Kristiansen et al., 1994; Daniel et al., 1995), cell lines derived from cervical neoplasms (Yee et al., 1985; Awady et al., 1987) and immortalized human keratinocytes (Barbosa and Schlegel, 1989), the viral DNA has been integrated into the host DNA. Integration of HPV DNA appears to be associated with the establishment of an immortalized phenotype in cultured cells (Yokoyama et al., 1995).

It has been suggested that integration of HPV into human chromosomes is not a random event (Rakoczy et al., 1993). It occurs preferentially at the E1-E2 ORFs levels, leaving intact the E6 and E7 ORFs (Awady et al., 1987; Rakoczy et al., 1993; Chen et al., 1994). Disruption of the E2 gene may lead to a loss of regulation by E2 of E6/E7 transcription, producing in consequence an overexpression of these oncoproteins (Saewha and Lambert, 1995). For this reason, integration of viral DNA is considered as an important step in the progression of HPV lesions to cancer (Kristiansen et al., 1994).

c) E4

Although E4 ORF is located within the early region of the papillomavirus genome, it is expressed in later stages of infection, along with the L1 and L2 late capsid proteins, when complete virions are being assembled (reviewed by Park et al., 1995). In some cases E4 is the most abundant constituent of the infected cell, comprising for instance up to 30% of the protein mass of HPV 1-induced skin warts (Doorbar et al., 1986).

The E4 protein does not seem to possess transforming properties, but is associated with the cellular membrane and accumulates in the cytoplasm (Brown et al., 1994). In human keratinocytes, E4 induces the collapse of the cytoplasmic cytokeratin network, suggesting that these changes facilitate the release of viral particles (Doorbar et al., 1991). Collapse of the cytokeratin network could cause the characteristic perinuclear clearing, or halos (koilocytosis), observed histologically and cytologically in HPV productively infected cells (reviewed by Park et al., 1995).

d) E5.

Some reports have described transforming activity for the HPV 16-E5 protein (Leptak et al., 1991; Straight et al., 1993). It is not clear if HPV E5 contributes to the development of human tumours, but taking into account its transforming potential in vitro, it is conceivable that it may play a role in the initiation of malignant transformation (reviewed by Arrand, 1994).

E) E6 and E7

The products of the E6 and E7 genes are the main transforming factors recognized in human papillomaviruses. The mechanisms by which HPV functions in malignant progression appear to be related to the activity of the two viral oncoproteins, E6 and E7, which form complexes with cell proteins normally involved in controlling cell growth. Expression of E6 and E7 is likely to overcome the regulation of cell proliferation normally mediated by proteins, p53 and Rb, allowing uncontrolled growth and providing the potential for malignant transformation.

Genital HPV encoded E6 and E7 proteins show some ability to disrupt the normal control of cell growth and contribute to cell transformation. In both rodent and human cells E6 and E7 encoded by low risk viruses (such as HPV 6 and HPV 11) showed a dramatically lower activity than those encoded by high risk viruses (such as HPV16 and HPV 18). (Halbert et al., 1992). Specific HPV 16 E6 and/or E7 sequence variations may be associated with a high risk for progression (Nindl et al., 1999).

1.4.3.1.3 Transformation capacities of HPV

Tissue culture studies have demonstrated that HPV E6 and E7 genes exhibit immortalizing properties in human foreskin keratinocytes (Pirisi et al., 1987; Hawley-Nelson et al., 1989; Kaur et al., 1989; Hudson et al., 1990). However, E6 and E7 genes alone are not sufficient to induce tumorigenicity, but are able to cooperate with an activated ras

oncogene to transform human cervical epithelial cells (Matlashewski et al., 1987; Crook et al., 1991).

a) E6

The HPV 16-E6 ORF encodes a protein of approximately 150 amino acids that is structurally related to SV 40 virus (Barbosa et al., 1989). All known papillomavirus E6 genes have the potential to form two large zinc fingers. The base of each finger contains four cysteines (Cys) in two pairs of the motif Cys-x-x-Cys, where x varies among the viruses. The putative E6 fingers consist of 29-30 amino acids, and have been shown to bind zinc (Barbosa et al., 1989).

The E6 proteins are 16 KDa in size and are localised primarily to the nucleus, but a significant subpopulation has also been found in cytoplasmic membranes (Androphy et al., 1987). It is importance is not known. The E6 protein is capable of binding to the product of the tumour suppresser gene p53 (Werness et al., 1990), which is normally involved in the suppression of cell transformation (Michalovitz et al., 1990).

The binding of E6 to p53 is dependent upon another cellular protein, E6-associated protein (E6-AP) (Huibregtse et al., 1991), and leads to an increased instability of the p53 protein (Scheffner et al., 1990). Consistently, E6 has been shown to inhibit p53 protein's transcriptional transactivation activity in tissue culture cells (Mietz et al., 1992).

Binding of HPV 16-or HPV 18-E6 protein to p53 results in loss of the p53 activity within the cell and stimulates degradation of cellular p53. (Thomas et al., 1999). Therefore, the

cellular levels of p53 are low in cells expressing HPV 16-or 18-E6. A carboxyl-terminal region was found to be important for p53 binding, while an amino-terminal domain did not mediate p53 binding, but appeared to be necessary for p53 degradation (Foster et al., 1994).

Compared with the E6 protein of HPV with low oncogenic potential (i.e., HPV 6, HPV 11), the E6 of oncogenic viruses (i.e., HPV 16, HPV 18) seems to bind p53 with a higher affinity and/or accelerates its degradation (Thomas et al 1999).

b) E7

The E7 ORF encodes the major transforming protein (E7) of genital HPV. The E7 oncoprotein is an acidic 98 amino acid phosphoprotein localized to the cell nucleus (Greenfield et al., 1991).

The E7 protein has been found in cervical carcinomas and cell lines derived from them (Smotkin and Wettstein, 1986; Smotkin and Wettstein, 1987; Seedorf et al., 1987), whereas an E7 mRNA has not been identified in lesions produced by some non-oncogenic HPV types (i.e. 11 and 6) (Chow et al., 1987) but requires a second oncogene to induce full malignant transformation (Storey et al., 1988; Bedell et al., 1989). In human cell models it has been shown that E7 together with E6 from oncogenic HPV are necessary for immortalization of primary keratinocytes (Kaur and McDugall, 1988; Barbosa and Schlegel, 1989; Munger et al., 1989).

E7 has been demonstrated to bind the retinoblastoma gene product (Rb) protein and the Rb related proteins p107 and p130 (Dyson et al., 1992).

In normal cells, Rb, p107 and p130 form complexes with transcription factors of the E2F family in the G0 and G1 phases of the cell cycle (Hinds and Weinberg, 1994) These complexes negatively regulate cell growth by repressing transcription of E2F-dependent genes, whose products are proteins required for DNA synthesis. As cells progress through the G1/S cell cycle boundary, the E2F-Rb complexes dissociate, and free E2F becomes available to stimulate the transcription of E2F-dependent genes and allow DNA replication (reviewed by Park et al., 1995).

The E7 protein of HPV 16 alters this cellular growth control mechanism by binding to Rb and dissociating the E2F-Rb complex (Chellappan et al., 1992), causing the release of transcriptionally active E2F and consequent replication of cellular DNA. E7 protein from HPV 6 has a lower Rb binding affinity than that observed for the E7 of HPV 16 and 18. The difference in Rb binding affinity of oncogenic and non-oncogenic HPV, is due to a single amino acid substitution found in the E7 protein of oncogenic HPV types. In fact, it has been demonstrated that mutation of the E7 of HPV 6, to induce this particular amino acid substitution, leads to a dramatic increment in the Rb binding affinity of this protein. The mutant also showed an increased in vitro transforming activity (Heck et al., 1992).

1.4.3.1.4 Late Region.

The late gene region of papillomavirus contains two separate ORFs, termed L1 and L2, which encode the viral capsid proteins. The L1 ORF encodes the major capsid protein of the mature papillomavirus virions. The virus particle consists of an icosahedral array of major (11) and minor (l2) capsid proteins.

The L1 protein of HPV16 contains 4 potential N-linked glycosylation sites. However, the majority of the L1 was glycosylated and localized to the nucleus. These observations suggest that the glycosylated form of L1 is unlikely to be an important part of the virion (Zhou et al., 1993)

The L2 ORF encodes the minor capsid protein. L2 may play a role in the stabilization of the capsid structure (Hagensee et al., 1993)

1.4.4 Role of HPV testing in the screening for cervical cancer

To date there are no reports on the value of HPV testing in the prediction of the treatment outcome of CIN.

It is possible that HPV testing could assist in the detection of women who are at risk of developing cervical cancer even before their smear is positive (Duncan, 1997).

Currently in the U.K, HPV testing does not have an established role in routine clinical practice. HPV testing may play a role in the management of women with borderline or mildly dyskaryotic smears as testing for HPV DNA from women with abnormal smears can help to determine which women have CIN (Nuovo, 1990). Cuzick et al (1994) showed that HPV testing from cervical smears could help predict the severity of the underlying CIN lesion in women attending for colposcopy because of an abnormal smear. Manos et al. (1999) concluded that HPV testing from cervical smears could predict the majority of high-risk cases. Major randomised trials are currently underway in Sweden, Netherlands and another one will be in U.K.

Reports have suggested that HPV testing may be of value as primary screen. Koutsky et al (1992) reported a high predictive value of HPV testing for development of CIN II-III in cytologically negative women.

HPV testing may be of value as primary screen in older women as the high prevalence of HPV occurs in younger age group and persistence of HPV infection has been associated with persistent of CIN (Ho et al., 1995).

Cruickshank et al (1999) suggested testing for HPV 18 & 16 in combination did not increase the sensitivity and specificity of repeat cytology for borderline or mildly dyskaryotic smears .

1.5 Smoking, genetic polymorphism, DNA adducts and CIN

1.5.1 Introduction

Metabolism in the body apparently plays a dual role in the handling of and protection against exogenous compounds. In most cases, toxic (chemical reactive) intermediates will be detoxified or bioinactivated at their site of formation, but under circumstances of inefficient detoxification, they can induce biochemical and physiological changes which may lead to toxic effects such as cell damage, cell death or tumorigenesis. (Caldwell and Jakoby, 1983; Anders, 1985)

Expression of the varied biological effect of chemical carcinogens depends upon their interaction with cell constituents. Generally, this occurs via covalent reactions that lead to the formation of carcinogenic adducts. In some cases, carcinogens may be spontaneously reactive in biological media, but for the most part they are inert and require conversion to a reactive intermediate. A category of enzymes collectively referred to as the mixed-function oxadises converts these xenobiotics to highly reactive electrophilic agents that will react instantaneously with nucleophilic sites in substrate molecules (Miller, 1978).

The distribution of these enzymes within the body may play a large part in determining the tissue-specific action of many carcinogens. Similarly, the distribution of enzymes responsible for conversion of carcinogen to less harmful metabolic products may lead to

adduct formation e.g. S- (2-chloroethyl) glutathione. (Cmarik et al., 1992).

Over the past two decades, DNA has become universally recognised as a critical, if not the critical, target for the action of carcinogens. Realisation of the importance of reaction with proto-oncogenes, tumour-suppresser genes and growth- factor genes and studies of mechanisms of carcinogen action have focused increasingly upon the role of DNA adducts and their ability to introduce changes into DNA. (Cooper et al., 1995)

1.5.2 Biotransformation reactions and enzymes

In general terms, biotransformation is the conversion of lipophilic xenobiotics into more hydrophilic, water-soluble metabolites, thereby serving to (1) reduce the biological half-life of the xenobiotics, (2) reduce the duration of the exposure to xenobiotics, (3) avoid accumulation of the parent xenobiotics in the organism, (4) change the biological activity of the xenobiotics and (5) change the duration of the biological activity of the xenobiotics. Although other organs and tissues are also relevant, the liver is quantitatively the most important organ in the process of biotransformation, a process which may be divided into phase I, phase II and phase III metabolism.

Phase I metabolic transformations include reduction, oxidation and hydrolysis reactions in order to reveal or introduce functional or reactive groups in the molecules. Phase II transformations are generally conjugation reactions of the parent xenobiotics or of phase I metabolites with, for example, inorganic sulphate, amino acids, glucuronic acid or

glutathione. Conjugation reactions facilitate transport and enhance elimination via the renal and biliary routes. Phase III metabolism constitutes the further metabolism of the products derived from phase II conjugation reactions. Phase III reactions are catalysed by enzymes which are also active in phase I and/or phase II reactions. Basically , phase III reactions differ from phase I and phase II reactions only in the fact that the substrates are products of previous phase I or phase II metabolic reactions. (Pitot, 1988)

1.5.3 The role of molecular biomarkers in epidemiological research

The term "Molecular Epidemiology" was defined as the use of molecular biomarkers in epidemiological research (McMichael, 1994). In cancer epidemiology, biomarkers are used to measure, the variation in individual susceptibility to carcinogens caused by genetic polymorphism of metabolising enzymes, the level of exposure at the target tissue (DNA) and early biological effects such as cytogenetic damage and mutations. The main objective for using biological markers to screen a population is to identify high cancer risk groups and hence design and evaluate intervention strategies and/or early treatments.

1.5.4 Metabolic polymorphism in relation to genetic susceptibility to cancer

Epidemiological and human genetic studies have identified different types of population "at risk," one consisting of individuals with heavy exposure to carcinogens, such as smokers and exposed workers, and the other consisting of carriers of cancer-predominating germ-line mutations in genes that because of high penetrance confer a very

high risk for cancer per se (Caporaso and Goldstien, 1995).

Genetically determined variation in responses to environmental exposures, that are associated with common malignancies, is an attractive mechanism to explain the differential individual susceptibility to tobacco carcinogens. Such individual susceptibility to CIN and cervical cancer due to environmental exposure to carcinogenic agents has been speculated to be modulated by host-specific factors. These include differences in metabolism, DNA repair and altered expression of tumour suppresser genes. The majority of environmental carcinogens are not capable of causing hazardous effects per se and metabolism of these compounds is a crucial part of the initial host response to environmental exposure. The efficiency of carcinogen metabolism could alter the relative risk of carcinogenesis, either conferring a protective effect, by the rapid clearing of carcinogens or their metabolites, or increasing risk through the activation of procarcinogens. Differences in the balance between activation and detoxification may thus explain, at least in part the individual variation in response to the exposure to carcinogens (Wolf, 1990).

One factor that is known to be able to affect metabolising enzyme activity is genetic polymorphism. This can occur in many xenobiotics metabolising enzymes leading to variation in the levels of enzyme expression and /or activity in vivo. Enzymes showing such polymorphism include the phase I enzymes: cytochrome P450 CYP1A1, CYP1A2, CYP2D6 and the phase II enzymes such as glutathione S-transferases (GST). Altered phenotypes and genotypes of these enzymes have been suggested to contribute to

individual cancer susceptibility as genetic modifiers of cancer (Kaderlik and Kadlubar, 1995; Raunio et al., 1995). The mutations which give rise to several of these polymorphism have been identified and genotyping assays for polymorphism in CYP2D6 ,GSTM1, GSTT1 genes have been developed. Many of the polymorphic genes of carcinogen metabolism show considerable ethnic differences in gene structures and allelic distribution (as detailed later).

1.5.4.1 Cytochromeoxidase 2D6 (CYP2D6)

There is a considerable interest in the 2D subfamily, due to the fact that about 30% of all currently used drugs are metabolised in man by the 2D6, which is known to exhibit genetic polymorphism in human ethnic groups. As the constitutive levels of 2D6 are low (0.5%) of human hepatic P450 complement and the enzyme is non inducible, any defect in 2D6 – dependant metabolism may have serious consequences with respect to an undesirable accumulation of the original pharmaceutical agent, leading to potential toxicity and possible adverse reactions. A particular characteristic of exogenous 2D 6 substrate is the presence of the nitrogen atom, which is likely to be protonated (and, therefore, positively charged) at physiological pH (Lewis, 1995).

A well-defined genetic polymorphism is at the cytochrome P450 CYP2D locus that is located on chromosome 22, that encodes cytochrome CYP2D6 (Wolf, 1990). As all substrates for this enzyme possess one or more basic nitrogens. CYP2D6 may play a role in the metabolic activation of the tobacco specific procarcinogen 4-(methylnitrosoamino)-

1-(3-pyridyl)-1-butanone (NNK) as well as the metabolism of nicotine to cotinine (Crespi et al., 1991).

The CYP2D6 polymorphism is perhaps the most studied human enzyme deficiency. The CYP2D6 enzyme exhibits polymorphic activity due to the presence of several disabling mutations in the CYP2D6 gene (Gough et al., 1990). Approximately 5-10% of Caucasians and <1% of the Chinese or Japanese population are unable to metabolise debrisoquine and are termed poor metabolisers (PM) (Gough et al., 1990; Wolf et al., 1992). The PM phenotype is caused by several mutant alleles of the CYP2D6 gene and is inherited as an autosomal recessive trait. (Gonzalez et al., 1988). Individuals with normal debrisoquine hydroxylase activity are designated 'extensive metabolisers' (EM), they show a broad spectrum of enzyme activity, from relatively low to ultra-high. In the last few years more than 90% of the mutations of the CYP2D6 gene that cause absence of the CYP2D6 protein and result in the debrisoquine poor metaboliser phenotype have been identified (Bartsch et al., 2000). Individuals with the PM phenotype (i.e., deficient in debrisoquine 4-hydroxylation) were first reported in 1984 to have a lower risk for lung cancer (Ayesh et al., 1984). The PM phenotype is attributable to several defective allelic CYP2D6 variants, three of which account for >90% of all PM individuals. The mutations that cause loss of gene function are attributable mainly to two main point mutations that result in CYP2D6*3 (formerly A) (consists of a one base pair deletion at exon 5, allele frequency in PMs about 5%), and CYP2D6*4 (B) alleles (the allele frequency in PMs is about 70-85%, characterised by a G to A transition mutation at the junction of intron 3 / exon 4) and deletion of the entire CYP2D6*5 allele (D) (allele frequency in PMs about 10-15%). A

CYP2D6*2xn (L2) allelle associated with 2-12 fold amplification of the CYP2D6 gene is found in carriers known as ultra metabolisers (reviewed by Bartsch et al., 2000) .

Several studies have shown that the EM phenotype is associated with an increased risk of various cancers, including lung (Ayesh et al., 1984), bladder (Kaisary et al., 1987) and liver cancer (Agundez et al., 1995) as well as pituitary adenomas (Perrett et al., 1995). Warwick et al. (1994b) studied the polymorphism at loci that encode carcinogen-metabolising enzymes such as CYP2D6 to determine susceptibility to CIN. The results suggested that women with CYP2D6 EM who smoked had increased susceptibility to CIN.

1.5.4.2 Glutathione S-transferases (GSTs)

GSTs are a multigene family of enzymes that catalyse detoxification of toxic or mutagenic electrophiles, which in most cases are generated by CYP 450, mediated oxidation reactions. This detoxification process involves conjugation of the electrophile to Glutathione, which precedes elimination from the host by excretion and as a consequence prevention of its reaction with critical target sites such as DNA. Tissues such as the testes and ovary contain high GST content, which would be compatible with a role in the protection of germ cells against toxins or mutagen. (Ketterer et al., 1988; Wolf, 1990). The expression of GST M1 has been detected in HPV16 transfected human cervical keratinocytes in culture (Chen and Nirunsuksiri 1999).

Several of the genes encoding for the above-mentioned GSTs enzymes are polymorphic in humans (summarised in TABLE 1.4). One of these genes is GSTM1 involved in detoxification of polycyclic aromatic hydrocarbons (PAHs) (Ketterer, 1988). About 50% of Caucasians are homozygous for a deletion in the GSTM1 gene (termed the null genotype) with a consequent absence of the enzyme activity. GSTM1 null frequencies exhibit ethnic variations and have been associated with increased susceptibility to different types of cancer. (Hirvonen et al., 1993, Zhong et al., 1993)

TABLE 1.4: Genetic polymorphism of some phase II metabolising enzymes.

Enzymes	Potential substrates	Polymorphism (allelic variant)	Phenotype	Prevalence
GST M1	PAHs Nitrosoureas	GST M1 wild type GST M1 null	Conjugator Non-conj.	40-50% GST M1 null
GST T1	Halogenated alkanes	GST T1 wild type GST T1 null	Conjugator Non-conj.	14-20% GST T1 null

1.5.4.3 Combinations of susceptibility genotypes and DNA adduct.

Cancer susceptibility due to chemical exposures is likely to be determined by an individual's genotype with respect to a number of enzymes, both activating and detoxifying, that are relevant to the chemical compound or mixture of compounds involved. Given the number of carcinogen metabolising enzymes now identified, the variability in expression of these enzymes and the complexity of chemical mixtures to which individuals may be exposed, the assessment of a single enzyme or genotype is unlikely to be sufficient to determine predisposition. Several recent reports have evaluated the combined effects of risk genotypes on cervical cancer susceptibility. For example, Warwick et al. (1994a) studied the combined effect of GSTT1, GSTM1 and CYP2D6 genotypes on the susceptibility to cervical neoplasia and they found an increased frequency of high grade CIN in GSTT1 null smokers with the CYP2D6 EM genotype.

Kato et al. (1995) have pointed to the importance of using molecular biomarkers for genetic polymorphism in combination with DNA adducts as an approach to predict disease risk on an individual basis rather than in general populations. Since DNA damage is one of the earliest events in the multistage process of carcinogenesis. it could be useful in predicting the risk before the onset of the clinical disease

No data are available regarding the relationship between genetic polymorphism, in carcinogen-metabolising enzymes, DNA adduct and susceptibility of treatment failure of CIN.

1.5.5 DNA damage and repair in relation to carcinogenesis

Exposure to environmental and occupational carcinogens is considered to be an important etiological factor in human cancer and molecular epidemiological techniques are being used for the identification and measurement of biomarkers that might predict cancer risk from such exposures. The biologically effective dose of a carcinogen in humans depends on exposure, uptake and activation and on the amount of the activated derivatives that reacts with the critical cellular targets (Harris, 1989). Biological markers such as DNA adducts are considered as indicators that the body has been exposed to carcinogenic agents and can be considered as an end point for research rather than other precancerous lesions (Bartsch et al., 2000). However, the biological consequences of such damage can also depend on DNA repair as well as DNA replication and programmed cell death pathways.

It is well established that DNA can be damaged by a very wide range of environmental agents including physical agents for example UV light and ionising radiation, and chemical agents for example alkylating and polycyclic aromatic hydrocarbons etc. Reaction of DNA bases with electrophiles, oxidising agents, or ultraviolet light produces covalently modified bases that are referred to as DNA adducts (Lawley, 1989). The formation of DNA

adducts is believed to be the earliest indicator of the potentially adverse biological effects arising from exposure to chemical carcinogens. (Wogan, 1988) DNA damage has been reported to interfere with cell signalling pathways and causes cell cycle arrest and if unrepaired, may induce apoptotic cell death (Enoch and Norbury, 1995).

The type of damage considered in the experimental work of this thesis is alkylation damage and other categories of damage therefore will not be discussed.

1.5.5.1 Carcinogenic compounds in tobacco and tobacco smoke.

The IARC monographs on Tobacco Smoking and Tobacco Habits other than Smoking have presented comprehensive reviews of the carcinogenic and toxic components of tobacco and tobacco smoke (IARC 1986). These monographs, summarise the known carcinogens in tobacco and tobacco smoke and give the ranges of their concentrations along with the evaluations of their carcinogenic activities where available. The diversity of carcinogenic compounds in tobacco and tobacco smoke may cause ambiguity as to which among them are most important.

The carcinogenic products of smoking can be classified into 5 main groups, which are Polynuclear Aromatic Hydrocarbons (PAH), N-Nitrosamines, Aromatic Amines, Aldehydes and a minor group of miscellaneous organic and inorganic compounds.
The carcinogenic properties of these compounds are related to their ability to cause DNA

alkylation damage.

Amongst the alkylating agents the most widely studied are the N-nitroso compounds (NOC) which include N-nitrosamines and N-nitrosamides. The N-nitrosamines require metabolic activation for expression of their biological effect, while the nitrosamides undergo spontaneous, base-catalysed hydrolysis leading to the formation of the alkylating species. The role of NOC in human cancer aetiology has been reviewed by Reed (1996). Humans are frequently exposed to nitrosamines through a variety of sources such as tobacco, diet, cancer chemotherapy and occupation (Hecht and Hoffmann, 1988). In addition to their presence in the environment, NOC can be formed endogenously. Humans are exposed to many nitrogen containing compounds and nitrosating agents which, can form NOCs.

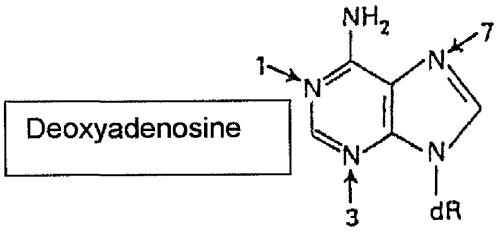
Alkylating agents have been shown to react with a wide range of cellular components, such as proteins and nucleic acids. However, DNA has been considered the most biologically important target of alkylation (Cooper et al., 1995).

Alkylating agents are able to react at many sites in DNA. The ring nitrogen and the exocyclic oxygen atom of the DNA bases and the oxygen atom of the phosphate internucleotide linkages are modified producing over a dozen DNA lesions (FIGURE 1.10) (Cooper et al, 1995). Alkylation occurs at the N-1, N-3, and N-7 position of adenine; The N-3, N-7, and O6 of guanine; the N-3, and O2 of cytosine; and the N-3, O4

and O2 of thymine. The extent of reaction with oxygen and nitrogen atoms is dependent on the mechanism by which the different agents react with DNA: agents that react via a unimolecular nucleophilic reaction (SN1) following first order kinetics (e.g. nitroso compounds) tend to react more readily with O atoms in DNA in comparison to those reacting via a bimolecular nucleophilic reaction (SN2) following second-order kinetics (e.g. methylmethanesulphonate MMS) (Cooper et al., 1995).

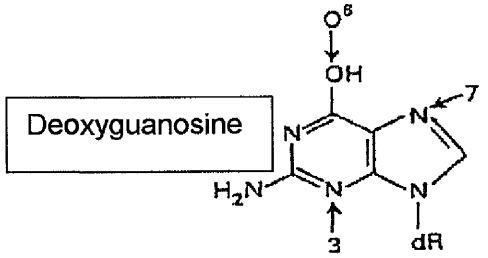
FIGURE 1.10 Sites of formation of the DNA adducts for both SN1 and SN2 reagents

% of adducts formed by S_N1 and S_N2 methylating agents



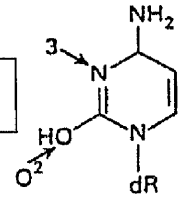
Deoxyadenosine

position	S_N1	S_N2
1-	1.0	1.5
3-	9.0	10
7-	2.0	1.5



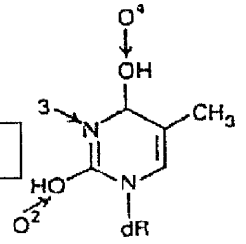
Deoxyguanosine

3-	0.8	0.6
7-	66	84
O^6-	7.5	0.3



Deoxycytidine

3-	0.2	0.1



Deoxythymid

3-	0.2	0.1
O^2-	0.1	nd
O^4-	<0.1	nd

Studies conducted in vivo and in vitro indicate that alkylation at the oxygen atoms of DNA bases is biologically more critical than alkylation at other positions in the mutagenic and carcinogenic effects induced by N-nitroso compounds (Cooper et al., 1995).

1.5.5.2 Biological effects of DNA adduct formation

The principal promutagenic base lesions are *O*6-meG which upon DNA replication can mispair with thymine, and O^4-meT which can mispair with guanine (Saffhill et al., 1985). Following two rounds of DNA synthesis these primarily lead to GC-AT and AT-GC transition mutations, respectively. Methylation of N7-meG also occurs at 10-100 times higher levels than O^6-meG and represents up to 70% of total methylation adducts following a single exposure to NOC (Saffhill et al., 1985). However, there is evidence that 7-meG lesion may also be promutagenic (Loeb, 1985).

Amongst the many biological effects of alkylating agents is their ability to induce sister chromatid exchanges (SCEs) in mammalian cells. There was evidence for a possible role of O^6-meG in the induction of SCE (White et al., 1986; Kaina et al., 1997). O^6-meG lesions have also been suggested to elicit the signal for apoptosis (Kaina et al., 1997; Meikrantz et al., 1998).

1.5.5.3 Repair of DNA alkylation damage

The repair of alkylation damage in DNA can occur by different mechanisms either by direct repair by reversal of damage in small lesions or base excision repair which takes place in "non bulky" DNA lesions where few bases are released and moved from DNA and replaced by new ones (Demple and Harrison, 1994; Dodson et al., 1994), or by nucleotide excision repair in which DNA damage is removed by incision of the damaged strand on both sides of the lesion, followed by repair synthesis. In humans the damage is excised in a 24-32 nucleotides fragment (Wood, 1996). Nucleotide excision repair is able to remove a broad spectrum of unrelated "bulky" lesions, especially pyrimidine photoproducts formed by UV light and adducts formed by polycyclic carcinogens such as benzo[a] pyrene (Petit and Sancar, 1999). It is reasonable to suggest, therefore, that some degree of functional overlap exists between the various repair systems

1.5.6 Epidemiological biomonitoring of exposure to alkylating agents

In epidemiological studies the quantitation of DNA adducts is considered an appropriate biomarker for assessment of human exposure to environmental carcinogens (Wild and Pisani, 1998). The levels of DNA adducts represent an integration of carcinogen exposure and metabolism, as well as DNA repair and cell proliferation (Shields and Harris, 1991).

1.5.6.1 Methods of detection of DNA adducts.

Many techniques have been developed for the detection and quantitation of minute levels of DNA adducts formed by a wide variety of chemical carcinogens in cellular DNA (Autrup, 1991). Five distinct approaches have been in use: (i) mass spectrometry (ii) immunoassays including competitive radioimmunoassay (RIA) and competitive or non-competitive enzyme linked immunosorbent assay (ELISA); (iii) fluorescence assays; (iv) 32P-postlabeling and (v) the Immunoslot-blot technique (for review see Ref from methodology). The most sensitive technique for detecting small amounts of adducts is the Immunoslot-blot technique.

Most of the above mentioned techniques have been applied to the quantitation of alkyl adducts including O^6-meG and N7-meG in human DNA. Because of its established promutagenicity, O^6-meG has been the most widely studied alkylation product in human DNA. Several projects have investigated target organ DNA alkylation in populations where an increased risk for cancer in certain tissues has been identified, and in some cases, where exposure to alkylating agents is known to occur (reviewed in Cooper et al., 1995). However, repair mechanisms for N7-meG in mammalian cells have limited capacity (Margison and Pegg, 1981) and the adduct can accumulate upon exposure to alkylating agents. This adduct, therefore, was considered to be a more suitable marker than the less prevalent and promutagenic but rapidly repaired O^6-meG as an indicator of exposure to methylating agents in epidemiological studies. Thus, N7-meG has been studied in human

tissue in relation to exposure to tobacco smoke and chemotherapeutic drugs (Copper et al., 1995)

1.5.7 The aetiology of smoking associated CIN.

The aetiology of CIN arising in association with HPV infection is thought to be multifactorial, involving initiation by a carcinogen followed by promotion and propagation of the neoplastic foci in the target tissue (Farin et al., 1995; Nakao et al., 1996). Numerous explanations have been offered for the proposed association. Generally, these can be categorised as involving exogenous and endogenous agents which induce DNA damage e.g. exposure to environmental carcinogens (arylamines and N-nitroso compounds), or alter host metabolism of chemical carcinogens, Most attention, however, has focused on the possible role of chemical carcinogens, particularly the N-nitroso compounds, in this process as cotinine and nicotine have been detected in cervical mucus (Hellberg et al., 1988) and the N-nitroso compounds have been detected in cervical mucus of smokers and non-smokers (Prokopczyk et al., 1997). Individual susceptibility to CIN, cervical cancer, as well as to other types of cancer, is thought to be modulated by host specific factors, such as proliferative activity and genetic polymorphism in phase I enzymes responsible for carcinogen activation e.g. cytochromes P450 and the phase II enzymes responsible for carcinogen inactivation e.g. glutathione S-transferases (GSTs) (Raunio et al., 1995) or in the level of expression of DNA repair enzymes (Spitz and Bondy, 1993).

1.5.8 Smoking, HPV and CIN

The first suggestion of cigarette smoking as a risk factor for CaCx was made by Winkelstein in 1977. A number of later investigations, in which it was possible to control for age at first intercourse, number of sexual partners, and/or social class, found the association with smoking to persist (Harris et al., 1980; Hellberg at al., 1983; Trevathan at al., 1983; Peters et al., 1986, Blomfied 1998). Although cigarette smokers are more likely to begin sexual intercourse at a younger age and to have a greater number of sexual partners, than non smokers, a significant increased risk remains after adjustment is made for these sexual variables. The estimated increased risk for smokers is around two fold, with the highest risk associated with long term and high- intensity use (Brinton and Hoover, 1992). When adjustment for other known risk factors for cervical cancer is made, the risk of cervical cancer is four times higher in women who smoke than it is in non-smokers (Winkelstein, 1990). It is possible that smoking acts in some way as a cofactor with sexually transmitted agents, probably HPV (Zur Hausen, 1982) since cotinine and nicotine have been shown to be strongly concentrated in cervical lavages (Schiffman et al., 1987). This increased risk may result from a local immunological effect of smoking on cervical epithelium (Barton et al., 1988) or by the formation of carcinogen-DNA adducts in the cervical tissue itself which may initiate neoplastic transformation (Phillips et al., 1990; Simons et al., 1993). The relation between smoking and CIN tends to become

stronger for the higher grade CIN (Cuzick et al., 1990). Schiffman et al (1993a) found a significant relationship of smoking with CIN Grade II/III in HPV positive smokers but not in those with low-grade disease. Kjaer et al (1996) found that smoking remained an independent risk factor for all grades of CIN even after controlling for HPV and the relation was stronger for high-grade disease. Table 1.3 summarises the case control studies which investigated the relationship between smoking and CIN.

Patel et al (1993) have demonstrated the expression of many of the cytochrome P450 isoenzymes in a histologically normal cervix. Susceptibility to low and high grade CIN is associated with the CYP2D6EM genotype, possibly as a result of the metabolism of cigarette smoke constituents by CYP2D6EM (Warwick et al., 1994b). Exposure to cigarette smoke may be a potential risk factor in the treatment failure of CIN since host - treatment epidemiological studies have shown a statistically significant association between smoking and cancer of the cervix and high-grade cervical intraepithelial neoplasia (Winkelstein et al., 1977).

On the other hand, such association was found to be restricted to squamous cell carcinomas, since it was not observed in either adenocarcinomas or adenosquamous carcinomas (Brinton et al., 1986b). Although smoking had generally been accepted as an associated risk factor for CaCx and CIN, other epidemiological case-controlled studies did not support this concept. No significant association between smoking CaCx was observed in a group of Finnish women (Lehtinen et al., 1996) or in studies relating to the risk of developing CIN II/ III (Koutsky et al., 1992). Additionally, three studies of cervical

neoplasia in Latin America did not find an association with smoking (Bosch et al., 1992; Munoz et al., 1992; Eluf-Neto et al., 1994). However, in different ethnic groups genetic polymorphism of the metabolising enzymes may be a factor, since, these studies were carried out in Latin America.

Glandular tumours do not appear to be promoted by the effect of smoking, as no increased risk has been noted for adenocarcinoma (Brinton et al., 1986b).

Smoking was associated with the occurrence of high grade CIN despite the presence of mild dyskaryosis and also with a significant increase in lesion size (Luesley et al., 1994). This risk is dose-dependant amongst women who smoke 20 or more cigarettes per day (Daly et al., 1998). A decrease in the lesion size of CIN is associated with cessation of smoking and indeed smoking reduction correlates with a reduction in the lesion size (Szarewski et al., 1996).

In the study performed by Cuzick et al (1990) the relation between smoking and CINI was not statistically significant. However for CIN III, it was statistically significant ($p<0.05$). The same correlation between smoking and the occurrence of high-grade disease has been confirmed by Schiffman (1993a).

Ho et al (1998a) concluded that infection with HPV 16 is associated with high grade CIN lesions, but an additional cofactor, such as cigarette smoking, may be required as a carcinogen to advance HPV-infected cells toward neoplastic progression. The mechanism

by which smoking influences the development of CIN is not well understood. It has been proposed that HPV may act synergistically with tobacco products (Zur Hausen, 1982), or that tobacco products may allow penetrance of HPV by causing local immunosuppression within the cervix (Barton et al., 1988).

Tobacco smoke contains many potential carcinogens including PAH (Poly Aromatic Hydrocarbons), N-nitrosamines and aromatic amines and aldehydes. The importance of tobacco derived compounds as local carcinogens is shown by the findings that nicotine and cotinine, which can be mutagenic, are concentrated in the cervical mucus (Winkelstein, 1990). In addition DNA from cervical epithelial cells of smokers contains adducts of the type expected from interaction with PAH and aromatic amines (Simons et al., 1993) and these adducts are similar to those of smoking related adducts found in the lung. (Phillips et al., 1990). Increased gene expression of several P450 enzymes (CYP1A1, CYP1A2, CYP2D6 and CYYP2E2) is potentially important in the activation of tobacco-specific carcinogens and has been demonstrated in HPV 16 immortalized cervical epithelial cells (Farin et al., 1995) while, human cervical cells immortalized with HPV 16 or 18 undergo malignant transformation after treatment with tobacco condensate. (Nakao et al., 1996; Young et al., 1996).

Also, the presence of smoking-related DNA adducts has been reported to be significantly higher in the cervical DNA of smokers than non-smokers (Simons et al., 1993). However, no significant differences in smoking-related DNA adducts have been found between HPV-positive and HPV-negative smokers, suggesting that smoking-related DNA damage

does not augment HPV infectivity, therefore this finding does not support the molecular synergism theory (Simons et al., 1995).

More recently, Kjellberg et al (2000) conducted a population- based case control study in northern Sweden which examined smoking, nutrition, parity and the use of oral contraceptives as major environmental risk factors for cervical cancer and they concluded that after taking HPV into account, smoking is the most significant environmental risk factor for CIN.

Currently there is no data to support or exclude any correlation between genetic polymorphism of the metabolising enzymes, the formation of DNA adducts and the treatment failure of CIN.

1.6 Management and treatment of CIN.

1.6.1 Principles of management .

The diagnosis of CIN requires either a directed or excisional biopsy. The treatment requires either destruction or excision. The simplicity of this statement does not reflect the complexity either may pose in clinical practice.

Directed biopsies should be taken from the most atypical part of the cervical TZ. The colposcopic features suggestive of CIN are mainly punctation, mosaicism, aceto white,

irregular surface and atypical vessels (Coppleson and Pixley, 1992). The whole TZ should be easily visible and if not such biopsies should be questioned. This may be the case after treatment of CIN due to cervical stenosis. Cone biopsy was primarily used when the upper limit of the squamous-columnar junction was not visible and therefore the degree of abnormality within the canal not assessable by colposcopy. Many of these have been replaced by alternative excisional procedures such as loop excision. However, it is still appropriate to consider conization as separate from TZ excision, as in the latter the intent is to excise as much tissue as one would normally have destroyed (if a destructive method had been employed). Conization is a procedure where there is deliberate intent to remove an additional segment of the endocervical canal. The indications for cone biopsy are suspected invasive disease, upper limit of the lesion of a suspected high-grade disease is not seen and previous treatment and persisting high-grade abnormality. In the latter situation, a large or deep loop or laser excisional cone may be used. All methods employing heat as a cutting tool will be associated with some thermal artefact at the margins and this could be of importance for interpretation (Luesley and Barrasso, 1998).

The treatment of choice consists of either surgical excision (cone biopsy, local excision or hysterectomy) or local ablation (cold coagulation, Laser). (FIGURE 1.11)

1.6.2 Treatment failure of CIN.

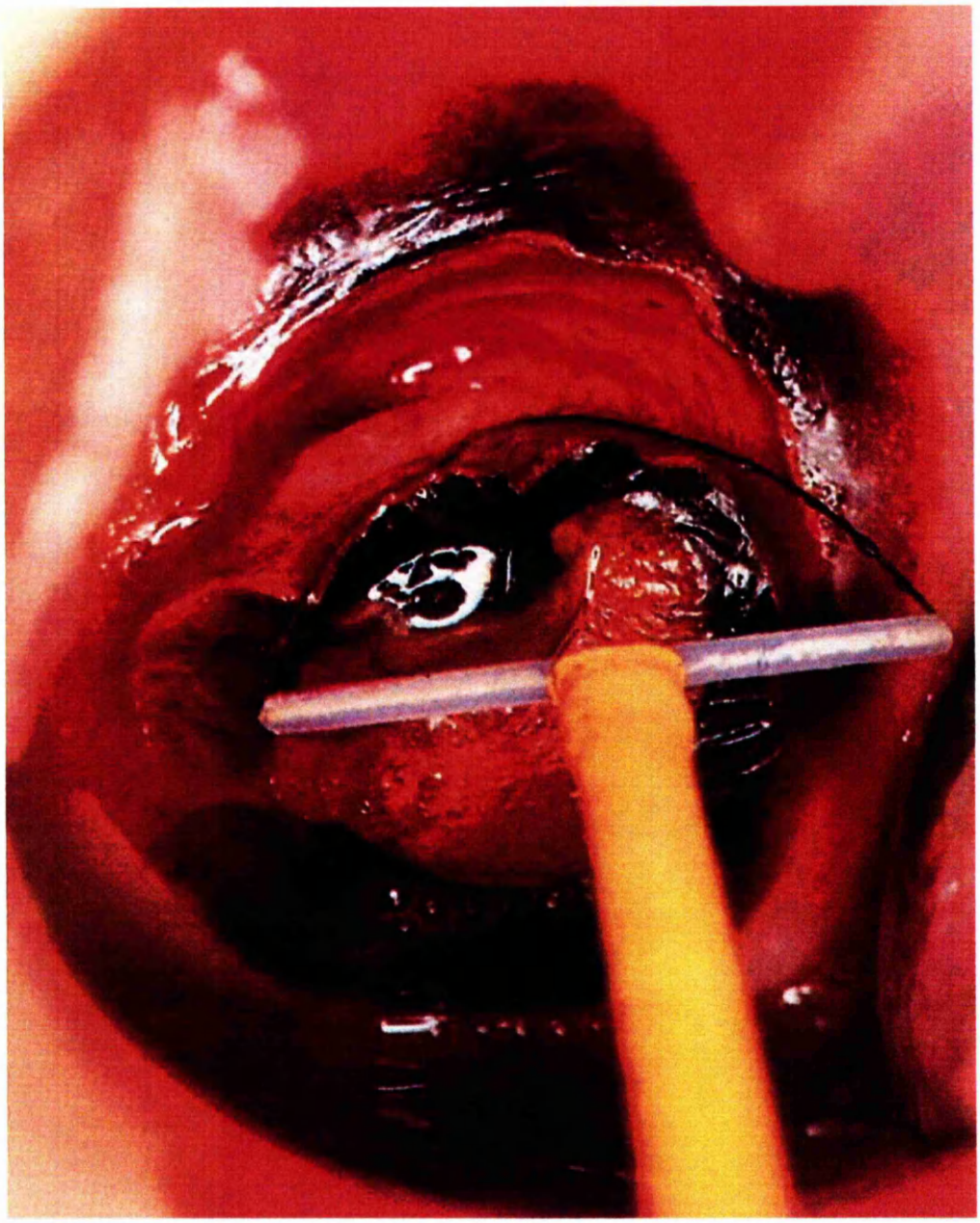
The treatment failure of CIN has been found to be associated with the method of treatment, the size of the lesion, incomplete excision of the margins and the degree of CIN

(Andersen et al.,1994; Shafi et al.,1993; Cox, 1999).

There is a wide variation in the reported failure rate in each treatment modality. The success rate of each treatment method has varied from around 55% to 95% (Yliskoski et al., 1989; Andersen et al., 1994; Shumsky et al.,1994). However the current methods of treatment in the United Kingdom (loop diathermy, cold coagulation, CO2 laser, cone biopsy) have been associated with a high success rate of about 90% (Andersen et al.,1994; Shafi et al.,1993).

The national guidelines of the British Society of Colposcopy and Cervical Pathology stated that the treatment failure rate should not exceed 10 %.

FIGURE 1.11 Loop diathermy in the treatment of CIN



The causes of the treatment failure of CIN are not consistent in all studies and there is a wide variation in the reported failure rate in each treatment modality. (Andersen et al., 1994; Shafi et al., 1993; Cox, 1999; Yliskoski et al., 1989; Shumsky et al., 1994). The factors that determine the treatment outcome of CIN other than treatment related factors are unknown these findings lead to the possibility that other factors such as infections, probably HPV, or life style risk factor such as smoking may be involved in the treatment failure of CIN.

The diagnostic process of treatment failure is initiated following the recognition of a cytological abnormality. As CIN is asymptomatic this rests on detection by screening. An abnormal cervical smear report should prompt further investigation and this will usually be a colposcopic examination. Neither colposcopy nor cytology can be regarded as diagnostic procedures but both will prompt a biopsy in certain situations. These have become the object of critical review as not all abnormal cervical smear reports will reflect underlying CIN. Minor degrees of CIN may resolve without any form of intervention. The screening procedure, whilst relatively sensitive, lacks specificity, particularly when minor cytological abnormalities are present. The interface between medicine and women in the screening context generates a considerable degree of stress and anxiety. This is true even in women whom eventually receive normal smear reports (Campion et al., 1988; Marteau et al., 1990).

1.6.3 Management of treatment failure of CIN.

1.6.3.1 Difficulties in the diagnosis of treatment failure.

Cervical stenosis following treatment of CIN may be asymptomatic but can be problematic during cytological and colposcopic follow up. It may postpone the recognition of the treatment failure. (Shafi and Luesley, 1995). (FIGURE 1.12)

A further problem is that of tubo endometrioid metaplasia, which may be confused with dyskaryosis and result in over treatment. (Hirschowitz et al, 1994). (FIGURE 1.13)

Following treatment for preinvasive disease the topography of the TZ has been altered (Soutter et al 1997) with the result that isolated iatrogenic skip lesions surrounded by columnar epithelium or covered by new squamous epithelium may occur. Consequently, the colposcopist cannot rely on visualization of the squamo- columnar junction excluding any abnormality further up in the cervical canal. (Hammond, 1999). Colposcopic appearance of ectopy of the cervix after treatment is shown in figure 1.14.

1.6.3.2 Preventive management (role of chemoprevention)

The aim of preventive management is to prevent the occurrence of CIN treatment failure by preventing the factors, which may lead to such a condition. The treatment related factors have been mentioned before and it is beyond the scope of this thesis to discuss it further.

One important aspect in the prevention of strategy is to address the life style risk factors e.g. smoking and informative advice of the patients about these risk factors and their effect on the treatment outcome. Life style risk factor prevention may be difficult to achieve.

FIGURE 1.12 Picture of cervical stenosis following treatment of CIN

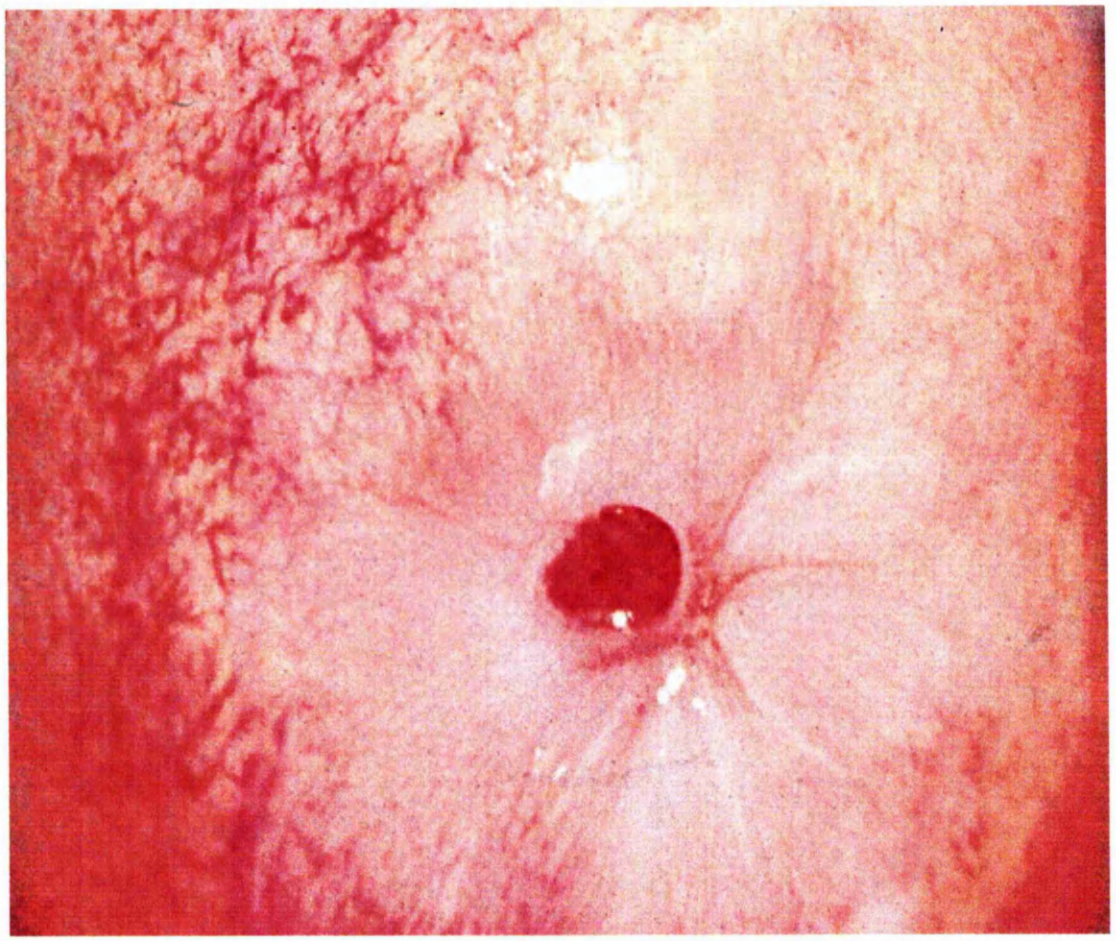


FIGURE 1.13 Tubo endometriod metaplasia following treatment of CIN

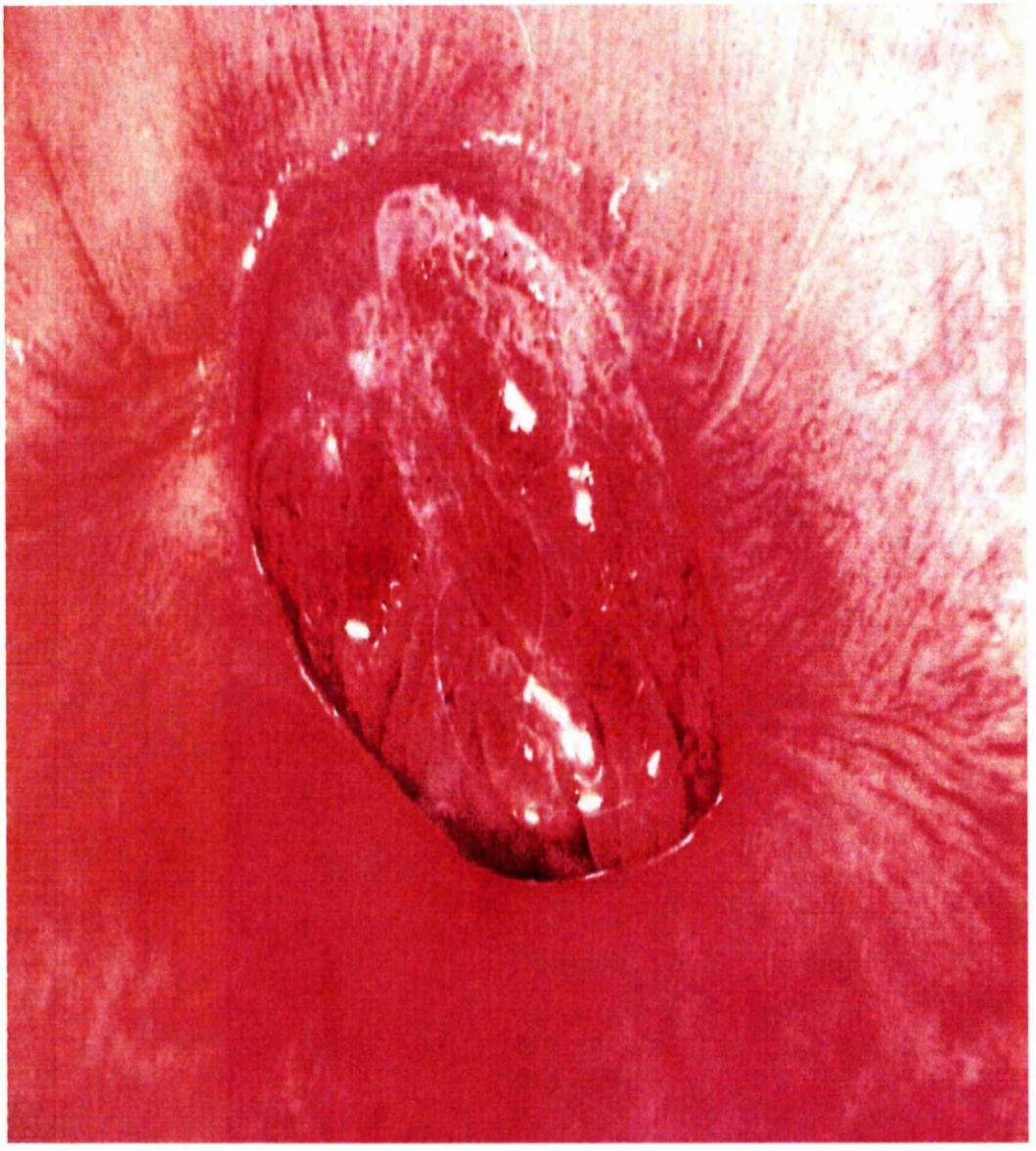
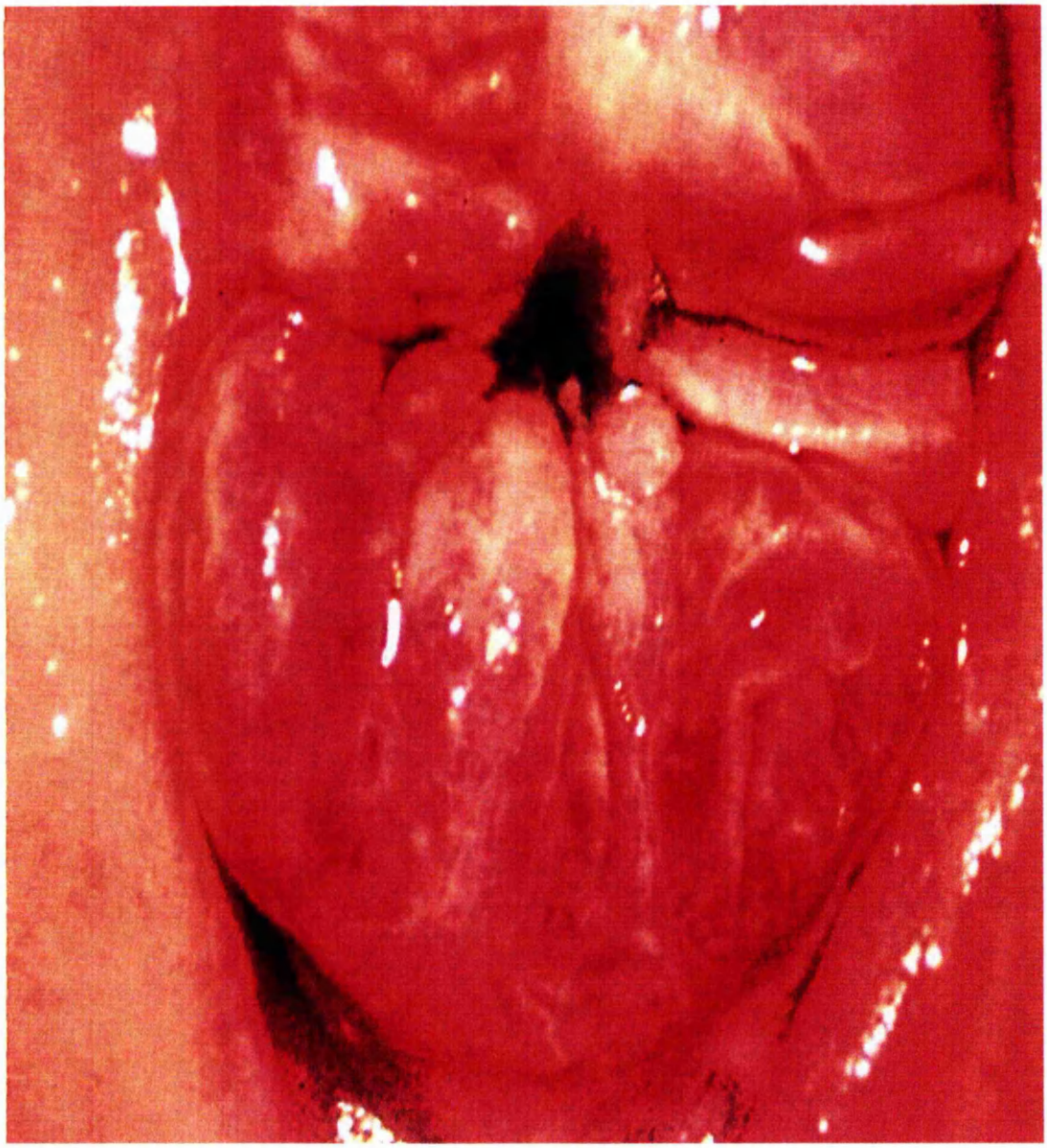


FIGURE 1.14 Colposcopic appearance of ectopy of the cervix following treatment of CIN



Another important aspect in the prevention of treatment failure of CIN is the development of chemopreventive substances. Chemoprevention refers to the use of chemical agents (micronutrients, pharmaceuticals) to prevent or delay the development of cancer in healthy population. (reviewed by Mitchell et al., 1995)

1.6.3.3 Curative management.

Following the colposcopic assessment, possibly including a further cytology specimen one has to make a decision on whether or not treatment is required, and, if so, the most appropriate technique. A number of important factors will require consideration including the patients age and desire for future pregnancy as will as her wishes regarding surgical intervention, along with the type of dyskaryosis and severity of disease, its location and the original treatment employed. (Hammond, 1999)

If the abnormal smear is moderate dyskaryosis or worse the likelihood is that treatment failure is present and unless the whole TZ can be scrutinized and seen to be normal, a further excision biopsy will be required. This ranges from loop excision to full conisation of the remaining cervix (Luesley and Barrasso, 1998).

Photodynamic therapy (PDT) is a medical treatment by which a combination of visible light and a sensitizing drug causes the destruction of selected cells. Because large loop biopsy is so effective there have been few attempts to develop other treatment techniques. CIN may be widespread making it extremely difficult to treat by excision. Excision biopsy may also be complicated by bleeding, pain and infection and these problems are less likely to occur with using PDT. (Gannon and Brown, 1999). PDT may play a role in the management of treatment failure in the future.

1.7 Aims of the study

Cancer is the result of complex interaction between multiple environmental factors, infection, and both acquired and inherited host factors.

The objectives of this study are

a) To determine whether detailed information regarding the smoking history, the sexual history and other known correlates of cervical neoplasia could be used to identify those who were likely to have treatment failure of CIN.

b) To study the effect of HPV detection in the treatment outcome of CIN

c) To identify any particular post-treatment advice which might influence the treatment outcome.

d) To analyse for known polymorphism in enzymes involved in carcinogenesis metabolism.

e) To quantitatively assess carcinogenic exposure by measuring the level of N7-m G.

This study should contribute to the identification of individuals at risk of treatment failure of CIN who will most benefit from prevention strategies which might include more frequent screening of some patients, adding some tests to the screening programme and offering specific post treatment advice .

Chapter 2 Materials and Methods

2.1 Case control study design

A prospective, multi-centre, study was conducted on a cohort of women recruited from Saint Mary's Hospital, Manchester, the Royal Bolton Hospital, Bolton and the Rochdale Royal Infirmary, Rochdale, all of which are in the North West of England. Approval was granted by the local research ethics committees. These women attended for colposcopic examination following abnormal cervical smear test results between 1st of August 1995 and the end of July 1999. They were interviewed and asked to complete a questionnaire (Appendix 1), which established whether they had ever (currently or previously) smoked. For those who had smoked, the length of time they smoked, the number of cigarettes per day, and the last time they smoked were also recorded. Women who reported that they had never smoked were noted. Additional information collected in the questionnaire related to age, number of children and/or previous miscarriages / abortions, sexual history (history of sexual transmitted diseases, number of partners in the last twelve months, number of lifetime partners and age at first sex). Informed consent was obtained from all patients participating in the study.

This cohort of women was used to perform a nested case-control study. The cases were defined as those patients who had treatment failure of CIN within the first two years following treatment. It was confirmed histologically. The controls were selected from

patients showing no sign of treatment failure during the two years following treatment. Their status was confirmed by two negative smears, the second of which being taken at least 24 months after treatment. Women showing other smear results during the course of follow-up (e.g. borderline, inadequate, abnormal results without histological confirmation or reverted to normal without treatment) were excluded. Pregnant patients, cervical cancer patients and those with conditions causing diminished immune competence(such as corticosteriod treatment, diabetes, human immune-deficiency virus (HIV) infection, organ transplantation, chemotherapy, radiotherapy) were also excluded from the study.

The size of the lesion was determined by assessment of the transformation zone size using the graph shown in figure 2.1. The method of assessing the size of the lesion was fully explained to the staff who performed the clinical examinations and who helped with patient recruitment .

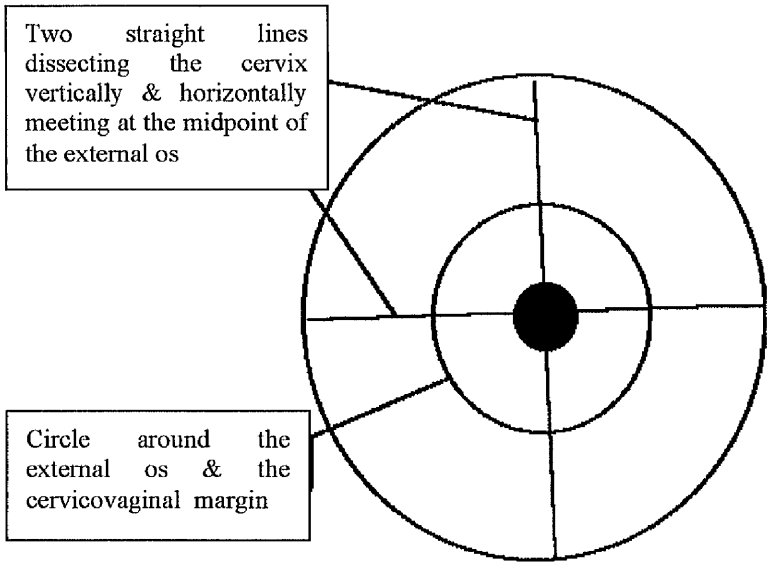
A pre-treatment cervical biopsy was taken from both cases and controls for HPV testing. During the course of the study (June, 1997) the protocol was amended to include an additional post-treatment cervical swab.

For every case two matched controls were selected. The matching of controls to cases was based upon exclusion all of treatment related factors i.e. the size of the lesion (±1 quadrants), the method of treatment, the degree of CIN (±1) and the excision of the margins status (for loop and cone only). The cervical biopsy, taken by punch biopsy forceps at the time of the initial diagnosis of CIN, was stored in dry ice and transferred in

liquid nitrogen to the virology department at Manchester Royal Infirmary where it was stored at –70°C. A Follow up cervical swab was taken 6 to 12 months after treatment, during the first follow up visit, and stored as before. This swab was taken using a cotton swab and placed in a virus transport media (Hanks BSS 85% v/v, Foetal Calf Serum 10% v/v, $NaHCO_3$ 0.1% w/v, Crystamycin 200 μg/ml, Fungizone 5μg/ml). The results of the cervical cytology obtained at the same time as the post-treatment HPV swab were recorded.

After identification of the cases and matched controls, DNA was extracted from the relevant samples and analysed firstly for the presence of HPV using the PCR technique, secondly for genetic polymorphism of the metabolising enzymes using the PCR technique and finally for the presence of the DNA adduct for N7-methylguanine using the immunoslotblot technique. The life style data obtained from the cases and controls were also analysed.

Figure 2.1 Semiquantitative assessment of the transformation zone



2.2 Statistical Methods

As is the usual practice, sample size calculations were based on the risk factors individually, based on McNemar test (Campbell, M.J. and Machin, D., 1993) as the corresponding analytic technique (Machin, D et al.,1997). An initial audit of treatment failure was performed in the units concerned and an overall treatment failure rate of approximately 10% was found. We therefore estimated that we would observe up to 100 treatment failures if around one thousand women had treatment for CIN in the participating hospitals over the study period. There are no data regarding the prevalence of HPV, lifestyle risk factors, genetic polymorphisms or DNA adducts in CIN treatment failure patients.

As HPV positivity has been found to be the most important factor associated with CIN and cervical cancer, this study was powered primarily to investigate this risk factor. The prevalence of HPV positivity among CIN patients has been reported to be between 63% and 94% in the natural history studies (See Table 1.2). Using a conservative estimate of 65% HPV positivity amongst those with treatment failure and assuming a 20% difference between cases and controls (i.e. 45% HPV positivity amongst controls), a sample size of 85 matched groups (1:2 matching) was chosen as this would give 85% power for a two-sided test at the 5% significance level. 74 matched groups (1:2 matching) would yield 80% power for this set of parameters.

As a secondary factor we used data on the percentages of smokers with CIN grades I-III from Grail and Norval (1988) to estimate that 66.9% of our cohort would be current smokers. Our chosen sample size of 85 matched groups would give 80% power for detecting an odds ratio of 5.0 (current to ex/non-smokers), for which 92% of cases (treatment failures) and 64.3% of matched controls would be current smokers (using a two-sided test with a 5% significance level).

Data from the matched cases and controls were analysed using SPSS (Version 6.0) and STATA (Version 6.0). Characteristics of cases and controls were summarised in terms of frequencies and percentages for categorical variables and in terms of medians, inter-quartile ranges (IQRs) and ranges for quantitative variables. Treatment failure of CIN was modelled using multiple conditional logistic regression. Odds ratio estimates with 95% confidence intervals (CIs) were obtained.

The effect of being HPV positive was then investigated. The effect of pre-treatment and post-treatment test results was assessed independently both in the absence and presence of the significant lifestyle risk factors collected pre-treatment from the women via the questionnaire. Odd ratios were estimated as earlier. The sensitivity, specificity, positive and negative predictive values of post-treatment HPV testing were estimated using the known sampling fractions for cases and controls.

The effect of DNA adduct formation and different genotypes of the metabolising enzymes GSTM1, GSTT1 and CYP2D6 on treatment failure of CIN were assessed independently and in combination with each other. Odd ratios were estimated with 95% confidence intervals (CIs) obtained. The interaction between them and smoking and HPV status was examined using multiple conditional logistic regression.

2.3 DNA EXTRACTION METHODS

2.3.1 DNA extraction from Biopsy tissue

DNA extraction was performed by the clinical virology unit at Manchester Royal Infirmary. The biopsy tissue was digested for 18 hours at 37°C on a rotary mixer in Proteinase K (Pk) Lysis buffer (20mM Tris/HCI pH 8.3, 2mM EDTA, 1% Triton x 100, 0.002% SDS, 500 μg/ml Pk). The DNA extract was then divided into two equal aliquots. One aliquot was transferred in liquid nitrogen to the Carcinogenesis Laboratory at the Paterson Institute for Cancer Research, Manchester, for analysis of genetic polymorphism of the metabolising enzymes using a PCR technique and for DNA adduct estimation by immunoslotblot technique. The DNA extracted for HPV PCR was then heated to 100 °C for 10 minutes to inactivate the Pk prior to analysis in the clinical virology unit.

2.3.2 DNA extraction from cervical follow up swabs

Virus transport medium containing the swabs was vortexed and 100 µl of the solution were incubated in Pk Lysis buffer as before.

2.4 HPV PCR method.

2.4.1 DNA amplification.

Prior to HPV PCR, the integrity of the DNA template was assessed by PCR of part of the GapDH (Glyceraldehyde-3 Phosphate De-Hydrogenase) gene (McLaughlan et al., 1997). Five microlitres of DNA lysate were added to 45 µl of PCR reaction mix to give the following final concentrations: 1x PCR buffer (67mM TRIS-HCl pH8.4, 16mM (NH4)2SO4, 2mM MgCl2, 0.1% gelatine), 200 µM each deoxynucleotide triphosphate (dNTP), 10pmols each primer (GapDH1, GapDH2) and 1 unit Thermus aquaticus (Taq) polymerase. Thermal cycling was performed as follows: 94°C for 7mins, 62°C for 1min, 72°C for 1min (1cycle); 94°C for 1min, 62°C for 1min, 72°C for 1min (49 cycles). Loading buffer (40% w/v sucrose, 0.1% bromophenol blue) was added to the amplified DNA (5 µl of the loading buffer to 50 µl of the amplified DNA).

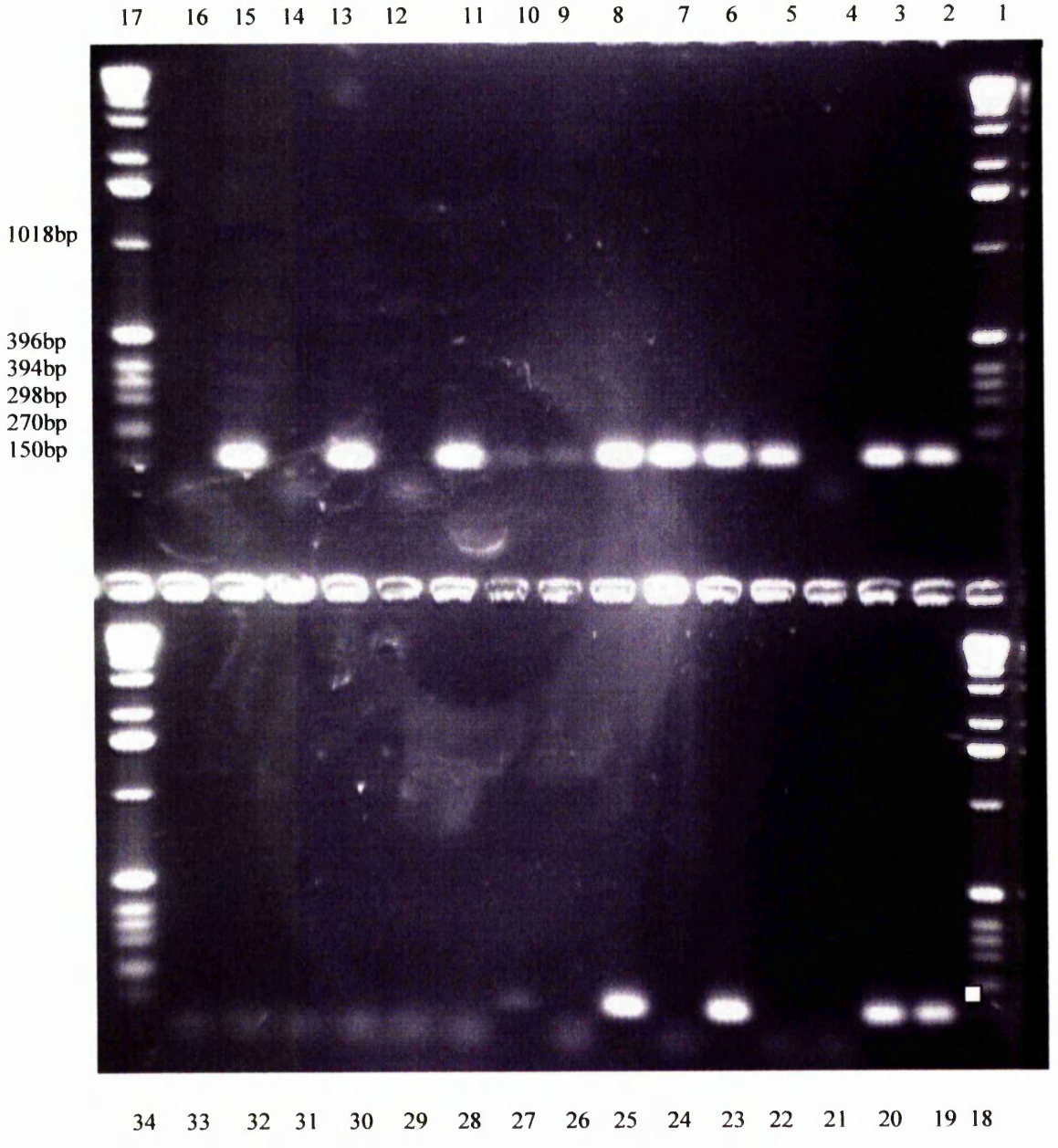
Amplified DNA was resolved in 1.5 % agarose in TAE x 1 electrophoresis buffer. following electrophoresis at 10 volts/cm in a horizontal electrophoresis plate. The 240 amplified DNA was visualised by ultra-violet illumination following ethidium bromide staining (1 µg/ml in TAE).

2.4.2 HPV PCR

All successfully extracted specimens were then screened for the presence of HPV DNA using the broadly reactive consensus primers GP5+/GP6+ which generate a product of 140-150 bp depending upon the HPV genotype (De Roda Husman et al., 1995). The appearance of one such gel for GP5+/GP6+ is shown in Figure 2.2. Five microlitres of DNA lysate were amplified with 50 pmols of each primer using identical concentrations of PCR buffer dNTPs and Taq polymerase to that described above. Thermal cycling was at 94°C for 7mins, 40°C for 2 minutes and 72°C for 1.5 minutes (1 cycle) followed by 94°C for 1min, 40°C for 2mins and 72°C for 1.5 mins (49 cycles). Amplified DNA was resolved on 2% agarose.

Those specimens giving a positive result with GP5+/GP6+ were genotyped by further PCR utilising individual type-specific primers for HPV types 6/11, 16, 18, 31 and 33. The results of a typical gel for HPV typing are shown in figure 2.3. Reaction mixes were prepared as previously described and 5 µl DNA lysate were amplified with 10pmols of type-specific primers for HPV types 6/11,16,18 (Soler et al., 1991), 31 (Van den Brule et al., 1992) and 30pmols type-specific primer for HPV type 33 (Vandenvelde et al., 1992). Thermal cycling was at 94°C for 7mins, 55°C for 1min, 72°C for 1min (1 cycle) followed by 94°C for 1min, 55°C for 1min, 72°C for 1min (44 cycles) and product bands were resolved on 2% agarose.

Figure 2.2 Picture of the HPV gel representing an example of the change of the HPV status (in matched pairs) pre and post treatment (amplified with GP5+/GP6+)



1,17,18,34 = 1Kb DNA Ladder (supplied by Bethesda Research Laboratories)

2, 3, 19, 20 = Positive Control 4,21 = Negative Control

5,6 and 7,8 and 9,10 = Persistence positive samples pre and post-treatment

11,12 and 13,14 and 15,16= Positive samples turn into negative post treatment

22,23 and 24, 25 and 26, 27 = negative samples turned into positive post treatment

28, 29 and 30,31 and 32, 33 = negative samples pre and post treatment

It was discovered during the course of the study that the HPV type 33 specific primers used here would also amplify HPV types 52 and 58. However, these were distinguished by restriction digestion of the PCR products with Rsa1 enzyme which was resolved in 15% polyacrylamide. The results of one of these gels is shown in figure 2.4.

All specimens positive using GP5+/ GP6+ but negative with type specific primers were classified as HPV positive (unknown type).

2.4.3 Southern transfer

All specimens amplified with GP5+/GP6+ were subjected to southern transfer. DNA was first denatured by immersion of the gel in denaturation solution (0.5N NaOH, 1.5M NaCl) for 30 mins with mixing followed by a similar period of treatment in neutralising solution (0.5M TRIS-HCl pH 7.4, 1.5M NaCl, 1mM EDTA). DNA was transferred to a positively charged nylon membrane pre-soaked for 30mins in 20x SSC (3M NaCl, 0.3M sodium citrate) by capillary action for 16 hours using 10 x SSC as the transfer buffer (Maniatis et al., 1982). The membrane was then washed briefly in 6 x SSC and baked for 2h at 80°C to immobilise the transferred DNA.

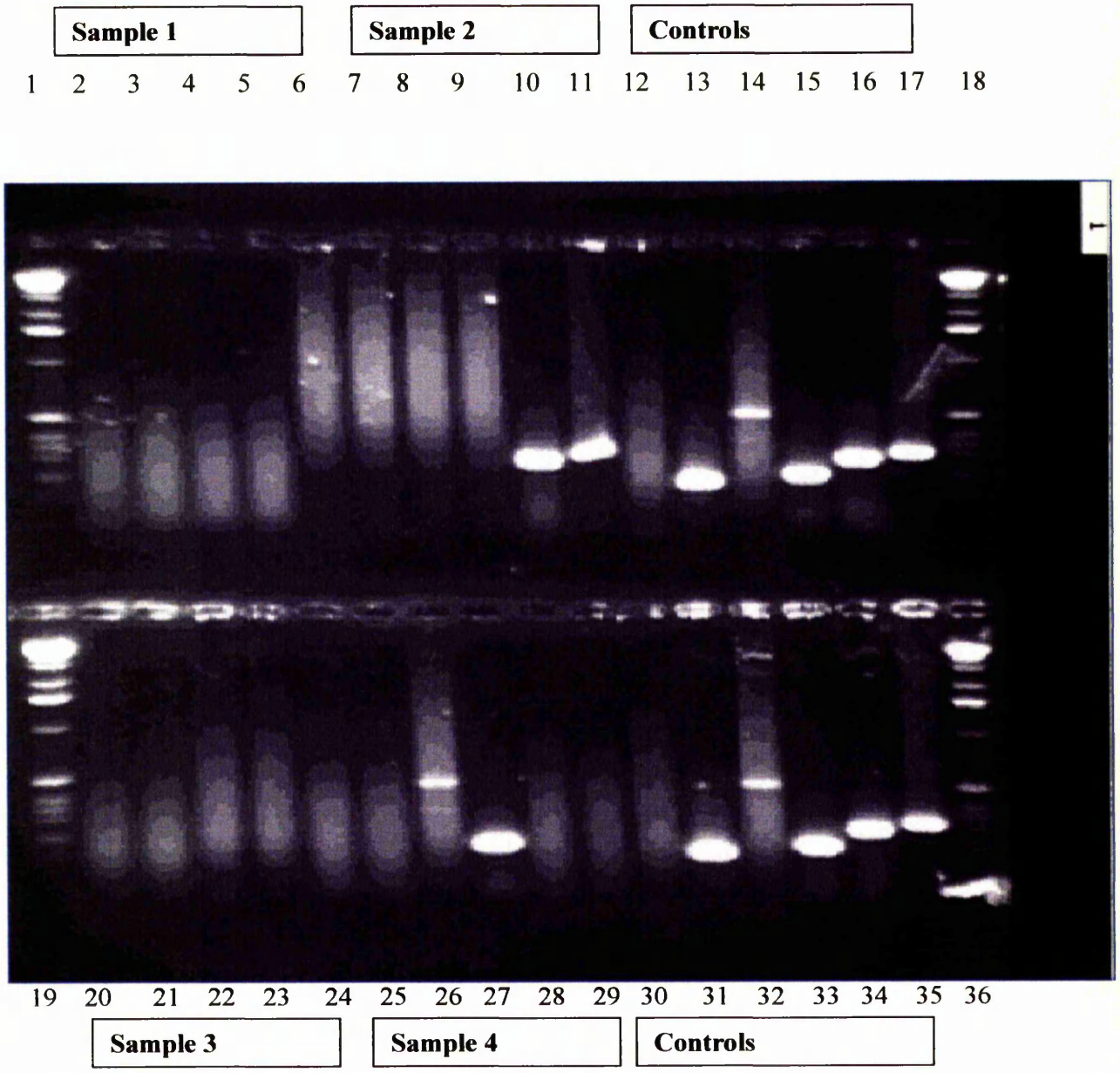
2.4.4 Probe labelling

A generic probe was prepared by labelling a cocktail of GP5+/GP6+ generated amplicons from HPV 6, 11, 16, 18 and 33 (Snijders et al., 1990) with digoxigenin (DIG) using the

DIG DNA labelling kit (BCL). Briefly, amplicons from the various HPV genotypes were pooled, denatured by boiling for 10mins, plunged immediately into ice for 1min and 10 μl added to a labelling mixture containing random hexanucleotides, dNTPs (containing DIG-11-dUTP) and Klenow DNA polymerase in a total volume of 20μl according to the manufacturer's instructions. Labelling was allowed to proceed for 16 hours at 37°C and the reaction terminated by the addition of 0.1 volume of 200 mM EDTA.

Unincorporated DIG-11-dUTP was removed by precipitation of the DNA with 0.1 volume 4M LiCl and 3 volumes of ice cold ethanol for 1 hour at -70°C. The DNA was pelletted at 13,000 g for 20 minutes, washed in 70% ethanol and finally dissolved in 50μl sterile distilled water. The approximate concentration of labelled DNA was calculated according to the protocol described in the DIG labelling kit by comparison with a dilution series of DIG-labelled DNA of known concentration.

FIGURE 2.3 An example of the HPV typing gel



1,18,19,36 molecular weight marker

6-2 (sample 1) negative

7-11 (sample 2) HPV6 /11, 16 positive

20-24 (sample3) negative

25-29 (sample 4) HPV 18,31 positive

Controls 1&2

17,35 HPV 6 /11 positive control

16,34 HPV 16 positive control

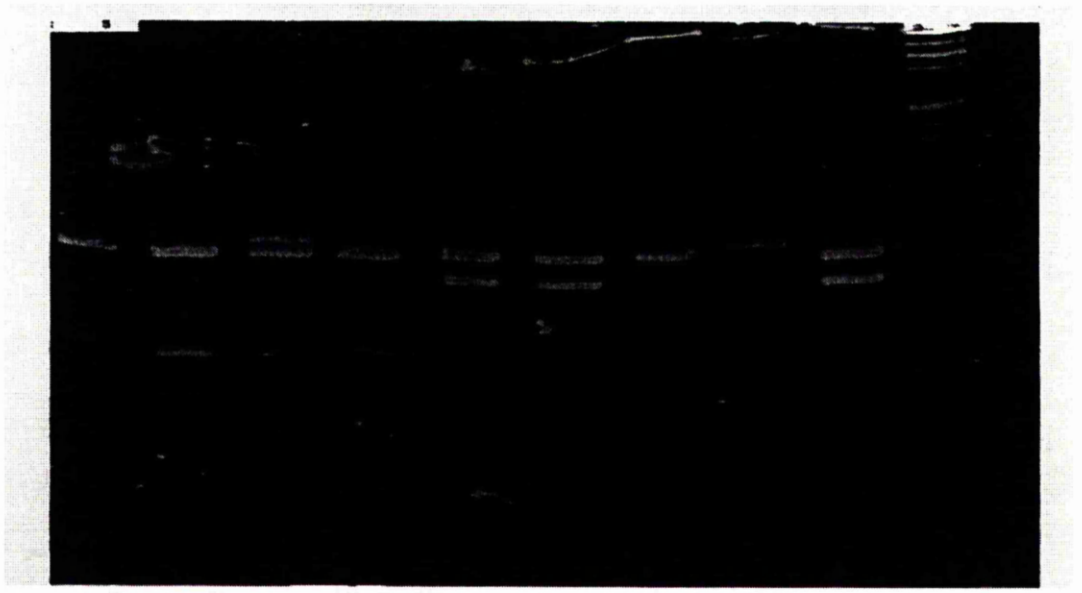
15,33HPV 18 positive control

4,32HPV 31postive control

13,31 HPV 33positive control

12,30HPV negative control

Figure 2.4 Picture of gel differentiating HPV TYPES 33, 52 and 58



10 9 8 7 6 5 4 3 2 1

1 Molecular weight marker

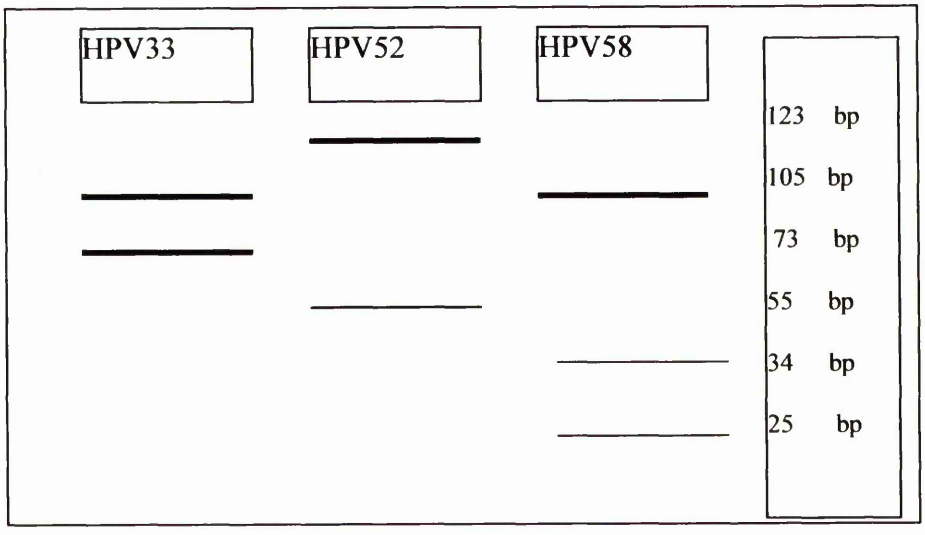
2,5 & 6 HPV type 33

3,10 HPV type 52

7,9 HPV type58

8 Dual infection of HPV type 52 and 58

Figure 2.5 The effect of the Rsa I digestion on HPV 33 PCR products.

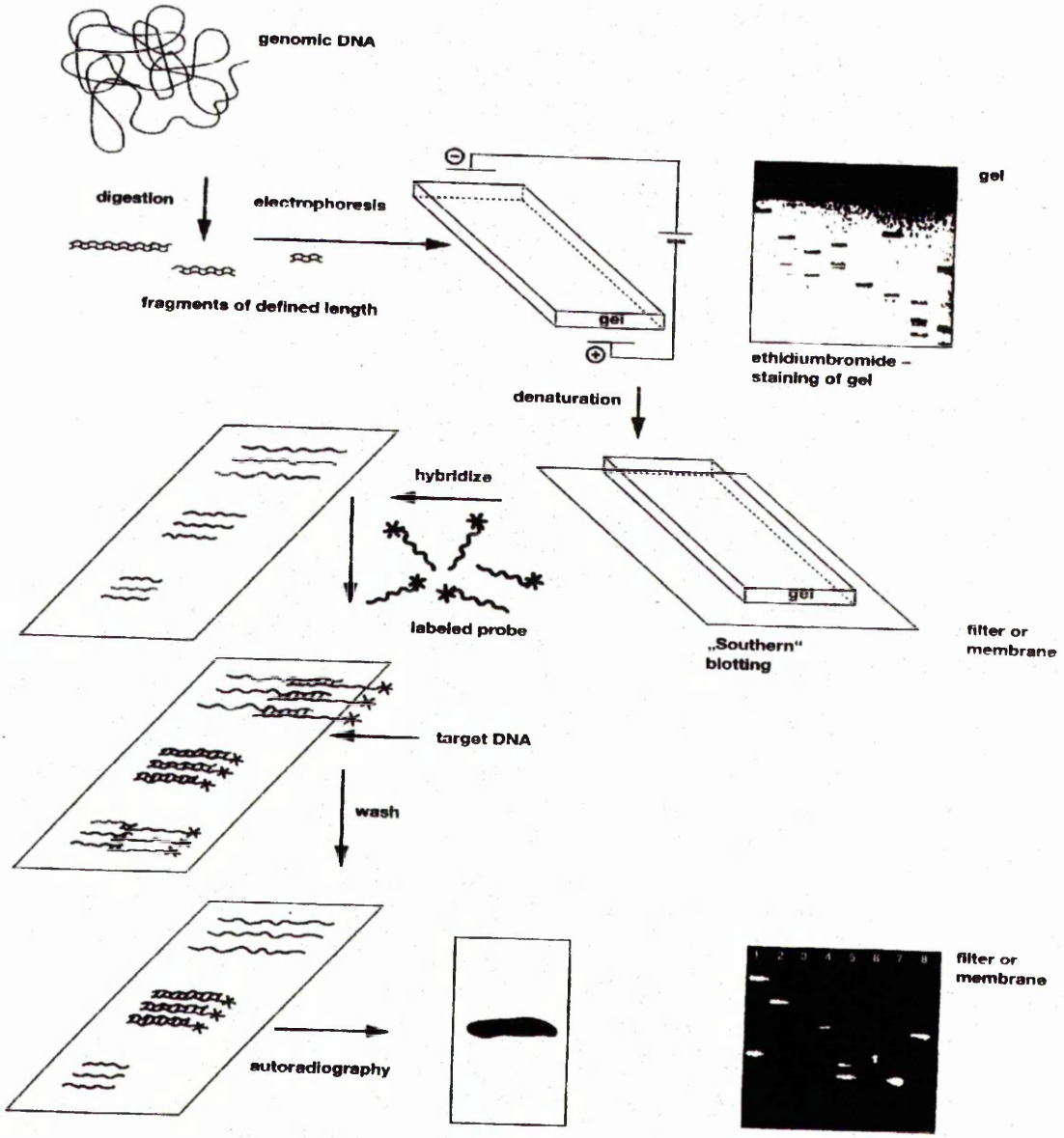


2.4.5 Hybridisation and detection of hybrids

Membranes were immersed for 3 hours in prehybridisation solution (5 x SSC, 0.1% N-lauryl sarcosine, 0.02% sodium dodecylsulphate (SDS), 2% DIG blocking agent) at 56°C, which was then replaced with 10ml fresh hybridisation solution containing 20pmol generic probe cocktail which had first been denatured by boiling for 10 minutes. Hybridisation was performed overnight at 56°C after which the membrane was washed for 2 x 15minutes in 3 x SSC, 0.1% SDS at 56°C.

Detection of hybrids was carried out according to the manufacturer's instructions. Briefly, the membrane was equilibrated for 1min in DIG buffer 1 (100mM maleic acid, 150mM NaCl pH7.5), blocked for 30mins in DIG buffer 2 (2% DIG blocking agent made up in DIG buffer 1), incubated with a 1/5,000 dilution (in DIG buffer 2) of anti-DIG-alkaline phosphatase conjugate for 30 mins, washed 2 x 30 minutes in DIG buffer 1 and colimetric substrate (NBT+BCIP) was then added. Colour was left to develop for 12-16h in the dark and the reaction was terminated by the addition of TE buffer (10mM Tris-HCl, 1mM EDTA). A summary of the HPV PCR technique is shown in figure 2.6.

FIGURE 2.6 Summary of the HPV PCR technique.



2.5 Genetic polymorphism and DNA adduct analysis

2.5.1 DNA purification

As the initial DNA extract from biopsy tissue contained RNA, proteins and viruses, it required purification before further tests could be performed. Further DNA purification was carried out with Qiagen columns using the supplied protocol. Briefly 4μl of RNase A stock solution (100mg/ml) was added to a 2ml aliquot of Buffer G2 (General lysis Buffer) (800mM guanidine HCl; 30 mM Tris-HCl, Ph 8.0; 30mM EDTA; 5%Tween-20; 0.5% Triton X-100). The DNA extract was transferred to a 10ml screw tube. Two ml of Buffer G2 (with RNase A) and 0.1ml of Pk stock solution were added to the initial DNA extract and incubated at 37°C for 1 hour. The DNA sample was then promptly applied to a QIAGEN Genomic-tip 20/G, pre-equilibrated with Buffer QBT (Equilibration Buffer) (750mM NaCl; 50mM MOPS, Ph 7.0; 15% isopropanol; 0.15% Triton X-100) and allowed to enter the resin. The resin was washed with Buffer QC (Wash Buffer) (1.0M NaCl; 50mM MOPS, Ph7.0; 15% isopropanol) twice to remove the contamination in the DNA sample. The genomic DNA was then eluted with Buffer QF (Elution Buffer) (1.25M NaCl; 50mM Tris-HCl, pH8.5; 15% isopropanol). The DNA was then precipitated by using isopropanol and collected as a pellet by centrifugation at 5000g for 15 minutes at 4°C.

The DNA pellet was washed with 1ml of cold 70% ethanol followed by centrifugation as described above. The pellet was then air-dried, the DNA re-suspended in 40 µl of autoclaved water and left to dissolve overnight.

2.5.2 DNA estimation by microtitre plate assay

The amount of the DNA was estimated by adding 10µl of the purified DNA solution to 100µl of Hoechst dye solution [1ml of TNE(0.2M NaCl, 10mM Tris-HCl, 1mM EDTA; pH 7.4 prepared as 10 x stock) + 9 ml of autoclaved water + 10 µl of Hoechst dye] and compared to DNA samples of known concentration. The amount of DNA in the sample was calculated by using a CCU fluorescence plate reader.

2.5.3 Immunoslotblot protocol

2.5.3.1 Chemicals and enzymes

Micrococcal nuclease, N-nitroso-N-methylurea (MNU), ribonuclease A (from bovine pancreas), Pk, calf thymus DNA (CT-DNA) and calf liver RNA (type IV) were purchased from Sigma (Dorset, UK.) All other reagents were analytical grade or higher. Double glass-distilled water was used.

2.5.3.2 Immunoslotblot

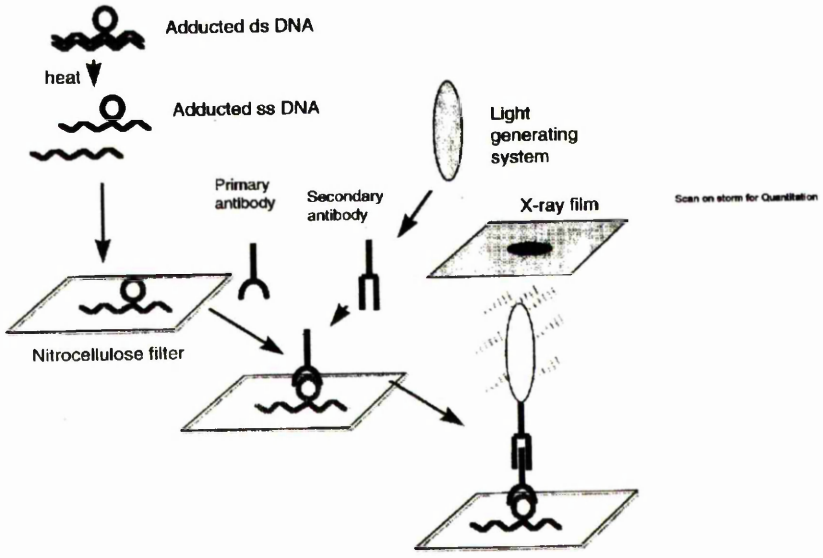
Immunoslotblot was carried out essentially as described by Mientjes et al., (1996) with some modifications. Briefly, MNU-modified CT-DNA, control CT-DNA and human samples (5 μg in 50 μl of 10 mM KH_2PO_4-KOH, pH 7) were sonicated on an ultrasonic sonicator at maximal amplitude for 10 seconds on ice to obtain DNA fragments of approximately 200-600 base pairs. The DNA concentration in each sample was then requantified using the Hoechst flourorescent dye method and the samples diluted with 10mM KH_2PO_4-KOH, pH 7 to 5 μg/100 μl. The MNU-modified CT-DNA was then diluted with unmodified CT-DNA in a ratio of 1:3 to obtain decreasing amounts (5.3 fmol) of adduct per 1μg DNA and used to generate 6 further dilutions i.e. 2.65, 1.325, 0.66, 0.33, 0.165 and 0.08 fmol to obtain a standard curve in the immunoslotblot assay (ISB).

Sonicated DNA samples were then alkali treated to open the imidazole ring of N7-meG adducts in the DNA. This was achieved by the addition of 2.35 μl 3M NaOH/100 μl DNA sample (final concentration 70mM NaOH) followed by incubation at 37°C for 30min and neutralization by the addition of 3 μl of 1M KH_2PO_4, 7.5 μl 1 M HCl and 37.5 μl PBS (pH 7.4) per 100μl of original sample volume and mixing after each addition. For non-ring opened samples only PBS (50.35ul) was added. DNA was then heat-denatured for 5 min by heating at 100°C cooled with ice for 10 min and mixed with 250 μl 2 M ammonium acetate.

Single stranded DNA (80 µl containing 1 µg DNA/sample) was immobilized on nitrocellulose (NC) filters (0.1 um pore size, BA79; Schleicher and Schuell, Dassel, Germany) wetted in 1 M ammonium acetate using a 48 well slot blot manifold (PR 600, Hoeffer, Amersham/Pharmacia, St. Albans, UK). The slots were rinsed with 200 µl 1 M ammonium acetate and the NC filters subsequently removed from the support and baked at 80°C for 90 min. The filters were then incubated in PBS containing 0.1% (v/v) Tween-20 (PBS-T) and 5% (w/v) fat-free milk powder (Marvel) for 1 hour at ambient temperature with continuous shaking. This was followed by an overnight incubation at 4°C with protein G purified anti-RON7-meG antibody (kindly provided by Rhoderick H. Elder (Elder et al., 1998) diluted 1:10000 in 20 ml PBS-T containing 0.5% fat-free milk powder. The NC filters were washed with PBS-T for 1 min and then twice more for 5 min. The filters were then incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (PO448; Dako, Glostrup, Denmark), diluted 1:10000 in 20 ml PBS-T containing 0.5% fat-free milk powder for 1 hour at room temperature. The NC filters were washed for 15 min in 100 ml of PBS-T and then twice more for 5min in 100 ml of PBS-T. Enzymatic activity was visualized by bathing the NC filters for 5 min in SuperSignal Ultra (Pierce and Warriner, Chester, UK), prepared just prior to use. Filters were then wrapped in cling film and exposed to a film (Super RX; Fuji medical, Fuji) and developed. The blacking of bands was quantified by scanning the X-ray films with an optical scanner (Storm 860; Molecular Dynamics) and then integrating the whole area of each band. Standard curves were generated using modified CT-DNA (volume of signal against amount N7-meG). Adduct levels in cervical sample DNA were quantified by referring to the N7-meG standard curve and following background subtraction were converted to nmol/mol G

assuming 1 μg of DNA contained 3124 pmol nucleotides. Samples that had adduct levels higher than the MNU modified DNA (20.24 fmol/μg DNA) were diluted with CT-DNA in the same manner as the generation of the standard curve. Summary of the technique is shown in figure 2.7

FIGURE 2.7 Summary of the immunoslotblot technique.



2.5.4 PCR technique for genetic polymorphism of the metabolising enzymes

2.5.4.1 Amplification of the DNA

The DNA was amplified by PCR for the genetic polymorphism of the metabolising enzymes. Specific sequences of DNA were amplified using heat-stable DNA polymerase (Taq) in a Hybaid Omnigene. A typical reaction premix contained the following: 10xTaq buffer (5μl), 2mM dNTPs (5μl), forward primer 3'primer (1μl of 0.1nM/μl), reverse primer 5'primer (1ulof 0.1nM/μl) and ddH_2O to 32μl. The amplification reaction was carried out in a 50μl volume containing 10-50 ng of genomic DNA as a template. A 1ml aliquot of DNA (0.4μg/μl) was added. The reaction was overlaid with mineral oil. After initial denaturation at 97°C for 5 min, the temperature was held at 90°C while 2.5 μl Taq were added to each tube. Following amplification (using one of the thermal cycling programs listed below for each genotyping assay) each aliquot was analysed by electrophoresis on 2% agarose gel containing ethidium bromide.

2.5.4.2 Identification of GSTM1 and GSTT1 genotypes

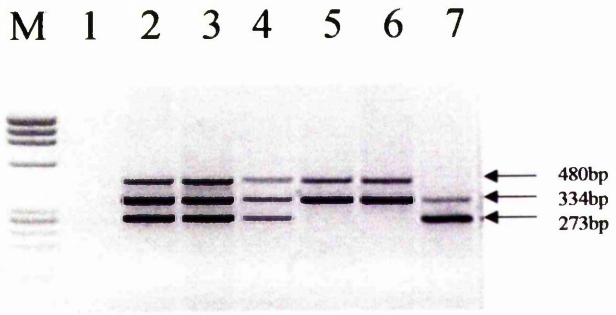
A multiplex PCR method was used to detect the presence or the absence of the GSTT1 and GSTM1 genes in DNA samples. Primer sets for both GSTs were included in the same reaction tube and as an internal control the exon3/ intron4 border of the CYP2D6 gene was also co-amplified. Products of amplification of GSTT1, GSTM1 and CYP2D6 genes

were resolved by electrophoresis on an ethidium bromide-stained 2.5% agarose gel and visualised using a UV transilluminator (320 nm). The absence of the PCR product of GSTT1 and/or GSTM1 was indicative of the null genotype (Figure 2.8). Positive and negative control samples were analysed with each experiment.

2.5.4.3 Identification of CYP2D6 polymorphism

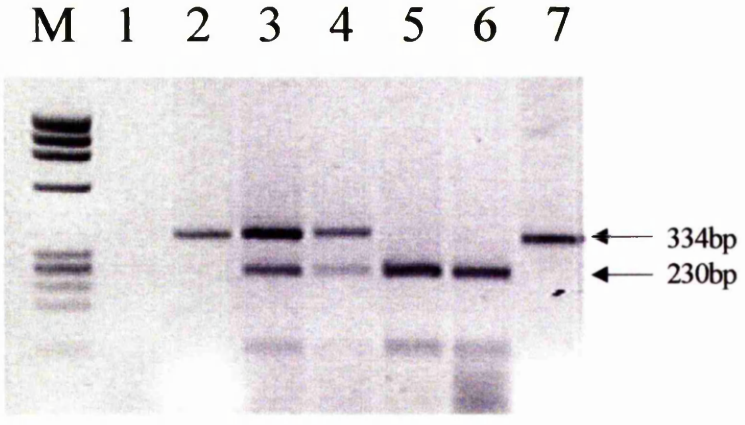
The CYP2D6 was analysed as described by Gough et al. (1990) to identify the G to A mutation at the intron 3/ exon 4 junction. The wild type is an extensive metaboliser (EM) phenotype, whilst the homozygous mutant is a poor metabiliser (PM). Genomic DNA was amplified using a pair of primers flanking the region of interest as illustrated in Figure 2.7 (Smith et al., 1992). Following the first denaturation step at 97°C for 5 min, 1U Taq was added in each tube then 30 reaction cycles each of 94°C 1min, 63°C 1min, 72°C 1 min were performed. Amplification generated a 334 bp product with a G to A transition at the junction of intron 3/exon 4 in affected individuals which renders the fragment resistant to digestion with the restriction enzyme *Bst*0. The wild type (EM) sequence is digested into 2 fragments of 229 bp and 105 bp. The size of the amplified products were determined by agarose gel electrophoresis. Restriction endonuclease analysis of PCR products with Bst 0 was performed at 55°C according to the method of Gough et al., (1990).

FIGURE 2.8 GSTT1/M1 gel picture



M) marker ΦX174 HaeIII (promega)
1) control no DNA
2) positive control using T10 DNA
3and4) GSTM1/T1 wildtype/wildtype
5and 6) GSTM1/T1 wildtype/null
7) GSTM1/T1 null/wildtype

FIGURE 2.9 CYP2D6 gel Picture



M) markerΦX174HaeIII
1) control no DNA
2) undigested DNA
3 and 4) HEM
5 and 6) EM
7) PM
All samples digested with BstO

Chapter 3 Analysis of the epidemiological data

3.1 Results

Nine hundred and fifty-eight patients were recruited into the study (638 from Bolton, 265 from Rochdale and 55 from St Mary's); 77 cases were identified and 154 controls were selected based on the matching criteria. The number of follow up swabs taken was 525 (385 from Bolton and 140 from Rochdale) i.e 433 patients did not have follow up swabs taken. The controls selected for matching were comparable with the rest of the potential controls in terms of age, smoking status and number of cigarettes smoked per day (by the smokers).The Characteristics of cases (n=77) and matched controls (n=154) are presented in table 3.1.

Frequencies and percentages of categorical lifestyle risk factors amongst the cases and matched controls, are presented in Table 3.2. Cases and controls were similar in terms of their distributions of age at first sex, and prevalence of genital warts. There was some disparity in the distributions of the other characteristics. Cases were more likely than controls to be using hormonal methods (oral contraceptive pills, progesterone only pills or injectables) of contraception (70.1% versus 57.1% of respondents) whereas 14.3% of controls and only 7.8% of cases used barrier methods. There was a greater likelihood of cases than controls having had two or more sexual partners in the last 12 months (27.4% and 17.0% of respondents, respectively) and, similarly, of having had more than 5 lifetime sexual partners (40.5% and 32.8% of respondents). At treatment, 71.4% of the treatment

failures but only 44.8% of the controls were current smokers (with 2.6% of each group failing to answer the relevant question).

The descriptive statistics for the quantitative characteristics are presented in Table 3.3, including some categorised in Table 3.2. The median age and the numbers both of children and abortions were the same amongst cases and controls, as was duration of smoking and the age at which smoking began amongst those who had ever smoked. Amongst the ever-smokers, both cases and controls smoked a median value of 10 cigarettes per day.

In the multiple logistic regression smoking status was found to be the only significant life style risk factor associated with treatment failure of CIN (p=0.0013). The odds of treatment failure were 3.17 times higher (95% CI between 1.68 -5.91) amongst smokers than non-smokers. The odds of treatment failure amongst ex-smokers relative to non-smokers were 1.53 which was not significantly different from 1 (95% CI between 0.49 - 4.76).

On investigating possible forms of the relationship between smoking and treatment failure, the number of cigarettes currently smoked led to a better fitting mode. There was no evidence of a non-linear trend in current cigarette consumption. This indicates an increasing trend in risk of treatment failure with increasing cigarette consumption as reported at the time of treatment. In particular, it is estimated that the odds of treatment failure of CIN increases by a factor of 2.58 (95% CI between 1.70 - 3.91) for each additional 10 cigarettes smoked per day (from 0 to 30 cigarettes). This indicates that at

the time of treatment increasing cigarette consumption is associated with an increasing trend in risk of treatment failure.

TABLE 3.1 Characteristics of cases and matched controls for study of CIN treatment failure. 1995-1999. Values are numbers (percentages) unless otherwise stated.

Characteristic		Cases (n=77)	Controls (n=154)
Site of recruitment	Bolton Rochdale St. Mary's	62 (80.5%) 11 (14.3%) 4(5.2%)	145 (94.2%) 8 (5.2%) 1 (0.6%)
Mean size of lesion (SD)		4.0 (1.7)	4.0 (1.7)
Size of lesion (quadrants of two concentric circles)	1-2 3-4 5-6 7-8	15 (19.5%) 46 (59.7%) 8 (10.4%) 8 (10.4%)	33 (21.4%) 88 (57.1%) 19 (12.3%) 14 (9.1%)
CIN severity at treatment	i i-ii, ii i-ii-iii, ii-iii, iii	12 (15.6%) 22 (28.6%) 43 (55.8%)	26 (16.5%) 44 (28.7%) 84 (54.6%)
Method of Treatment	Cone biopsy & Loop diathermy Laser & Cold coagulation	51 (66.2%) 26 (33.8%)	102 (66.2%) 52 (33.8 %)
Excision of margins (Cone/Loop diathermy only)	Complete Incomplete	32 (62.7%) 19 (37.3%)	64 (62.7%) 38 (37.3%)
Follow-up sample available for HPV testing		47 (61.0%)	69 (44.8%)

Note: Of the cases with follow-up sample available, 25 were matched with one control and 22 matched with two controls.

TABLE 3.2 Categorical characteristics of the life style risk factors of cases (n=77) and controls (n=154) in the study of CIN treatment failure.

Characteristic	Category	Cases		Controls	
		Number	%	Number	%
Age at first sex	Under 15	13	16.9	21	13.6
	15-19	55	71.4	119	77.3
	20-25	7	9.1	7	4.5
	Missing	2	2.6	7	4.5
Chlamydia	No	73	94.8	138	89.6
	Yes	3	3.9	13	8.4
	Missing	1	1.3	3	1.9
Current contraception method	Barrier	6	7.8	22	14.3
	IUCD	5	6.5	7	4.5
	None	4	5.2	15	9.7
	Hormonal	54	70.1	88	57.1
	Permanent	6	7.8	18	11.7
	Missing	2	2.6	4	2.6
Genital warts	No	69	89.6	137	89.0
	Yes	7	9.1	14	9.1
	Missing	1	1.3	3	1.9
Number of sexual partners in last 12 months	0-1	53	68.8	122	79.2
	2	15	19.5	20	13.0
	≥ 3	5	6.5	5	3.2
	Missing	4	5.2	7	4.5
Number of sexual partners during lifetime	1	9	11.7	14	9.1
	2-3	17	22.1	36	23.4
	4-5	18	23.4	42	27.3
	6-9	14	18.2	21	13.6
	10-19	8	10.4	16	10.4
	≥20	8	10.4	8	5.2
	Missing	3	3.9	17	11.0
Smoker status	Current	55	71.4	69	44.8
	Ex	5	6.5	13	8.4
	Never	15	19.5	68	44.2
	Missing	2	2.6	4	2.6

TABLE 3.3 Quantitative characteristics of life style risk factors of cases (n=77) and controls (n=154) in the study of CIN treatment failure.

Characteristic	Cases				Controls			
	Median	IQR	Range	n	Median	IQR	Range	n
Age (years)	27	9	19-55	77	27	9.25	18-57	154
Number of sexual partners in lifetime	5	5.25	1-70	74	5	4	1-25	137
Number of sexual partners in last 12 months	1	1	1-6	74	1	0	0-6	147
Number of abortions	0	0.25	0-4	74	0	1	0-3	149
Number of children	1	2	0-5	74	1	2	0-5	149
Time smoked (years)	10	5.5	3-30	57	10	10	1-29	77
Age started smoking (years)	16	4	10-35	55	16	3	7-35	75
Number of cigarettes smoked per day (current and ex-smokers)	10	10	5-30	57	10	10	2-25	81
Time since last smoked for ex-smokers (years)	5	-	3-8	3	3	5.5	0.33-16	12

IQR = Inter-quartile range

3.2 Discussion

Treatment failure of CIN has been found to be associated with the treatment modality, the size of the lesion, the margins excision status and the grade of CIN (Andersen et al., 1994; Shafi et al.,1993; Cox, 1999). The causes of treatment failure of CIN are not consistent in all studies and there is a wide variation in the reported failure rates associated with each treatment modality. The reported success rate of each treatment method has varied from around 55% to 95% (Yliskoski et al., 1989; Andersen et al., 1994; Shumsky et al., 1994). In this study all treatment related factors were controlled (size of the lesion, method of treatment, grade of CIN, margins excision) so as to allow other factors which may affect treatment outcome to be identified.

One of the objectives of this study was to identify any life style risk factor associated with treatment failure of CIN. These life style risk factors were identified in women attending four colposcopy clinics in the North West of England, by means of patient questionnaire. If such information was proven useful in predicting the outcome of CIN treatment, it would enable high risk patients to be identified and justify a more intensive programme of follow-up and post-treatment advice.

Smoking appears to be the most important factor affecting the progress of CIN after HPV infection (Kjellberg et al., 2000). Epidemiological evidence has already implicated cigarette smoking as a possible contributing factor to the development of cervical

neoplasia (La Vecchia et al., 1986). This has been confirmed in our study, in which the unadjusted odds of treatment failure amongst smokers was increased 3.4 fold compared with that of non-smokers. Smoking has been associated with the occurrence of high grade CIN in the presence of mild dyskaryosis and it has also been associated with significantly increased lesion size (Luesley et al., 1994). Decreased lesion size has been associated with the cessation of smoking, while smoking reduction correlated with a reduction in lesion size (Szarewski at al., 1996). Smoking risk is dose-dependent amongst women who smoke 20 or more cigarettes per day (Daly et al., 1998). This finding was confirmed in our study as the odds of treatment failure increase by a factor of 2.58 (95%CI 1.70-3.91) for each additional 10 cigarettes smoked. The effect of smoking on the risk of treatment failure of CIN has not previously been reported.

Since this study has shown smoking to be the most significant life style factor in treatment failure of CIN, women who smoke should be strongly advised to stop smoking after treatment. Ex-smokers appear to have a lower risk of treatment failure than smokers, but it is still higher than in non-smokers. This finding may be explained on a molecular basis since the repair of damaged DNA will occur after the cessation of smoking but may not be complete in all cases. Alternatively, or possibly additionally, the variation in individual susceptibility to carcinogens caused by genetic polymorphism of metabolising enzymes may play a role in the incomplete repair of the DNA. Although ex-smokers do not show a significant difference in the odds of treatment failure from the non-smokers, the number of ex-smokers in this study is small and a larger number would be required to confirm this

finding. It is likely that advising women to stop smoking, or at least to reduce their cigarette consumption, may decrease the risk of treatment failure.

This study has shown that the use of hormonal methods of contraception may increase the risk of treatment failure of CIN. Other reports on the effects of contraceptives in the development of cervical neoplasia are inconsistent (Piper, 1985; Brinton et al., 1986a; Beral et al., 1988; Berry et al., 1993). However, it appears that prolonged use of a combined oral contraceptive pill significantly increases the risk of cervical cancer (Beral et al, 1988; Berry et al., 1993) and dyskaryotic cytology (Blomfield et al., 1998). This is also true for the new generation of progestogen combined oral contraceptives (Bagshaw, 1995). The possible interaction of oral contraceptives and HPV may be important, since it has been suggested that sex steroid hormone receptors in HPV-induced cervical lesions may act as cofactors in promoting HPV-related cervical neoplasia (Monsonego et al., 1991). However, Ho et al. (1995) found no association between the persistence of HPV infection, cervical dysplasia and the use of combined oral contraceptive pills. The results of the present study are inconclusive on this matter and a further study is recommended.

The adoption of a prevention strategy to address such lifestyle risk factors as smoking is important. Advice about risk factors and their effect on treatment outcome should be given to all patients. However, improvements in a patient's lifestyle risk factor profile may be difficult to achieve. Primary health workers aware of this association, should advise their patients accordingly.

Although previous studies have shown CIN to be associated with sexually transmitted diseases (Thompson et al., 1994; Koffa et al., 1995), this finding was not confirmed in this study. This could be explained on the basis that successful treatment of STD before CIN treatment might reduce the incidence of treatment failure and justify screening of all CIN patients for genital infections before the onset of treatment.

Chapter 4 Role of Human Papilloma Virus testing in the treatment outcome of CIN

4.1 Results

The pre-treatment HPV frequencies and percentages, including those for the subtypes, are shown in Table 4.1. Six cases and 6 controls were GapDH negative. One case and one control each had four different types of HPV, two cases and one control had three different types of HPV and six cases and eleven controls each had two different types. The study protocol was amended 20 months into the study to include post-treatment swabs. This, together with loss of 20 of the follow-up swabs during transport, meant that post-treatment HPV results were available for only 47 cases and 69 controls. Pre-treatment samples were available from all of these patients. The HPV frequencies and percentages are shown in Table 4.2. One case had three subtypes (16, 18 and 33).

Twenty-nine controls were positive for HPV in the pre-treatment sample but negative in the post-treatment sample. Two controls were negative initially and positive at follow-up and another two controls were persistently positive. Among the cases, nine, which were initially positive, were found to be negative for HPV in the post-treatment sample. Eight cases were negative pre-treatment but tested positive in the post-treatment sample. Fourteen cases were positive both initially and at follow-up. Being HPV positive at the time of treatment had no significant effect on treatment outcome. Likewise, no type-specific pre-treatment HPV effect was detected. However, with regard to the follow-up

HPV data, a highly significantly adverse effect of being HPV positive was detected. On further investigation of the type-specific HPV effects, it was found that those with HPV 16 had odds of suffering treatment failure of CIN 23.3 times as great as those without HPV 16 (95% CI 3.15 to 172.1). No adverse effect was detected for any of the other HPV types, although the number of positive patients was very small. The sensitivity of the post-treatment HPV test was found to be 46.8% and the corresponding specificity 94.2%. Using the sampling fractions for cases and controls with post-treatment HPV results, the positive predictive value was estimated as 41.4% and the negative predictive value 95.3%.

The results of the cervical smear taken from the cases at the same visit were positive in 20 out of 43 (46.5%), while 19 of these cases (44.2%) were HPV positive at the same time (Table 4.3). However, in combination, 31 out of the 43 cases (72.1%) were positive for either HPV or cytology.

TABLE 4.1. Pre-treatment number positive/number tested (% positive) for various HPV types for cases and controls.

	Cases	Controls
Total HPV	37/71 (52.1%)	76/148 (51.4%)
HPV 16	21/71 (29.6%)	38/148 (25.7%)
HPV 18	8/71 (11.3%)	10/148 (6.8%)
HPV31	2/71 (2.8%)	7/148 (4.7%)
HPV 33	6/71 (8.5%)	9/148 (6.1%)
HPV52	1/71 (1.4%)	1/148 (0.7%)
HPV58	1/71 (1.4%)	3/148 (2.0%)
HPV6/11	3/71 (4.2%)	5/148 (3.4%)
HPV unclassified	8/71 (11.3%)	19/148 (12.8%)

TABLE 4.2 Post-treatment number positive/number tested (% positive) for various HPV types for cases and controls.

	Cases	Controls
Total HPV	22/47 (46.8%)*	4/69 (5.8%)
HPV 16	18/47 (38.3%)#	1/69(1.4%)
HPV 18	1/47 (2.1%)	0
HPV31	0	0
HPV 33	1/47 (2.1%)	0
HPV52	0	0
HPV58	0	0
HPV6/11	1/47 (2.1%)	0
HPV unclassified	3/47 (6.4%)	3/69 (4.3%)

* p= 0.002, OR 23.49 (95% CI 3.2 to 172.5)
\# p= 0.007, OR 21.3 (95%CI 2.34 to 194)

Note: Of the cases with follow-up sample available, 25 were matched with one control and 22 matched with two controls.

TABLE 4.3 HPV and cytology results at the first follow-up visit after treatment

	Smear		
HPV	Positive	Negative	Total
Positive	8	11	19
Negative	12	12	24
Total	20	23	43

4.2 Discussion

Most HPV infections disappear within months to a few years of diagnosis (Hildesheim et al., 1994). Persistence of HPV infection (high-risk types) has been found to be associated with progress from low to high-grade disease (Ho et al., 1998b). In HPV carriers, the risk of progression from cytologically normal to biopsy-confirmed CIN II-III is increased by a factor of 10 compared with HPV DNA negative patients. Progression was largely attributable to HPV 16 (Koutsky et al., 1992). Wallin et al. (1999) concluded that a single positive finding of HPV DNA in a pap smear would increase the risk of future invasive cervical changes. These findings support the present results that HPV infection, especially the high-risk types, plays a major role in the treatment outcome of CIN. Such a role was confirmed in this study since post treatment HPV PCR testing had a high predictive value for the outcome of treatment of CIN. This observation was particularly evident with HPV type 16.

There was no significant difference between pre-treatment HPV PCR testing or individual HPV types between cases and controls. However, post-treatment HPV positivity was highly significantly when associated with treatment failure of CIN, although the limited amount of data gave a very imprecise estimate of the odds ratio ($p= 0.002$, OR 23.49 with 95% CI 3.2 to 172.5). However, these results indicate that HPV positivity confers an odds of treatment failure at least 3.2 times greater than HPV negativity, (with 95% confidence). Whilst there may be some bias introduced by the unbalanced follow-up of

cases and controls, it would appear unlikely to explain all this increase. A large prospective study should be carried out to confirm this finding and obtain a more precise and unbiased estimate of this odds ratio.

These findings confirm the previous observation that persistence of HPV is associated with the persistence of cervical dysplasia (Ho et al., 1995) and support the observation that the median duration of HPV infection is around 8 months and that 12 months after infection 70% of women are no longer infected (Ho et al., 1998b).

The majority of cases (14/22) showed persistence of HPV infection while HPV disappeared from the majority of controls (only 2/31 remained positive). Eight cases acquired HPV infection post-treatment. These findings suggest that the persistence or presence of HPV infection in post-treatment cervical swabs was an early indicator of treatment failure of CIN. This information was available before the cytology became positive for 11 of the cases who were HPV positive in their first follow-up after treatment. However, another 12 cases had an abnormal smear at the same visit. (TABLE 4.3) As only 8 cases were positive for both HPV and smear at this visit, it indicated that HPV status and smear result should be used in conjunction when determining possible early treatment failure. Also, four controls were tested positive for post-treatment HPV, two of whom were initially negative. These two patients would appear to have contracted HPV infection after treatment and therefore may be at an early stage of the pathogenesis of the disease.

HPV type 16 appears to be the type most commonly associated with treatment failure of CIN. This observation supports the finding of Koutsky et al. (1992) and adds further weight to the suggestion that HPV testing could assist the detection of women who are at risk of developing cervical cancer even before their smear is positive.

Ninety-five percent of CIN treatment failures occur within the first 24 months (Gordon, 1991; Paraskevaidis et al.,1991). It is therefore reasonable to suggest that those patients with negative smears after their first follow-up visit (6-12 months after treatment) and who remain negative for a further 18-24 months may require less frequent follow-up smears than those with persistent HPV infection. This latter group probably require colposcopic follow-up.

Since several authors have reported a high false-negative rate of cytology (Cuzick et al., 1995; Szarewski et al., 1991), follow-up of patients after treatment by smears alone may be insufficient because this group has a higher risk of developing cervical cancer (Soutter et al., 1997).

This study has shown a high specificity and a high estimated negative predictive value of post-treatment HPV testing. Therefore, its use, in combination with cytology, should help the clinician to decide the most appropriate management of all CIN patients and ought to decrease patient anxiety.

Since 72.1% of the cases were positive for either HPV or cytology in the first follow up

after treatment, the combination of both tests following treatment appeared to be effective in the prediction of the treatment outcome. This confirmed the previous findings of Flannelly et al. (1997) who showed that 47% of the treatment failure group had abnormal colposcopy and proven CIN while concurrent smear cytology did not show dyskaryosis. A post-treatment positive HPV PCR test should indicate early referral for colposcopy rather than routine follow up colposcopy as occurs in some centres.

A cytological report of borderline or mild dyskaryosis after treatment of CIN, might present a difficult management decision since a subsequent colposcopic examination is often difficult due to the distortion of the squamo-columnar junction. Since 10 to 25% of women with borderline or mildly dyskaryotic smears will have underlying high-grade CIN lesions, HPV testing would help in the evaluation of this group of patients. HPV positive patients require further action, such as a cone biopsy, while HPV negative patients need only repeat cytology. The current approach of colposcoping all such women may result in over-treatment and unnecessary anxiety.

The development of PCR technology has provided a highly sensitive and specific method for detecting HPV DNA, even in very small samples. However, PCR has the disadvantage of susceptibility to contamination, which has occasionally led to an over-estimation of the prevalence of HPV in the normal population (Tidy et al., 1989; Young et al., 1989). To solve this problem a number of precautions were taken while performing PCR assays in this study. These included the use of different rooms or containers for the preparation of

samples, electrophoresis and PCR solution preparation. All these precautions were applied during the analysis of the study samples.

This study has shown that HPV testing after treatment will identify the great majority of women who are at low risk of developing treatment failure. Women with negative cytology and no evidence of HPV infection should need less frequent post-treatment screening. Adoption of such a management protocol would improve the specificity and cost effectiveness of cervical screening.

Chapter 5 Analysis of genetic polymorphism and DNA Adduct in the treatment outcome of CIN

5.1 Results

5.1.1 Results of the DNA adduct study

Only a limited number of biopsy samples (61) were available for this study: 20 from cases and 41 from controls. The Immunoslotblot assay used to determine the DNA adduct content of the samples, requires at least 1 microgram of DNA for each single assay. Such an assay is done in triplicate. Biopsy samples containing less than 3 micrograms of DNA were excluded from the study. Again only a limited number of these controls (21) were used in the multiple conditional logistic regression analysis. Those who did not matched to a case could not be included. However, the results of all the available controls as well as those in the matched subset, are presented in this thesis. Another comparable study encountered similar problems with regard to DNA extraction from the cervical tissue. (Harrison and Povey, personal communication)

Despite the limited data on DNA adducts, a significant effect of the log of N7-methylguanine content on the risk of treatment failure was detected ($p=0.030$). Lack of data precluded an investigation of the joint effect of smoking and the level of N7-

methylguanine on the risk of treatment failure. However, there is no significant difference between the median N7-methylguanine levels for the current and non/ex-smokers ($p=0.21$). The effect of the level of N7-methylguanine itself was less significant ($p=0.040$) then that of the log level of N7-methylguanine. Since the distribution of the level of N7-methylguanine was positively skewed, the effect of log N7-methylguanine is probably more appropriate in this analysis.

The N7-methylguanine results for all of the tested samples are summarised in table 5.1 Those from the matched subset (for the 20 cases and 21 controls) are summarised in table 5.2. The different values of the matched N7-methylguanine levels are shown in figure 5.1 (a similarly shaped distribution was found in the matching and unmatched controls). The distribution of the amount of N7-methylguanine (μmol/mol G) detected in the cases (20) and *all* controls (41) are shown in tab 5.3.The mean value of N7-methylguanine of the cases was 1.80 ± 1.57 SD and of the control was 0.65 ± 0.82 SD.

TABLE 5.1 Comparison of the levels of N7-methylguanine amongst cases (n=20) and *all* controls (n=41).

DNA adduct	Cases			Controls		
	Median	IQR	Range (Min to Max)	Median	IQR	Range (Min to Max)
N7-methylguanine (μmole / mole guanine)	1.42	2.75	4.78 (0.05 to 4.83)	0.42	0.71	4.10 (0.05 to 4.15)

TABLE 5.2 Comparison of the levels of N7-methylguanine amongst cases (n=20) and *matched* controls (n=21)

DNA adduct	Cases			Controls		
	Median	IQR	Range (Min to Max)	Median	IQR	Range (Min to Max)
N7-methylguanine (μmole / mole guanine)	1.42	2.75	4.78 (0.05 to 4.83)	0.42	0.71	2.87 (0.05 to 2.92)

N.B.: The detection limit of the assay is 0.05 micromole N7-methylguanine/mole guanine (μmol/mol G); those samples with non-detectable levels have been given the value 0.05.

IQR = Inter-quartile range

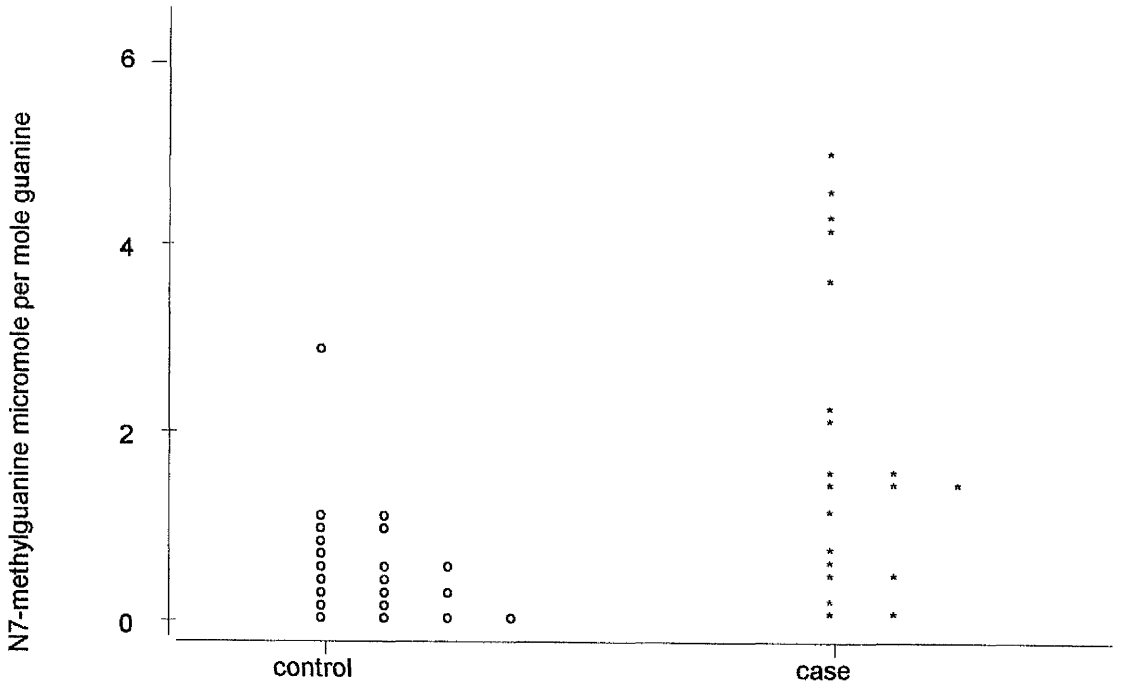


FIGURE 5.1. The amount of N7-methylguanine (μmol/mol G) detected in the cases (*) and *matched* controls (o).

Table 5.3 Distribution of the amount of N7-methylguanine (μmol/mol G) detected in the cases (20) and *all* controls (41).

μmol/mol G	Cases (20)	Controls (41)
<0.5	5	24
0.5- 1.0	2	10
1.1- 2.0	7	4
2.1- 4.0	2	2
>4.0	4	1

5.1.2 Analysis of genetic polymorphism in relation to treatment failure of CIN

PCR-based techniques were used to identify genetic polymorphism in the glutathione *S*-transferase genes (GSTM1, GSTT1), and in the cytochrome P450 genes (CYP2D6) as possible host factors modulating CIN treatment failure risk.

5.1.2.1 Frequency of glutathione *S*-transferase genetic polymorphism

The genotypes of GSTM1 and GSTT1 were analysed in the genomic DNA isolated from frozen CIN biopsy tissue of 45 cases and 110 controls.

Genetic polymorphism at the GSTM1 and GSTT1 loci, encoding for carcinogen-detoxification enzymes, were studied to see whether the non-conjugate phenotype was more abundant in the treatment failure cases. This phenotype (characterised by the absence of GSTM1 and/or GSTT1 activity) is due to homozygosity for an inherited deletion of these genes (termed the null genotype).

The frequency and percentages of each genotype in treatment failure cases and in controls are given in Tables 5.4 and 5.5. In a conditional logistic regression analysis, there was no significant effect of either the GSTM1 genotype ($p=0.57$) or the GSTT1 genotype ($p=0.26$) on the odds of treatment failure of CIN.

TABLE 5.4 Frequency (%) of GSTM1 genotypes in CIN treatment failure cases and controls.

	Wild	Null	Total
Cases	25 (55.6%)	20 (44.4%)	45 (100%)
Controls	51 (46.3%)	59 (53.6%)	110 (100%)
Total	76 (49.0%)	79 (51.0%)	155 (100%)

TABLE 5.5 Frequency (%) of GSTT1 genotypes in CIN treatment failure cases and controls.

	Wild	Null	Total
Cases	31 (68.9%)	14 (31.1%)	45 (100%)
Controls	80 (72.7%)	30 (27.3%)	110 (100%)
Total	111 (71.6%)	44 (28.4%)	155 (100%)

TABLE 5.6 Frequency (%) of each combination of GSTM1 and GSTT1 genotypes in CIN treatment failure.cases and controls and corresponding odds ratio (95% C.I.)

Group	No. of individuals with combined genotypes GSTM1/ T1			
	+/+	+/-	-/+	-/-
Controls (n=110)	39 (35.5%)	12 (10.9%)	41 (37.3%)	18 (16.4%)
Cases (n=45)	20 (44.4%)	5 (11.1%)	11 (24.4%)	9 (20.0%)
Odds Ratio (95% C.I.)	0.79 (0.41 to 1.50)	0.58 (0.17 to 1.96)	0.37 (0.16 to 0.83)	1.0

The combined genotypic distribution of GSTM1 and GSTT1 was also examined. The frequencies of the different combinations of GSTM1 and GSTT1 genotypes in CIN treatment failure cases and controls are given in Table 5.5. When the effect of the different combined genotypes was considered in the matched analysis, no significant effect was found (p=0.12).

5.1.2.2 Frequency of Cytochome 2D6 genetic polymorphism

Results were available for 60 cases and 125 controls. Table 5.6 shows the different genotypes and their respective frequencies in the CYP2D gene amongst CIN treatment failures and the corresponding control population. No significant effect (p=0.51) of CYP2D6 polymorphism on treatment failure was detected in a univariate conditional logistic regression analysis.

TABLE 5.7 Frequencies (%) of genotypes for CYP2D gene in CIN treatment failure cases and controls and corresponding odds ratio (95% CI)

Group	No. of individuals with genotypes		
	EM	HEM	PM
Controls (n=125)	82 (65.6%)	38 (30.4%)	5 (4.0%)
Cases (n=60)	42 (70.0 %)	14 (23.3%)	4 (6.7%)
Odds ratio (95% C.I.)	1.0	0.64 (0.30 to 1.38)	1.01 (0.26 to 3.97)

5.1.2.3 Interactions between the GSTM1, GSTT1 and CYP2D genotypes in the treatment outcome of CIN

The role of genetic polymorphism in the CYP2D6 gene in association with that of the GSTM1and GSTT1 genes was investigated and the results are presented in table 5.8.

There was no evidence of a different effect of the CYP2D genotypes on treatment failure for either the GSTM1 (p=0.63) or GSTT1 (p=0.46) genotypes.

TABLE 5.8 Genotypic distribution of combined CYP2D6, GSTM1 and GSTT1 in CIN treatment failure cases and controls

CYP2D genotypes		No. of individuals	GSTT1 genotypes	
			Wild type	Null
EM	Controls	70	52	18
	Cases	29	22	7
HEM	Controls	32	22	10
	Cases	9	4	5
PM	Controls	5	4	1
	Cases	4	3	1
			GSTM1 genotypes	
			Wild type	Null
EM	Controls	70	30	40
	Cases	29	17	12
HEM	Controls	32	16	16
	Cases	9	4	5
PM	Controls	5	3	2
	Cases	4	2	2

5.1.3 Interaction between genetic polymorphism and the levels of N7-methylguanine in treatment outcome of CIN

The possible influence of GST and CYP450 genotypes on the level of N7- methylguanine was examined. (The mean values are summarised in Tables 5.9). The individual values are shown in Figures 5.2, 5.3 and 5.4. There was some limited evidence to support the hypothesis of an increased risk of treatment failure in the absence of GSTM1 combined with increased levels of N7-methylguanine. However, the lack of data precluded formal testing of this hypothesis.

N7-methylguanine adduct levels were also analysed in relation to the different CYP2D6 genotype (Table 5.9). No significant differences were found between the median adduct levels for any of the examined mutations (p=0.51) The distribution of the N7-methylguanine levels in association with CYP2D6 polymorphism are shown in Figure 5.4, with the additional information on whether an individual was a case or control. There is a clear indication that those with high levels of N7-methylguanine amongst the excessive metabolisers tend towards treatment failure. An unmatched analysis (n=57) was performed to investigate the possible interactive effect of N7-methylguanine with the CYP2D6 genotype. It was found that the interaction was highly significant (p=0.007) and, moreover, that the estimated odds of treatment failure was greatly increased when the excessive metabolisers had higher levels of N7-methylguanine . Also included in Table 5.9 are statistics relating to the values of N7-methylguanine for the different combinations of CYP2D6 and GSTM1.

The following observations relating to the excessive metabolisers can be made.-

- The two cases with null GSTM1 have N7-meG values of 1.10 and 3.59. Both are higher than all seventeen controls with null GSTM1.
- The observed median value (2.35) is somewhat higher for the two cases with null GSTM1 than the corresponding median for the ten cases with wild-type GSTM1.

TABLE 5.9 N7- methylguanine in relation to GST and CYP2D6 polymorphism and its combination

Genotypes	μmoles of N7-meG / mole G median (IQ range) [n]	
	Controls	Cases
GSTM1		
Wild type	0.64 (0.66) [n=20]	1.42 (1.82) [n=13]
Null	0.30 (0.36) [n=18]	2.35 (3.47) [n=4]
GSTT1		
Wild type	0.40 (0.67) [n=32]	1.54 (2.62) [n=13]
Null	0.46 (0.62) [n=6]	1.01 (3.05) [n=4]
GSTM1/T1		
+/+	0.64 (0.71) [n=18]	1.54 (2.12) [n=11]
+/-	0.71 (-) [n=2]	1.01 (-) [n=2]
-/+	0.27 (0.33) [n=14]	2.35 (-) [n=2]
-/-	0.42 (0.74) [n=4]	2.25 (-) [n=2]
CYP2D6		
EM	0.37 (0.53) [n=31]	1.54 (2.06) [n=13]
HEM	1.00 (2.50) [n=6]	0.20 (1.10) [n=4]
PM	1.73 (-) [n=2]	0.74 (-) [n=1]
CYP2D6/ GSTM1		
EM/+	0.62 (0.63) [n=13]	1.55 (2.14) [n=10]
EM/-	0.29 (0.35) [n=17]	2.35 (-) [n=2]
HEM/+	1.00 (2.50) [n=6]	0.05 (-) [n=2]
HEM/-	- (-) [n=0]	0.35 (-) [n=1]
PM/+	0.53 (-) [n=1]	0.74 (-) [n=1]
PM/-	2.92 (-) [n=1]	- (-) [n=0]

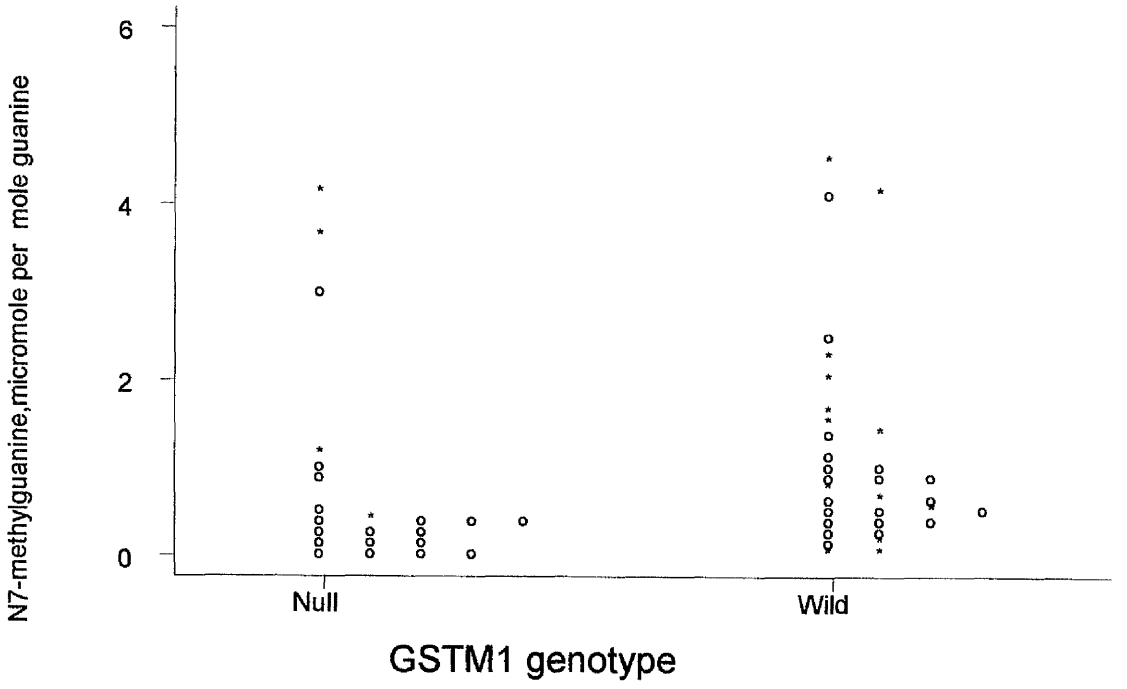


FIGURE 5.2. Distribution of N7-methylguanine (μmol/mol G) in cases (*) and controls (o) by GSTM1 genotype

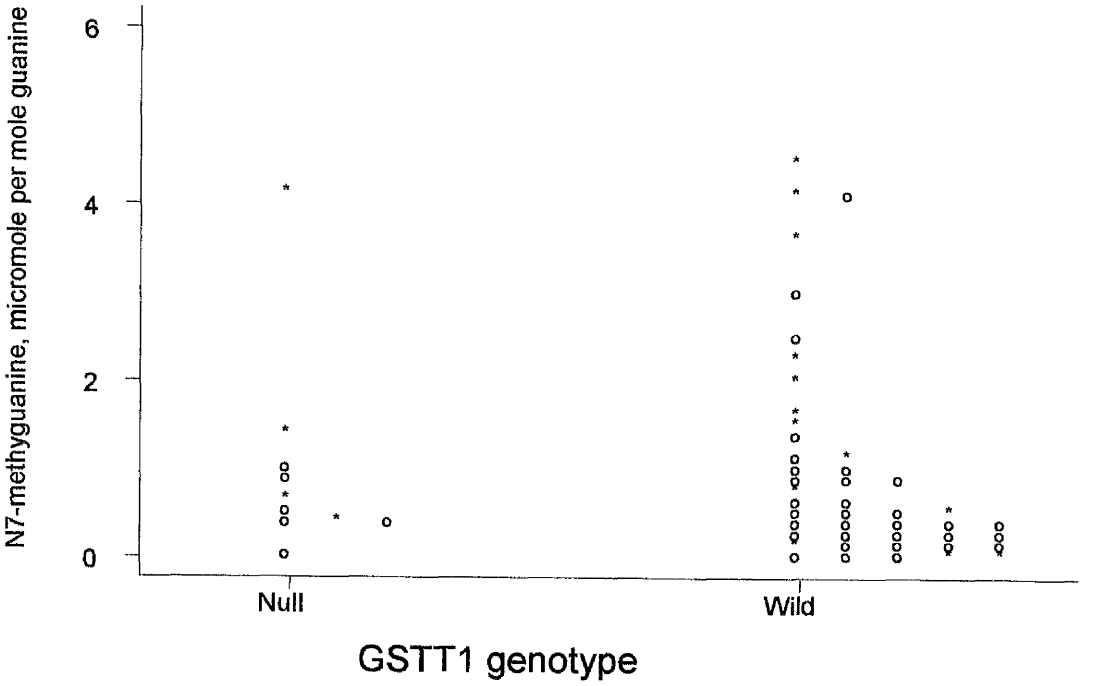


FIGURE 5.3 Distribution of N7-methylguanine (μmol/mol G) in cases (*) and controls (o) by GSTT1 genotype

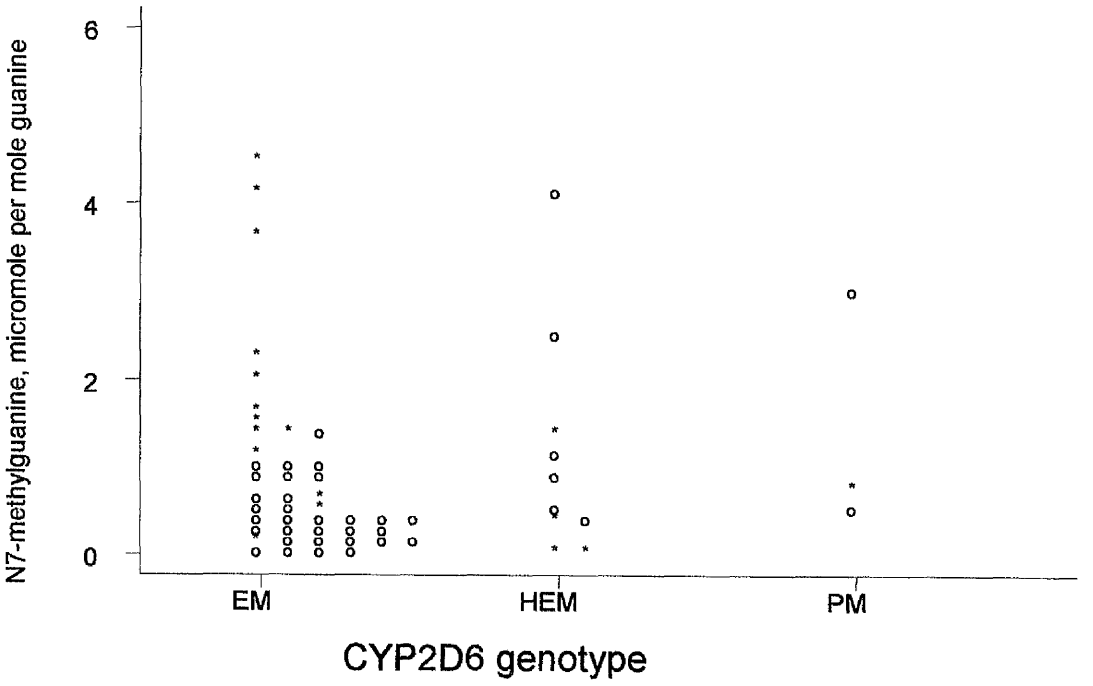


FIGURE 5.4 Distribution of N7-methylguanine (μmol/mol G) in cases (*) and controls (o) by CYP2D6 genotype

5.2 Discussion

The hypothesis that susceptibility to treatment failure of CIN is influenced by multiple allelism at gene loci encoding detoxifying enzymes and the formation of DNA adducts has been examined in this chapter. Environmental carcinogen exposure, DNA damage and genetic predisposition to cancer are important cancer risk factors. Host factors govern DNA adduct levels through carcinogen activation and detoxification and also through DNA repair and replication processes.

5.2.1 Relation between N7-methyl guanine and CIN treatment failure

N7-methylguanine is the major, persistent DNA base lesion generated by methylating agents e.g. smoking carcinogens. Several methods have been applied to the analysis of N7-methylguanine in human samples resulting from environmental exposure (see 1.5.6.1). The levels of N7-methylguanine have been quantitated in different types of human tissues (e.g., lung, larynx and blood) by using different techniques (Kato et al., 1993; Szyfter et al., 1996). No reports were available on the levels of N7-methylguanine in cervical tissues.

A sensitive and specific method for the measurement of N7-methylguanine in human DNA is the immunoslotblot test (Mientjes et al., 1996). In this study, N7-methylguanine was measured, using this recently developed immunoslotblot assay method, as a biomarker of

exposure of CIN treatment failure patients and controls to environmental and endogenously generated alkylating agents.

The study of DNA adduct formation in this thesis has been limited as the amount of DNA extracted in many of the cervical punch biopsies has fallen below the minimal required amount for the immunoslotblot assay. The amount of DNA required by this assay is, however, lower than other assays quantifying N7-methylguanine. Difficulties in extracting DNA from cervical biopsies have been previously experienced and may be related to the high elastic tissue content of the biopsy sample. (Povey and Harrison personal communications)

This study showed no significant relation between smoking habit and the proportion of DNA adducts in cervical epithelium, although the graphs indicate a possible weak relationship. However, any difference is too small to be detected from this sample, (Further discussion in chapter 6)

With the procedure used here, immunoslotblot assay, we have identifed alkylated DNA modifications. Tobacco smokers inhale a complex mixture of chemicals including tobacco-specific nitrosamines that can alkylate DNA (descried before), many of which have been shown to be carcinogenic in experimental animals (IARC,1986) and which therefore were implicated as causative agents in lung cancer (Phillips et al., 1988). On the other hand, other environmental agents can also cause such a modification in the DNA. This finding has been confirmed in this study. Even in the cases which the patient declared that

they were non-smokers, the DNA adduct level were high (Figure 6.1).

Analysis of N7-methylguanine adducts indicated that among the cases, the overall median value was 1.42 μmoles/mole G (range 0.05-4.83), while N7-meG among the matching controls was 0.42 μmoles/mole G (range 0.05-4.15). The wide interindividual range in the adduct levels most likely reflects interindividual variability in exposure, metabolic activation / inactivation and DNA repair (Harris, 1989; Nebert, 1991).

Since N7-methylguanine was detected in the majority of the cases (18 out of 20), it is reasonable to suggest that DNA adducts are one of the contributing factors in treatment failure of CIN. The cases may be exposed, probably continuously, to environmental and /or endogenous alkylating agents (e.g. N-nitroso compounds) (Cooper et al., 1995).

Based on the 10 to 1 ratio of N7-meG to O^6-meG formed in DNA by S_N1 methylating agents (Shields et al., 1990), the levels of N7-meG observed in this study suggested mean levels of O^6-meG of 0.065 ± 0.08 μmoles/moleG for control group and 0.18 ± 0.157 μmoles/mole G for the cases. That level for the cases closely corresponds to that reported for urinary bladder tumour which had a mean level of 0.126± 0.1 μmoles/mole G (Badawi et al.,1992).

The reason for the differences between the observed N7-meG levels in the cases and the controls in this study, could be due to the increased ability of the controls to repair N7-

meG lesions efficiently in the absence of HPV infection. This speculation remains to be experimentally proven.

5.2.2 Relation between different GST and P450 genotypes and treatment failure of CIN

Several research groups have examined and evaluated the possible role of genetic polymorphism in the genes encoding several Phase I and phase II metabolising enzymes as a determinant of interindividual differences in susceptibility to different types of cancer, especially in populations exposed to a wide range of environmental carcinogens (Wolf, 1990; Spitz and Bondy, 1993; Raunio et al., 1995; Warwick et al., 1994a).

The aim of this part of the study was to examine the occurrence of polymorphism in GST and CYP2D6 genes in relation to CIN treatment failure, using a molecular genetic approach. The power of combined analysis of GST and CYP2D6 polymorphism has, until now, not been applied to the study of susceptibility of treatment failure of CIN.

PCR approach was used to genotype for GSTM1, GSTT1 and CYP2D6 polymorphism and to determine whether these genes were associated with susceptibility to CIN treatment failure. Also, their interaction with HPV status and smoking history of the individuals was examined (joint effect of HPV and smoking will be discussed in chapter 6).

GSTM1 and GSTT1 may be important aetiological factors in CIN treatment failure

because they provide potential protection against DNA damage induced by carcinogens from tobacco smoke and other environmental sources. Deletion polymorphism of the GST genes, especially the GSTM1, has been found to be associated with a variety of cancers e.g. lung (Hirvonen et al., 1993) and colon (Zhong et al., 1993). A relation was found between GSTT1 null and the occurrence of CIN. (Warwick et al.,1994a).

In the present study individuals were genotyped for CYP2D6 polymorphism. The frequency of the extensive metabolisers (EM) among controls (65.6 %) was similar to that found among cases (70 %) (Table 5.6). A similar low level of poor metabolisers (PM) was detected in cases and controls (6.7%, 4% respectively). Thus CYP2D6 activity alone was not associated with CIN treatment failure risk.

The role of genetic polymorphism in the CYP2D6 gene in association with that of the GSTM1and GSTT1 genes was studied (Table 5.7). There was no evidence of a different effect of the CYP2D genotypes on treatment failure for either the GSTM1 ($p=0.63$) or GSTT1 ($p=0.46$) genotypes.

5.2.3 The levels of N7-meG in CIN treatment failure population in relation to different GST and CYP2D6 genotypes

The aim of this part of the work was to determine whether the susceptibility to CIN treatment failure was related to the joint effect of GST, CYP2D6 genotypes and DNA

adduct formation, in cervical tissue.

The possible influence of GST and CYP2D6 genotypes on the level of N7-meG was studied. The mean values are summarised in Tables 5.9 and the individual values are shown in Figures 5.2, 5.3 and 5.4.

Although the numbers are small for many combinations, The following observations related to the EM were made:-

- Two cases with null GSTM1 have N7-meG values of 1.10 and 3.59 (μmol/mol G), which are higher than all the seventeen controls with null GSTM1.
- Observed median (2.35μmol/mol G) is somewhat higher for the two cases with null GSTM1 than the corresponding median for the ten cases with wild-type GSTM1.

These observations may highlight the fact that environmental exposure to carcinogen, DNA damage and genetic predisposition to CIN treatment failure are important risk factors. Host factors control levels of DNA adducts through carcinogen activation, detoxification and DNA repair and replication processes.

The combination of GSTM1 null and CYP2D6 EM in these patients may have lead to increased exposure to carcinogens with subsequent increase in the level of DNA adducts in the cervical tissue particularly when associated with deficiency in DNA repair mechanisms.

Chapter 6 Matching of the data results and general discussion.

6.1 Results

6.1.1 Effect of joint HPV infection and smoking in the treatment outcome of CIN.

There was no significant effect of being HPV positive at the time of treatment, either independently or in conjunction with the number of cigarettes smoked, on treatment failure. Likewise, no type-specific pre-treatment HPV effect was detected. However, on considering the follow-up HPV data, a highly significantly adverse effect of being HPV positive was detected, both independently ($p=0.001$) and in conjunction with the number of cigarettes smoked ($p=0.002$). There were insufficient data to investigate the possibility of an interaction between an individual's follow-up HPV status and their pre-treatment smoking status in the effect on the chance of treatment failure.

TABLE 6.1 Unadjusted and adjusted odds ratios (ORs) of CIN treatment failure by smoking status and number of cigarettes smoked, (§ The odds ratio presented is for an increase in 10 cigarettes smoked per day).

Variable	OR	95% C.I.	OR adjusted for post-treatment HPV infection	95% C.I.
Current smoking status				
Never smoked	1.00	-	1.00	-
Ex-smoker	1.53	(0.49, 4.76)	2.28	(0.18, 28.51)
Current smoker	3.17	(1.68, 5.98)	18.73	(1.65, 212.2)
Cigarettes smoked per day now (units of 10)§	2.58	(1.70, 3.91)	4.43	(1.6, 12.25)

6.1.2 Joint effect of the level of N7-methylguanine and smoking on the treatment outcome of CIN

The limited amount of information available to assess the joint effects of smoking and log N7-methylguanine on the risk of treatment failure of CIN means that the p-values for smoking (present vs. ex/non smokers) and logN7 are 0.23 and 0.076 respectively. The corresponding odds ratio estimates are 2.88 (95% C.I. 0.51 to 16.21) for smokers relative to ex/non-smokers and 1.74 (95% C.I. 0.94 to 3.22) for unit increase in log N7-methylguanine. However, it can be observed from Figure 6.1 that the higher N7-methylguanine concentrations are amongst current smokers, particularly those with subsequent treatment failure. It is interesting to note that the two non/ex-smokers who have high N7-meG concentration are both cases, whereas the majority of the rest are controls.

The N7-meG levels in the treatment failure patients and controls with respect to smoking history are also presented in Table 6.2.

TABLE 6.2: N7-meG levels in treatment failure patients with respect to smoking status at time of treatment for CIN

	μmoles of N7-meG / mole G median (IQR) [n]	
	Controls	Cases
Current smoking status		
Non-smoker	0.48 (0.65) [n=19]	1.98 (-) [n=3]
Smoker	0.38 (0.81) [n=21]	1.42 (2.38) [n=17]

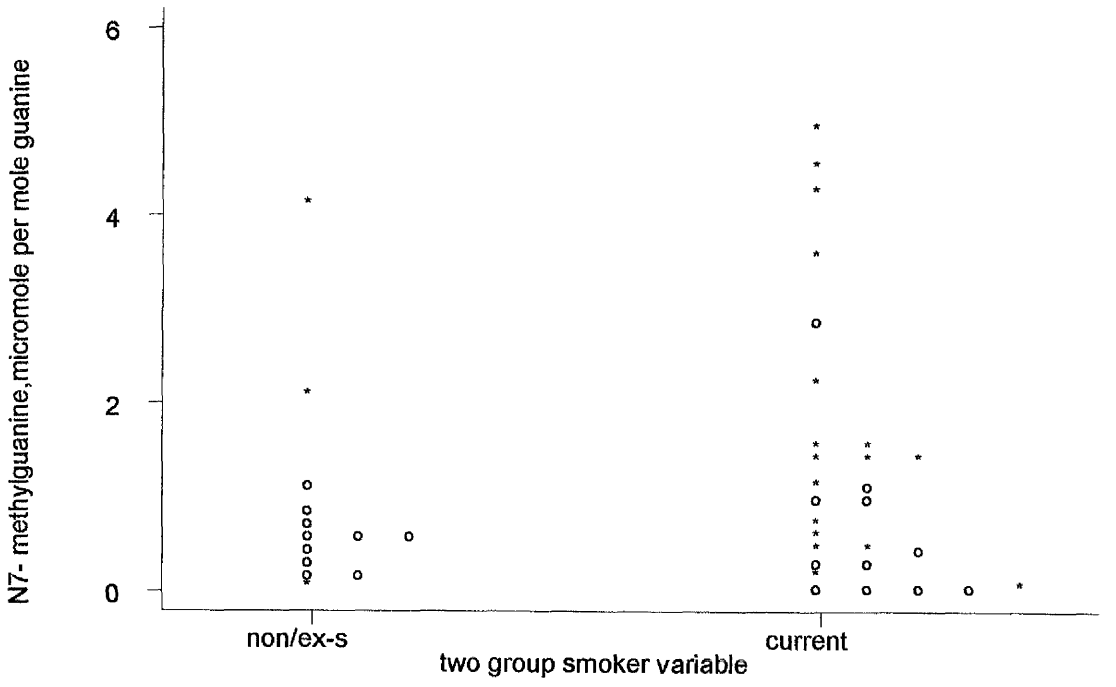


FIGURE 6.1 Level of N7-methylguanine concentration amongst current smokers versus non/ex-smokers in cases (*) and controls (o).

6.1.3 Effects of log N7-methylguanine and HPV status

There are insufficient data to assess the joint effects of log N7-methylguanine and post-treatment HPV status on the risk of treatment failure of CIN (a total of only 10 cases and matched control pairs would be available for this investigation).

On considering the joint effect of pre-treatment HPV status and log N7-methylguanine level on subsequent treatment failure, the levels of N7-methylguanine found amongst the positive and negative controls were similar. For the cases, the corresponding N7-methylguanine levels for those negative for pre-treatment HPV and those positive for pre-treatment HPV tended to be at a moderate or high level, with no real indication of a relationship between the HPV status and N7-methylguanine level.

When both pre-treatment HPV status and log N7-methylguanine were considered in a multiple conditional logistic regression model, the effect of pre-treatment HPV status on treatment failure remained non-significant ($p=0.114$) and that of log N7-methylguanine remained significant ($p=0.043$). There were insufficient data values available to investigate any potential interaction between pre-treatment HPV status and log N7-methylguanine on the odds of treatment failure.

TABLE 6.3 N7-meG levels in treatment failure patients with respect to pre-treatment HPV status.

	μmoles of N7-meG / mole G median (IQR) [n]	
	Controls	Cases
Pre-treatment HPV status		
Negative	0.34 (0.76) [n=10]	1.90 (3.82) [n=10]
Positive	0.43 (0.76) [n=31]	1.23 (1.12) [n=10]

6.1.4 Interaction between metabolic genotypes and smoking history

Potential associations between the GSTs and CYP450 genotypes and smoking habits of the studied individuals were also examined.

Among the cases for which smoking and GSTM1 data were available, 33/43 (76.7%) were smokers (i.e. current smokers) of whom 15 (45.4%) were GSTM1 null individuals. A similar finding was apparent for the controls. Fifty-four of the 108 controls were smokers, of whom 27 (50.0%) were GSTT1 null (Table 6.4). Consequently, no significant effect of smoking on the odds of treatment failure among individuals with the GSTM1 null genotype was detected (p=0.97). For the same set of individuals, 10 of the 33 (30.3%) smoking cases were GSTT1 null individuals whilst 17 of the 55 (30.9%) of the smoking controls were GSTT1 null individuals. As with the interactive effect of GSTT1

null with current smoking, no significant effect of smoking on the odds of treatment failure among individuals with the GSTM1 null genotype was apparent (p=0.64).

The distribution of the different genotypes among the cases and the controls according to their smoking history is also presented in Table 6.4. The association between the studied CYP2D6 genotypes (EM versus HEM or PM) and treatment failure risk did not appear to be modified by the smoking habits of the patients (p=0.31), although the number of matched case-control groups with other than Excessive Metabolisers was low for both current and ex/non-smokers.

TABLE 6.4 Genotypic distribution of GSTM1, GSTT1 and CYP2D6 by smoking status in CIN treatment cases and controls

		No. of individuals	Smoking status	
GSTM1 genotype			Non/ex-smoker	Smoker
Null	Controls	59	32	27
	Cases	19	4	15
Wild	Controls	49	22	27
	Cases	24	6	18
GSTT1 genotype				
Null	Controls	30	13	17
	Cases	13	3	10
Wild	Controls	78	41	38
	Cases	30	7	23
CYP2D genotype				
EM	Controls	80	41	39
	Cases	40	10	30
HEM	Controls	37	19	18
	Cases	14	6	8
PM	Controls	5	1	4
	Cases	4	0	4

6.1.5 Interaction between genetic polymorphism and post-treatment HPV status in the treatment outcome of CIN

It was hypothesised that those positive for HPV at follow-up, with GSTM1 null genotype and of CYP2D6 genotype EM might be at increased risk of treatment failure. Lack of data meant that no detailed investigation of this hypothesis was possible. However, it should be noted that of 9 individuals with this profile, 7 (77.8%) had treatment failure.

6.1.6 Interaction between genetic polymorphism, the level of N7-meG and smoking status in the treatment outcome of CIN

Although it would have been of interest to investigate the relationships between genetic polymorphism and the levels of N7-meG and smoking in the treatment outcome of CIN, the limited number of matched groups with levels of N7-meG recorded in the case and at least one of the controls precluded any investigation of interactions between N7-meG using a matched analysis.

6.2 Discussion

Treatment failure of CIN may result from either persistence of the disease or development of a new one (recurrence). From the clinical point of view; it is difficult to differentiate between persistence and recurrence.

Because of the cost implications of following up large numbers of women after treatment, it is important to document when treatment failure occurs in order to concentrate surveillance to the period of maximum risk of treatment failure of CIN. This involves the analysis of large number of cases. As previous reports showed that 95% of the treatment failures occurred in the first 2 years following treatment (Paraskevaidis et al., 1991; Flannelly et al., 1997), in this study follow up of the patients was carried out for that period.

In 1986, the International Agency for Research on Cancer determined that there was not enough evidence to conclude that smoking is a cause of cervical cancer. In this study, the findings are consistent with the proposal put forward by Winkelstein and Simons that smoking is a causative agent of cervical cancer (Winkelstein et al., 1990; Simons et al., 1993).

It is possible that in the progression of CIN, smoking acts in some way as a cofactor with sexually transmitted agents, probably HPV (Zur Hausen, 1982). Cotinine and nicotine, metabolic products in smokers, have been shown to be strongly concentrated in cervical lavages (Schiffman et al., 1987). This increased risk may result from a local immunological effect of smoking on the cervical epithelium (Barton et al., 1988) by the formation of carcinogen-DNA adducts in the cervical tissue itself which may initiate neoplastic transformation (Phillips et al.,1990; Simons et al., 1993). The present study has shown that smoking is an independent risk factor in the treatment failure of CIN and that this risk persists after controlling for HPV. This supports previous reports that smoking remains an independent risk factor for all grades of CIN after controlling for HPV (Cuzick et al., 1990; Kjaer et al., 1996). In addition, the present study provides some degree of support for the synergism theory between smoking and HPV (Schiffman et al., 1993), since each increases the effect of the other. Schiffman et al. (1993) found a significant association between smoking and CIN Grade II/III in HPV positive patients.

Despite the limited amount of information available to assess the joint effects of smoking and log N7-methylguanine on the risk of treatment failure of CIN, it can be observed from Figure 6.1 that higher N7-methylguanine concentrations are amongst current smokers, particularly those with subsequent treatment failure. It is also interesting to note that the two non/ex-smokers who have high N7-methylguanine concentrations are both cases, whereas the majority of the rest are controls.

The results showed no significant relation between smoking habit and the proportion of DNA adducts in cervical epithelium. However, any difference is too small to be detected from this sample (Table 6.2), although the graph (Figure 6.1) indicates a possible weak relationship.

Immunoslotblot assay was the procedure used to identify alkyl-DNA modifications. Tobacco smokers inhale a complex mixture of chemicals including tobacco-specific nitrosamines that can alkylate DNA (described before). Many of these chemicals had been shown to be carcinogenic in experimental animals (IARC, 1986) and therefore were implicated as causative agents in lung cancer (Phillips et al 1988). On the other hand, few environmental agents can also cause such modification in the DNA. These findings were confirmed in this study. Even in the cases which the patients declared that they were non-smokers, the DNA adduct level was high (figure 6.1).

The presence of detectable levels of DNA adducts in the non-smokers may be due to passive smoking. Deception by patients in hospital when reporting on smoking habits is

well recognised. (Simons et al., 1993).

The presence of HPV was the strongest predisposing factors for treatment failure. However, it has been shown that the functional inactivation of p53 is not invariably required for the induction of malignant transformation in the genital tract and thus other genetic events can also significantly participate in genital carcinogenesis (Curving et al., 1994).

This study showed that :-

- Smoking was a strong epidemiological factor independently and in association with HPV.
- Increased level of DNA adduct formation was found amongst treatment failure patients even when they failed to declare smoking.
- Nine individuals with GSTM1 null genotype and of CYP2D6 genotype EM, 7 (77.8%) had treatment failure.
- The combination of GSTM1 null and CYP2D6 EM was associated with an increase in the level of DNA adducts in the cervical tissue

These findings may make it possible to hypothesise that positive post-treatment HPV, smoking, high level of DNA adduct and combination of GSTM1 null and CYP2D6 EM genotypes are expected to be associated with increase risk of treatment failure of CIN. However, this hypothesis needs a larger study.

A future chemoprevention trial using isothiocyanates, which occur in vegetables and which has been shown to be broad-spectrum inhibitors of the metabolic activation of nitrosamines may be of value in the prevention strategy of CIN treatment failure. Phenethyl isothiocyanate was proven to be effective in the reduction of the level of O^6–methylguanine (Hecht, 1994).

Chapter 7 Conclusion

This is the first large prospective study to look at the value of HPV testing in the prediction of the treatment outcome of CIN. Pre-treatment HPV testing does not appear to influence the treatment outcome of CIN. Persistence of HPV appears to be the main factor influencing the treatment outcome of CIN. The sensitivity of the post-treatment HPV test was found to be 46.8% and the corresponding specificity 94.2%. Cigarette smoking is a factor, which, independently of HPV infection, influences the treatment outcome of CIN. Smokers should be encouraged to stop smoking after treatment for CIN.

The main point of weakness in this study is that the protocol was amended to take post treatment swab after the start of the study. Despite the presence of significant p-values in the post-treatment HPV swabs between cases and controls, this finding should be handled with caution because the sample size is smaller than it should be and the confidence interval is wide. Also, the limited amount of DNA which can be extracted from the cervical biopsies should be considered in any future study.

In an effort to minimize intervention as well as the diagnostic difficulties after treatment, there is scope to develop referral polices to colposcopy based on screening for both cervical cytology and/or HPV testing.

The use of hormonal methods of contraception might slightly increase the risk of treatment failure of CIN and the results are inconclusive on this matter .

DNA-adduct detection as a biomarker strategy for measuring exposure in this population could be facilitated by screening for N7-meG and other adducts in exfoliated cervical cells however a special attention such be given to the amount of DNA required for such a test. It would also be useful to examine, in conjunction with the N7-methyl adduct, the activity of its specific repair enzyme e.g. ATase enzyme.

Ideally the observation made during this study should be confirmed by more extensive study. A future large muticentre nation wide study is recommended to look at the value of post treatment HPV testing using different techniques of HPV detection.

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Appendix : **Patient questionnaire**

(Colposcopy Clinic)

PATIENT QUESTIONNAIRE

This form is absolutely confidential and for the purpose of research
Answers will only be seen by the researchers
Any particular questions can be omitted

Hospital number: Date:

Date of Birth:

Number of Children:

Previous Miscarriages or Terminations:

Number of Sexual Partners in the last 12 Months: Male/Female

Lifetime number of Sexual Partners: Male/Female

Previous genital warts in any
Of the your sexual partners: _ Yes _ No

Age of 1st sexual activity (1st intercourse):
younger than 15 _
15 - 20 _
20 - 25 _
25 - 30 _
30 and over _

Smoking Status:
Current _
Ex-smoker _
Never smoked _

If Current or Ex Smoker:

No of cigarettes smoked per day
No of years smoked
Age started smoking

If Ex-smoker: How long since smoking

Current prescription drug history (name and dose)

Any previous sexually transmitted diseases: _ Yes _ No

If yes:

Gonorrhoea _ Trichomonas _
Chlamydia _ Herpes(cold sores) _
Syphilis _ Other Specify.....
Yes but I do not know _

Current Method of Contraception :

Pills _

Injection _

Condoms _

IUCD _

Diaphragm _

Cap _

Others _ Please specify.....

None _

Previous methods of contraception and duration of use:

Pills _ dates.....

Injection _ dates.....

Condoms _ dates.....

IUCD(Coil) _ dates.....

Diaphragm _ dates.....

Cap _ dates.....

Others _ Please specify.....

None _

Ethnic origin

White (Caucasian) _

Black Caribbean _

Black African _

Asian _

Others _ Please specify.....

THANK YOU FOR COMPLETING THE QUESTIONNAIRE

For official use only

Biopsy Results :

Normal _

HPV only _

Borderline _

CIN1 _

CIN2 _

CIN3 _

Microinvasive _

Invasive _

Glandular Neoplasia _

Other Please State.....

Date of treatment:

Type of treatment:

Size of the lesion : 1 2 3 4 5 6 7 8