THE EPIDEMIOLOGY AND NATURAL HISTORY OF PAPILLOMAVIRUS INFECTION OF THE NORMAL CERVIX UTERI

By

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ABSTRACT

The epidemiology of cervical cancer indicates that some sexually transmitted agent is responsible and Human papillomavirus (HPV) is the currently favoured agent. This study was designed to assess whether HPV16 could be identified by filter in-situ hybridisation (FISH) in the normal cervix and, if present, whether the woman was placed at an increased risk of developing cervical neoplasia.

To examine this question, 1412 women, attending for routine cervical cytology, were studied. HPV16 DNA was found in the cervical scrapes in 23% of this population when assayed by FISH. There was no difference in the positivity rates between those with normal or abnormal cytology. A group of women (427) from this cohort, who were all cytologically normal, were colposcoped and a significant association between cervical disease, missed by cytology, and HPV16 positivity was found ($p = 0.01$). A follow-up case control study of those with a normal cervix with or without HPV16 DNA was conducted. Regular review over the ensuing 2 years with repeat cytology, colposcopy and FISH was undertaken. Only nine of these women developed any evidence of CIN over this period and this was not associated with HPV16 DNA. The epidemiologic factors were also compared between the cases and the controls.

162 cervical scrapes were used to compare the FISH method with Southern blotting and a weak association was found (Kappa=0.32), indicating the limitations of FISH for this type of study. The serial FISH results from each colposcopy clinic visit were assessed and also showed weak association.

To Ann, my wife:

the woman behind the man,

for her love, help and constant support.

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TABLE OF CONTENTS

		Page
	TITLE	1
	ABSTRACT	2
	ACKNOWLEDGEMENTS	4
	TABLE OF CONTENTS	6
	LIST OF TABLES	10
	LIST OF GRAPHS	16
	LIST OF ILLUSTRATIONS	16
	ABBREVIATIONS	17
	PREAMBLE	20
SECTION ONE	**INTRODUCTION**	**2 1**
1.2	The Transformation Zone	24
1.3	Papillomaviruses	26
1.4	Biology of Papillomaviruses	26
1.5	Classification of Papillomaviruses	27
1.6	Pathogenesis of Papillomaviral Infection	27
1.7	Papillomavirus and Carcinogenesis	29
1.8	Papillomavirus and Cervical Neoplasia	31
1.9	Clinical Aspects of HPV Infection	33
1.9.1	Genital Warts (Condyloma acuminata)	33
1.9.2	Subclinical Lesions and Colposcopy	34
1.10	Detection of HPV	35
1.10.1	Cytology	35
1.10.2	Histology	37
1.10.3	DNA-DNA Hybridisation	37
1.10.4	Southern Blotting Hybridisation	39

1.10.5	Filter in-situ Hybridisation (FISH)	40
1.10.6	Polymerase Chain Reaction	41
1.11	Cervical Intraepithelial Neoplasia (CIN)	42
1.12	Natural History of CIN	44
1.13	Koch's Postulates	46
1.14	Risk Factors for CIN	47
1.14.1	Sexual Factors	48
1.14.2	Male Factor	52
1.14.3	Smoking	53
1.14.4	Contraception use	55
1.14.5	Social Class	56
1.14.6	Other Infectious Agents	57
SECTION TWO	**MATERIALS AND METHODS**	**5 9**
2.1	Study Design and Aims	60
2.2	General Screening	61
2.3	Colposcopy Clinic	63
2.3.1	The Clinic	63
2.3.2	Counselling and Examination	64
2.3.3	Records and Results	64
2.3.4	Treatment of Abnormalities	65
2.4	Sample Collection	65
2.5	Cytology	67
2.6	Bacteriology	69
2.7	Histopathology	71
2.8	Filter in-situ Hybrisation	73
2.9	Random Primer Oligolabelling with Linear HPV16 DNA	74
2.9.1	Prehybridisation Washing	74

2.9.2	Labelling of Linear HPV	75
2.9.3	Checking Incorporation	76
2.9.4	Separation of unincorporated nucleotides	77
2.9.5	High Stringency Wash	78
2.9.6	Autoradiographs	79
2.9.7	Developing the autoradiographs	79
2.9.8	Interpretation	80
2.10	Southern Blotting	80
2.10.1	Extraction	80
2.10.2	Dialysis	81
2.10.3	Restriction Endonuclease Analysis	82
2.10.4	Depurination	83
2.10.5	Setting up the Southern Blot	83
2.10.6	Hybridisation	84
2.11	Preparation of Linear HPV 16 DNA	84
2.12	Solutions	88
2.13	Statistical Methods	93

SECTION THREE RESULTS 9 7

3.1	Screening results	98
3.2	Abnormal Cytology results	115
3.3	Results from the Study Population	130
3.4	Prevalence of cervical infections in the Colposcopy Clinic population.	150
3.5	Logistic Regression analysis of the study population.	153
3.5.1	Normal versus presence of disease	154
3.5.2	Normal versus HPVI alone	155

3.5.3	Normal versus CIN	157
3.5.4	HPVI alone versus CIN	158
3.5.5	Normal versus major grade disease	159
3.5.6	Normal versus minor grade disease	160
3.5.7	Minor versus major grade disease	161
3.6	Comparison of Filter in-situ hybridisation and Southern Blotting	162
3.7	Dilution Experiment results	182
3.8	Results of Subgroup of patients used in serial analysis	187
3.9	Serial HPV16 results using FISH method and Transitional Modelling	189
3.10	Progression to disease	198
3.10.1	Normal cases developing CIN	203
3.10.2	HPVI cases developing CIN	205
3.10.3	Cases developing CIN from the original study population	206

SECTION FOUR DISCUSSION 209

4.1	Screened population	210
4.2	Colposcopy population	214
4.3	Comparison of methodologies	217
4.4	Progression of disease and HPV16 positivity	220
4.5	Concluding comments	222

REFERENCES 225

APPENDIX 248

LIST OF TABLES

TABLE		PAGE
SECTION 3.1		
1.	Age Range of the screened population	98
2.	Cytology Results	100
2.1	Summary of cytology results	100
3.	Kruskal-Wallis test for equality of ages - all cytology results	101
4.	Kruskal-Wallis test for equality of ages - summary of cytology results	102
4.1	P values for pair wise Wilcoxon comparisons of Table 4	102
5.	Cytology versus HPV16 results by FISH	103
5.1	Summary of cytology versus HPV16 status	103
5.2	Normal and abnormal cytology and HPV16 status	103
6.	Kruskal-Wallis test for equality of ages - HPV16 results	105
6.1	P values for pair wise Wilcoxon comparisons of Table 6	105
7.	Smoking history and cytology result	106
8.	Smoking history and HPV16 result	107
9.	Current contraception use against cytology.	108
10.	History of OCP usage	109
11.	History of OCP usage and Cytology result	109
12.	HPV16 status against history of OCP usage	110
13.	Kruskal-Wallis test for OCP usage time compared to cytology	110
13.1	P values for pair wise Wilcoxon comparisons of Table 13	111

14. Time of OCP exposure in the screened population 112

15. Time of OCP exposure compared to cytology result 112

16. Kruskal-Wallis test of OCP usage time and HPV16 status. 113

16.1 P values for pair wise Wilcoxon comparisons of Table 16 114

SECTION 3.2

17. Age distribution of the abnormal cytology group 115

18. Cytology result with HPV16 status. 117

19. Summary of outcome of these patients 117

20. Cytology results against outcome 118

21. HPV16 status compared to outcome 119

22. Kruskal-Wallis test for age and HPV16 status 120

22.1 P values for pair wise Wilcoxon comparisons of Table 22 120

23. Kruskal-Wallis test for age and cytology result 121

23.1 P values for pair wise Wilcoxon comparisons of Table 23 121

24. Kruskal-Wallis test for age against outcome 122

24.1 P values for pair wise Wilcoxon comparisons of Table 24 123

25. Outcome versus smoking history 123

26. History of OCP usage and outcome 124

27. Kruskal-Wallis test for OCP usage time in ever users 125

27.1 P values for pair wise Wilcoxon comparisons of Table 27 126

28. Cytology result with reference to OCP usage 126

29. Time of OCP exposure compared to colposcopic outcome 127

30. HPV16 status with reference to OCP usage 127

SECTION 3.3

31. Number of women colposcoped 130

32. Age range of Colposcopy population 131

33. Kruskal-Wallis test on the median ages depending on HPV16 status 131

33.1 P values for pair wise Wilcoxon comparisons of Table 33 131

34. Current contraception 133

35. OCP use 134

36. Kruskal-Wallis test on time of OCP usage 134

36.1 P values for pair wise Wilcoxon comparisons of Table 36 135

37. Kruskal-Wallis test on coitarche 136

37.1 P values for pair wise Wilcoxon comparisons of Table 37 136

38. Kruskal-Wallis test on median number of sexual partners 137

38.1 P values for pair wise Wilcoxon comparisons of Table 38 137

39. History of Genital infection against HPV16 status. 138

39.1 Any infection (excluding candida) 138

39.2 Candida 138

39.3 NSU (Chlamydia) 139

39.4 Trichomonas vaginalis 139

39.5 Genital warts 139

39.6	Herpes simplex	140
39.7	Neisseria gonorrhoea	140
40.	Total number of pregnancies against HPV16 status	141
41.	Number of terminations per woman against HPV16 status	141
42.	Number of children per woman against HPV16 status	142
43.	Kruskal-Wallis test on age of first pregnancy and HPV16 status	142
43.1	P values for pair wise Wilcoxon comparisons of Table 43	143
44.	Smoking history & HPV16 status	144
45.	Cigarette consumption.	145
45.1	P values for pair wise Wilcoxon comparisons of Table 45	145
46.	Repeat cytology in the study population	146
47.	Colposcopic findings against HPV16 status	147
48.	FISH results on initial screening and at the time of first colposcopy	148
48.1	Kappa results and time interval between visits	149

SECTION 3.4

49.	High vaginal and cervical swab results	150

SECTION 3.6

50.	Amount of DNA extracted for Southern blotting	162
51.	Colposcopic findings	162
52.	Amount of DNA extracted per group	163
52.1	Range of amount of extracted DNA	163

52.2	Distribution of samples where amount of extracted DNA was less than 10μg	164
53.	Kruskal-Wallis test on median amounts of DNA extracted in the different colposcopic groups	168
53.1	P values for pair wise Wilcoxon comparisons of Table 53	168
54.	FISH results compared to Southern blotting	169
55.	Kruskal-Wallis test on the median amount of DNA extracted within each group	170
55.1	P values for pair wise Wilcoxon comparisons of Table 55	170
56.	Comparison of amounts of DNA extracted relative to the Southern blot result	171
56.1	All Southern blot results	171
56.2.	Range of the amount of DNA extracted for Southern blotting	172
56.3	Southern blot results compared to the amount of DNA extracted	172
57	FISH results relative to the amount of DNA extracted for the Southern blot	173
58.	Comparison of amounts of DNA extracted and the Southern blot results within each group	174
58.1	Normal	174
58.2	HPVI	174
58.3	CIN	175
59.	Comparison of FISH and Southern blot result in the colposcopically Normal group	176
60.	Comparison of FISH and Southern blot result in the HPVI group	177

61. Comparison of FISH and Southern blot result in the CIN group 177

62. Comparison of FISH and Southern blot result for any grade of abnormality 178

63. Comparison of the degree of FISH positivity and the Southern Blot result 180

64. Colposcopic findings and FISH results - summary 180

65. Colposcopic findings and Southern blot result 181

SECTION 3.7

66 Cell content per nitrocelluose filter 182

SECTION 3.8

67. Referral cytology and findings 187

68. Original FISH findings for HPV16 188

SECTION 3.9

69. Number of observed and expected positive results for HPV16 per number of colposcopy visits 189

69.1 Goodness of fit for the binomial distribution 190

70. Number of observed and expected positive results for HPV16 per number of subsequent results - excluding the initial result 191

70.1 Goodness of fit for the binomial distribution 192

71.1 Transitional modelling using all HPV16 results 194

71.2 Transitional modelling using HPV16 results excluding original screening values 195

72. HPV16 positivity rates amongst the study population 196

73. Subsequent HPV16 results in women of the Colposcopy study population 196

SECTION 3.10

74.	Progression of disease of the colposcopically normal women	198
75.	Summary of HPV16 results of the normal cases developing disease	199
75.1	CIN 1	199
75.2	CIN 2	199
76.	Features of women developing CIN compared to those remaining normal	200
77.	Follow-up results from those women with HPVI alone within the original study group.	204
78.	Gravidity in Normals and those developing CIN	207
79.	Parity in normals and those developing CIN	207

GRAPHS

1.	Age distribution of screened population & HPV16 status	99
2.	Age distribution of women & cytology result	116
3.	Age distribution of colposcopy population & HPV status	132
4.	Range of amount of extracted DNA	165
5.	Amount of extracted DNA per colposcopic group	166
5	Distribution of samples where amount of extracted DNA was less than 10μg	167

ILLUSTRATIONS

Figure 1.	Examples of FISH results	87
Figure 2.	Dilution experiment - Filter autoradiographs	185
Figure 3.	Reconstruction experiment - Southern blot autoradiographs	186

ABBREVIATIONS

Av	Average
BSA	Bovine serum albumin
CIN	Cervical Intraepithelial Neoplasia
CIS	Carcinoma in situ
cm	centimetre
CMV	Cytomegalovirus
CTP	Cytosine triphosphate
d.f.	degrees of freedom
DNA	Deoxyribonucleic acid
Dysk	Dyskaryosis
E	Expected
ECNS	Endocervical cells not seen
Eq	Equivocal
FISH	Filter in-situ hybridisation
F.P.C.	Family planning clinic
F.U.	Follow-up
gm	gram
G.P.	General Practitioner
GUM	Genito-Urinary Medicine
H.C.	Health Centre
HPV	Human Papillomavirus
HPVI	Human Papillomavirus infection
HSV	Herpes simplex virus
HVS	High vaginal swab
IU	International units
IUCD	Intrauterine contraceptive device
K	Kappa
kb	kilobase

M	molar
mg	milligram
ml	millilitre
mm	millimetre
mmol	millimole
mths	months
ng	nanogram
nm	nanometre
NaCl	sodium chloride
NaOH	sodium hydroxide
Neg	Negative
N/K	Not known
n or no.	number
NSU	Non-specific urethritis
O	Observed
Obs	Observations
OCP or OC's	Oral contraceptive pill
OD	Optical density
OPCS	Office of Population & Census Surveys
ORF	Open reading frame
p	Probability
PBS	Phosphate buffered saline
pcg	picogram
PCR	Polymerase chain reaction
PHLS	Public Health Laboratory Service
Pos	Positive
py	Pack years
RCOG	Royal College of Obstetricians & Gynaecologists
RNA	Ribonucleic acid

RNH	Royal Northern Hospital
r p m	revolutions per minute
RR	Relative risk
s.d.	standard deviation
SDS	Sodium dodecyl sulphate
SMR	Standardized mortality rate
SSC	Salt sodium citrate
SSST	Single stranded salmon testes (DNA)
STD	Sexually Transmitted Disease
Tm	Melting temperature
TZ	Transformation Zone
v / v	volume for volume
WHO	World Health Organisation
w / v	weight per volume
y r s	years
µg	microgram
µl	microlitre
µm	micrometre

PREAMBLE

In this introduction, I have attempted to demonstrate the incidence of cervical neoplasia, both malignant and premalignant. I have discussed the relevance of the development of the Transformation Zone of the cervix, making it susceptible to abnormal changes. The association of papillomavirus with dysplastic change in the cervix is briefly mentioned before reviewing the Papillomavirus species with regard to their relevant biology, classification and pathogenesis of disease. I have presented evidence for the association of papillomaviruses and carcinogenesis in general terms and then go on to discuss the evidence for a possible causal association between Human Papillomavirus (HPV), especially HPV16, and cervical neoplasia.

The clinical aspects of Human Papillomavirus Infection (HPVI) both overt and subclinical are discussed, followed by a review of the various methods of HPV detection cytologically, histologically and by DNA-DNA hybridisation. I have reviewed the concept of Cervical Intraepithelial Neoplasia (CIN) and its continuum, as well as evidence for the natural history of the disease. It seemed appropriate to mention Koch's postulates and their limitations with regard to viral disease, before reviewing other risk factors in patients developing CIN.

The hypothesis that I am, therefore, trying to test is that if HPV16 is a causative agent in the development of CIN, is it possible to identify women who are cytologically and colposcopically normal but positive for HPV16 by a DNA hybridisation technique (filter in-situ hybridisation). If that is possible, are these women at an increased risk of developing CIN compared to a control population and are there any other features in their history prediposing them to this risk?

SECTION ONE
INTRODUCTION

INTRODUCTION

Cervical cancer and precancer form a disease continuum ranging from cervical intraepithelial neoplasia (CIN) grades 1 to 3, through microinvasion to invasive carcinoma. The great majority (90-95%) of the cancerous lesions are squamous cell carcinomas (Anderson, 1985) and arise within the Transformation Zone of the cervix (Coppleson & Reid, 1967). In 1984, there were 4,043 cases and 1,899 deaths from carcinoma of the cervix reported in England and Wales. These figures revealed that cancer of the cervix was the eighth most common cancer in women and accounted for approximately 4% of all cancers in women (OPCS, Cancer Statistics Registration, 1984). More recent unpublished OPCS data shows a mortality rate of 7.4/100,000 in 1987, compared with an average mortality of 9/100,000 between 1968-78 (Gardner et al, 1983).

On a worldwide scale, the situation is very different. In 1975, it was estimated that an equal number of women developed genital (mainly cervical) cancer as developed breast cancer, and this amounted to approximately half a million in each group (Parkin et al, 1984). This figure accounted for about 15% of all cancers diagnosed in women, though the geographical distribution was markedly different. In the developed world, cancer of the cervix has been eclipsed by other malignancies, namely of breast and lung. In the developing world, cervical cancer remains the most frequent neoplastic cause of death and accounts for 80% of all the cases of cervical cancer occurring worldwide (Parkin et al, 1988). Overall, the cervical cancer mortality rate is declining in most parts of the world (Cuzick & Boyle, 1988), though the rate has been increasing in younger women in the 20-35 year age group in a few places, notably Australia and Britain (Elliott et al, 1989).

The incidence of precancerous lesions is much more difficult to assess, as problems arise from inadequate registration of these lesions. Most estimates are based on cohort studies of screened women. In a cervical screening programme conducted in California during the late 1970's, 34,318 women were screened and 166 (0.5%) were found to have biopsy proven neoplasia, of which 157 were precancerous (Fasal et al, 1981). Meisels and colleagues (1977), in an earlier study in a 'highly homogeneous population' of French Canadians, found a dysplasia rate of 2.53% amongst a population of 153,231 being screened for the first time. Problems arise with the interpretation of these figures through not knowing:

1. the age distribution of the population.
2. the nature of the unscreened women in the same district, and
3. the number of false negatives.

In the United Kingdom, the cervical cytology service has developed over the past 20-25 years. In 1976, just over 2.92 million smears were taken and this had increased by 63% to over 4.75 million in 1987 (Social Trends, 1989). Cook and Draper (1984) presented data which strongly suggested a true increase in the incidence rates of premalignant lesions between 1972-74 and 1978-80. Soutter and his colleagues (1984) reported a cytology abnormality rate of 1.21% in the Gateshead area in 1983. These figures contrast with those of the London district of Islington, where the number of cervical smears performed rose by 36% between 1972 and 1987, with an increase in the percentage abnormal rates from 0.2% to 12.2% over the same period (Hollingworth & Cuzick, In press). These figures may be explained by a possible lowering of the threshold for reporting abnormalities, or a possible increase in the prevalence of wart virus infection of the cervix. Indeed, Drake et al (1987) stated that reporting wart viral was not initiated until 1978, a review of cervical smears from

1965 was performed and HPVI changes were found though the significance of these changes had not been originally appreciated. However, these same figures showed an almost 8-fold increase in the rates of severely dyskaryotic changes over the same period, which is more likely to reflect a genuine rise in the prevalence of cervical intraepithelial neoplasia. The main concern from these figures is the notable rise in abnormality rates in the 15-35 year age groups.

1.2 THE TRANSFORMATION ZONE

The neonatal cervix is almost completely covered by non-keratinising stratified squamous epithelium, with columnar epithelium lining the cervical canal. The effect of increasing concentrations of ovarian hormones during adolescence results in an increase in the bulk of the cervix and eversion of the columnar epithelium. It is this area of everted columnar epithelium that is of paramount importance in the pathogenesis of cervical neoplasia (Coppleson & Reid, 1967). Squamous metaplasia occurs due to reserve cell proliferation. The origin of these cells is unknown but they are subcolumnar and thought to be stimulated by the relative acidity of the vaginal environment compared to that of the cervical canal (Burghardt & Ostor, 1983). There is gradual replacement of the columnar epithelium and the resulting squamous epithelium may be normal or abnormal. It is in this Transformation Zone (TZ), where change from columnar to squamous epithelium occurs, that CIN may arise via the process of squamous metaplasia. There are two theories for the development of precancer. One suggests a unicellular origin with horizontal spread to replace adjacent normal epithelium (Richart, 1967; Ferenczy, 1977) and the other multicellular theory which assumes that neoplasia arises in predetermined fields with all cells transforming simultaneously (Coppleson & Reid, 1967; Johnson, 1969).

The basal cells of the squamous epithelium divide mitotically and give rise to 3 types of cells:

1. those that will be capable of further mitotic divisions and so guarantee the survival of the epithelium (immortal cells);

2. those that provide anchorage to the basal lamina;

3. the rest, which terminally differentiate and form the bulk of the epithelium and lose mitotic capability - Mortal Cells. (Burghardt, 1986)

In the sequence of events leading to malignant transformation, some genetic error has to occur at the level of the immortal cell. The resulting genetic disturbance may be perceived at the level of the mortal cells, which results in their acquisition of the capability for further mitotic division. This newly acquired mitotic capability lacks the usual regulatory mechanism and may give rise to an abnormal DNA content in the daughters. Such abnormal cells were named FITTER cells by Cairns (1975), because they appear better capable of survival than the mortal cells. The ultimate fate of these fitter cells is unsure; they may be transient and disappear or they may persist and eventually progress to invasive cancer.

The relationship between sexual behaviour and cervical cancer was first observed as early as 1842 by Rigoni-Stern (see section 1.14.1). This association (Rotkin, 1973; Kessler, 1974) has led to the search for a sexually transmitted agent, especially viral in nature. Human papillomavirus was suggested as the causal agent (zur Hausen, 1977), which may act at the cellular level and promote dysplastic change within the epithelium of the transformation zone.

1.3 PAPILLOMAVIRUSES

Papillomaviruses are a group of species-specific viruses that infect man as well as many animals. They have been known to cause warts for many years (Cuiffo, 1907; Rowson & Mahy, 1967), but more detailed information was difficult to obtain because of their inability to be propagated in cell culture (Butel, 1972). Research over the past 10-15 years has been stimulated by the development of DNA recombinant technology and the recognition of an association between the presence of specific human papillomavirus (HPV) types and lower genital tract neoplasia in men (Zachow et al 1982; McCance et al, 1986c) and women (Gissmann et al, 1983; Durst et al, 1983; Boshart et al, 1984; McCance et al, 1985).

1.4 BIOLOGY OF PAPILLOMAVIRUSES

Papillomaviruses are members of the Papovaviridae family, which comprises two genera: Papillomavirus and Polyomaviruses (Melnick, 1962). Papilloma virions (52-55nm in diameter) are simple assemblies of a double strand of DNA and histones surrounded by an icosahedral capsid (Williams & Fisher, 1974). There is a major HPV capsid protein with a molecular weight of approximately 55,000 daltons plus a minor one of approximately 76,000 daltons (Komly et al, 1986). The capsid proteins of all animal and human papillomaviruses are antigenically cross-reactive and this provided the basis for immunocytochemical detection. (Broker, 1987). The cross reactivity is due to the major capsid protein (Jenson et al, 1987).

These capsids, 45-55nm in diameter, are made of 72 capsomeres. Within this covering lies the genome which is a supercoiled double stranded circular DNA molecule of about 7,800-8,000 base pairs (Pfister, 1984). The molecular weight of these molecules is in the region of 5,200,000 daltons.

1.5 CLASSIFICATION OF PAPILLOMAVIRUSES

Within the genus, papillomaviruses are classified into types and subtypes on the basis of DNA homology or similarity. This is determined by reassociation of heterologous DNA in liquid phase followed by S1 nuclease digestion. Different types share less than 50% DNA homology and different subtypes share greater than 50% but less than 100% DNA homology. (Coggin & zur Hausen, 1979). The nucleotide sequences of the DNA's from human and animal PV's show a strikingly similar organisation of the protein coding areas (open reading frames, ORF), which have historically been divided into Early (E) and Late (L) regions. The Early regions (E1-7) code for episomal replication of viral DNA, high copy maintenance of viral plasmids, transactivation of viral transcription and activation. The Late regions (L1-2) code for the structural proteins of the virus.

Sixty HPV types have been identified and at least 20 have been associated with genital tract infection (de Villiers, 1989). The more common genital types include HPV 6 and 11, which infect both sexes and produce elevated lesions (condyloma acuminata or genital wart) which are usually benign (Gissmann et al, 1983). HPV types 16 (Durst et al, 1983) and 18 (Boshart et al, 1984) are associated with flat dysplastic lesions which, when they develop on the transformation zone of the cervix, have a greater malignant potential than when found in other sites of the genital tract. The other HPV types associated with genital infection make up less than 10-15% of lesions.

1.6 PATHOGENESIS OF PAPILLOMAVIRAL INFECTION

The sexual route of transmission of HPV was reported by Barrett et al, (1954), with an incubation period varying from between a few weeks to

several months (Oriel, 1971). Epithelial proliferations of skin or mucosa may result, which show limited growth and often regress spontaneously. Papillomaviruses induce hyperplasia in the lower epithelial layers, but the formation of complete viral particles appears to be restricted to the upper layers of the epidermis (Koutsky et al, 1988). This would suggest that viral replication and transcription could only be supported at a certain level of differentiation (Broker, 1987). Natural infection is thought to occur as a result of injury to the epidermis, whether by microlesions or local abrasion (White et al, 1963). This allows direct physical access of the virus particles to the basal cells of the epithelium and the wound healing may assist the infection through accelerated basal cell division, stimulation of capillary growth and an augmented blood supply at the site of healing (Broker, 1987). Within a lesion, the infection is highly focal and may involve only a small group of cells.

The primary site of infection is the basal cell layer, which provides a reservoir of virus (Jenson et al, 1987). As the epithelial differentiation progresses, so these more superficial cells become more amenable to viral DNA replication, which can be demonstrated by in situ hybridisation in the suprabasal layers. Structural proteins and mature virus particles appear in the upper epidermal layers (Jenson et al, 1984).

There are three clinicopathological groups:

1. cutaneotropic viruses, which occur in the individual with normal immunity. The vegetative growths are benign and may regress spontaneously;

2. cutaneotropic viruses, which may lead, in individuals with epidermodysplasia verruciformis or in immunocompromised patients, to

malignant lesions. These viruses do not appear to cause lesions in immunocompetent individuals;

3. mucosotropic group of viruses, which affect the genital, oral and respiratory epithelia, independent of immune status.

1.7 PAPILLOMAVIRUSES AND CARCINOGENESIS

The first evidence that papillomavirus could induce tumours, which in turn progress to malignancy, was demonstrated in the cotton tail rabbit (Shope papillomavirus). Rous and Beard (1935) showed in animals with long persisting papillomas, malignant tumours developed in approximately 25% of infected animals. It was later shown that the frequency and speed of the malignant conversion was affected by exposure of the papillomas to chemical carcinogens (Rous & Friedewald, 1944). However, even without any obvious promotion, the papillomas rarely remained benign after 18 months.

This rabbit system has become the model for papillomaviral oncogenesis. The viruses seemed only weakly oncogenic by themselves and a particular genetic background or additional exposure to physical or chemical co-carcinogens such as ultraviolet light, X-rays or diet was required. The use of X-ray treatment in juvenile respiratory (laryngeal) papillomas, which led to an increasing risk of subsequent malignant conversion (Zehnder & Lyons, 1975), was an unfortunate example.

In man, the disease epidermodysplasia verruciformis represents an excellent model in studies on malignant conversion of papillomavirus induced tumours. Due to a genetic predisposition, the patient develops skin lesions during childhood which are induced by papillomaviruses not usually observed in the normal population (Jablonska et al, 1972). The

lesions do not regress but persist for life, gradually spreading over the entire body. Between one quarter and one third of these patients develop cancer, after a lag phase of 20-25 years of persisting disease. There is a preference of these lesions for the sun exposed regions (Ruiter, 1973). These patients have an impairment of their cell mediated immune status, though malignant conversion seems to be more related to the specific viral types, namely HPV 5 and 8 (Orth et al, 1978; Pfister et al, 1981).

Kreider et al (1985) transplanted small pieces of human cervix or foreskin under the renal capsule of immunologically incompetent nude mice. The transplanted tissue grew and differentiated normally. However, if the tissue was exposed to HPV 11 prior to transplantation, the resulting tissue that grew under the capsule showed histological evidence of human papillomavirus infection (HPVI). The usefulness of this model has been limited in that it has only been successful with HPV 11, but it does show that HPV causes lesions which may be part of the continuum leading to CIN and invasive cancer (McCance, 1988).

In-vitro evidence for the causal association of HPV's with disease has been shown by the ability of HPV 16 and 18 to immortalize human foreskin keratinocytes (Durst et al, 1987; Pirisi et al, 1987). If these transfected human keratinocytes are placed in a collagen raft system, they stratify and subsequently develop the histological features of CIN 3 (McCance et al, 1988).

At the molecular level, the E6 and E7 proteins of HPV16 are regularly expressed in cervical cancers. E6 is an oncoprotein which cooperates with E7 to immortalize primary human keratinocytes (Hawley-Nelson et al, 1989). Wild type p53 gene has tumour suppressor properties (Finlay et al,

1989) and is a target for several of the oncoproteins encoded by DNA tumour viruses. E6 has been found to bind to p53 and stimulates p53 degradation, thereby removing the suppressor gene effect (Scheffner et al, 1990). This selective degradation of cellular proteins such as p53, which has a negative regulatory function, may provide a mechanism of action for HPV16.

1.8 PAPILLOMAVIRUS AND CERVICAL NEOPLASIA

In the early 1980's, two novel HPVs were identified namely HPV16 and HPV18, and their DNAs were molecularly cloned from two cervical carcinoma biopsies (Durst et al, 1983; Boshart et al, 1984). HPV 16 and 18 are the commonest viruses worldwide associated with premalignant and malignant disease of the cervix. They have been found in a large number of genital cancer biopsies (Durst et al, 1983; Boshart et al, 1984; McCance et al, 1985; Prakash et al, 1985; Riou et al, 1985; Scholl et al, 1985; Lorincz et al, 1986; Villa & Lopes, 1986). Subsequently other viral types HPV 31 (Lorincz et al, 1986), 33 (Beaudenon et al, 1986), 35 (Lorincz et al, 1987), 39, 42 (Beaudenon et al, 1987), and 45 (Naghashfar et al, 1987) were identified in a lower percentage of genital cancer biopsies.

Papillomaviruses have also been found in cervical dysplasias by electron microscopy (Della Torre et al, 1978; Laverty et al, 1978; Morin & Meisels, 1980), by using species-specific antiserum (Ferenczy et al, 1981; Kadish et al, 1986) and by DNA hybridisation (McCance et al, 1985; Kadish et al, 1986). The types most frequently associated with preinvasive disease are HPV 6, 11, 16 and 18. The frequency of HPV DNA detection varies depending on the severity of the lesion, with HPV 16 being detected with increasing frequency as the severity of the lesion increases (McCance et al, 1985).

HPV16 DNA has also been found in the normal cervix. In the study by de Villiers et al (1987), normality was equated with normal cytology, while others have relied on cytology together with colposcopy (McCance et al, 1985; Meanwell et al, 1987). The latter study suggested that the prevalence of HPV 16 also increased with advancing age. The situation is further confused by the method of hybridisation, many of the larger studies have used a filter in-situ (FISH) method, which is not as reliable as the labour intensive Southern blotting method. Southern blotting sensitivity is such that it will detect an average 0.1-1.0 viral genomes per cell, DNA from approximately one million cells being required. The FISH method is much less sensitive, requiring at least some cells to contain one thousand viral genomes (Lancaster & Norrild, 1989). The specificity of both methods will be dependent on the stringency of the procedure, though in the case of FSIH both sensitivity and specificity may be affected by background contamination. With the advent of the polymerase chain reaction (PCR), the prevalence of any HPV DNA may be found to be, in varying amounts, almost ubiquitous (Young et al, 1989; Tidy et al, 1989). The value of these findings is, at present, unclear because of the possibility of contamination affecting the results of such a sensitive method.

In preinvasive disease of the cervix, the various DNA types are found mainly in episomal or extrachromosomal form and in high copy number (50 to >1000 copies per cell in the cells nearer the epithelial surface). They replicate free of the cellular chromosomes (McCance, 1988). By the time the disease has progressed to invasion, the viral DNA has been integrated into the cellular chromosomes of the host (Durst et al, 1985). The copy number in tumour cells is lower (1-100), though some of the DNA may remain free or episomal. This poses the question: is integration a cause or effect of malignancy?

The fact that the vast majority of biopsies from invasive tissue have detectable integrated sequences, would suggest that this is an essential step in the progression to malignancy, as viral DNA would be expected to be randomly lost from cells if it were unable to replicate in the undifferentiated cancer cell, or if other factors were of major importance in progression (McCance, 1988).

Even if papillomavirus is an important aetiological agent in tumorigenesis, it is unlikely to act alone in the immunocompetent host. This is reflected in the lag time between infection and malignant conversion, and the spontaneous regression of many primary lesions. A long latent period and the existence of cofactors (see section 1.14) in the development of malignancy are consistent with a multistep mechanism of carcinogenesis. A recent review by Munoz et al (1988), concluded that 'while experimental data suggest an oncogenic potential for HPV, the epidemiologic evidence implicating it as a cause of cervical cancer is still rather limited'.

1.9 CLINICAL ASPECTS OF HPV INFECTION

1.9.1 GENITAL WARTS (CONDYLOMA ACUMINATA)

Clinical warts on the external genitalia are the most obvious signs of HPV infection. The prevalence of the condition is difficult to assess as not all cases are notified. However, the minimum incidence of genital warts in the United Kingdom has more than doubled from 1971 to 1981, when they were diagnosed in 33,480 out of 523,319 new patients attending STD clinics (Report of the Public Health Laboratory Service, 1983). The difficulty in assessing the extent of the problem arises because many patients may never present themselves to a Genito-Urinary Medicine clinic but may be

treated by a gynaecologist, dermatologist or a G.P. Epidemiological evidence has shown a significant association between genital warts and cervical dyskaryosis (Franceschi et al, 1983; Walker et al, 1983). In a study in London on perianal and anal warts, HPV 6 DNA was found in 66% and HPV 11 in 17% of cases. HPV 16 was found in only 4.6% of specimens (McCance et al, 1986d).

As regards the cervix, overt condyloma acuminata were once regarded as one of the rarest of gynaecological disorders (Wharton, 1921). They are readily recognised as papillary epithelial proliferation, often with irregular vascular loops beneath the translucent surface epithelium (Coppleson, 1987). They appear in the transformation zone and ectocervix and are very often due to infection with HPV 6 and 11 (McCance, 1986). It is important to bear in mind that 20% of women with clinically apparent cervical condylomata will have coexistent cervical intraepithelial neoplasia, making biopsy of such lesions mandatory (Campion, 1987).

1.9.2 SUBCLINICAL LESIONS AND COLPOSCOPY

Most of the lesions on the cervix, however, are subclinical (Laverty et al, 1987; Meisels et al, 1977; Purola & Savvia, 1977) and only become visible colposcopically after the application of 5% acetic acid (Reid et al, 1982). Subclinical HPV infection is one of the commonest sexually transmitted diseases (Becker et al, 1987; Campion, 1989). It is identified colposcopically as either indistinct acetowhitening or a shiny snow-white lesion with irregular outline, whose margins are jagged, angular or feathered (Coppleson, 1987). Satellite lesions may be present which extend beyond the transformation zone (TZ). These lesions are flat, have the same histological features of exophytic warts and are found in the TZ as well as on the mature squamous epithelium of the ectocervix. The capillary

patterns may be pronounced and can often be confused with mosaicism and punctation, which is the hallmark of CIN 2-3 (Coppleson, 1987). The vascular pattern may show uniform, fine calibre vessels, loosely and randomly arranged, often as a horizontal mesh. Non-dilated loops may also run vertically towards the surface, maintaining a uniform vessel calibre throughout their course. Application of Lugol's iodine to the cervix may be positive or show partial staining, suggesting some glycogenation present within the epithelium, which is usually lost in the more severe degrees of CIN (Reid & Scalzi, 1985). These subclinical lesions are now usually referred to as human papillomavirus infection (HPVI), but were previously diagnosed as mild dysplasia (Meisels & Fortin, 1976). They are frequently benign proliferations though may mark exposure to higher risk HPV types (Koutsky et al, 1988). In approximately 40% of these flat condylomas, HPV 6 and/or 11 has been identified and HPV 16 has been detected in up to 17% of these lesions (Pfister, 1987).

1.10 DETECTION OF HPV

1.10.1 CYTOLOGY

As most HPVI is subclinical, an aid to diagnosis is the cytopathic effects of the virus, which can be identified on routine cervical cytology. Two abnormal types of cells are taken as diagnostic of the condition namely the koilocyte and the dyskeratocyte (Meisels & Fortin, 1976; Purola & Savia, 1977). The prevalence of HPV cytopathic effects is not exactly known, though Drake and colleagues (1984) found that 3.7% of routine cytology taken in the Australian state of Victoria showed these features.

The koilocyte is central to the cytological identification of HPVI of the cervix. The name was initially proposed by Koss and Durfee in 1956. The koilocyte is a squamous cell of superficial or intermediate type which may

or may not be enlarged. It usually contains one nucleus but may frequently be binucleate and occasionally multinucleate. The nuclear chromatin is usually dense and opaque in appearance, though may be dense and granular. The nuclei are surrounded by an irregular area that varies in size from a small halo to a large apparent space occupying most of the cytoplasmic volume. The cytoplasm which is peripheral to the clear area is dense and refractile and characteristically varies in staining reactions.

The dyskeratotic cells may be seen singly or as small aggregates with hyperchromatic, usually irregular nuclei. The cytoplasm frequently has a dense refractile eosinophilia.

There are other cytological features associated with HPVI and these include parakeratosis and hyperkeratosis, karyorrhexis (non-specific nuclear degeneration), binucleation and multinucleation, and perinuclear halos in immature cells may be seen in the parabasal or immature metaplastic cells (Drake et al, 1987).

These changes described above occur in the squamous cells, but changes can also occur in the endocervical cells that are typical of HPVI (Drake et al, 1987). These include:

1. Repair reaction with slightly enlarged nuclei and prominent, frequently multiple nucleoli. The nuclei are a similar size and shape with uniform thickness of the nuclear membranes and a relatively smooth outline.

2. Nuclear enlargement may occur within clumps of endocervical cells. The nuclei may be markedly enlarged and the cytoplasm may be conspicuously absent.

3. There may be nuclear crowding within the sheets of the endocervical cells, with no visible nucleoli and a complete absence of cytoplasm.

4. There may be marked nuclear abnormalities within the sheets of endocervical cells in which the large nuclei have blurred or granular chromatin showing nuclear crowding and some moulding.

The degree of nuclear atypia may be marked and lead to an erroneous diagnosis of dysplasia (Meisels et al, 1977).

1.10.2 HISTOLOGY

The main histological features of subclinical HPVI of the cervix are similar to the features seen on cytology. They are: koilocytotic atypia; individual cell keratinization; multinucleation; parakeratosis; acanthosis and papillomatosis (Kirkup et al, 1982; Meisels et al, 1982; Reid et al, 1982). The koilocytotic atypical cells have a prominent clear halo around the nucleus, which is hyperchromatic and irregular, (similar to the description in the cytology section above). These cells are usually in the upper layers of the epithelium, the viral induced changes not being expressed until the cell has achieved a certain level of maturity (Anderson, 1985).

1.10.3 DNA-DNA HYBRIDISATION

Problems arise with the detection of the papillomavirus as there are no serological tests for the individual viruses (Lorincz, 1987). The typing is confounded by an inability to isolate the virus from clinical specimens by cell culture (Butel, 1972). At present, hybridisation analysis of viral nucleic acid (DNA or RNA) is the best method available for diagnosis of HPV. These methods are highly sensitive and offer the only means of identifying

individual HPV types. The different types are defined according to their degree of homology under standardized hybridisation conditions (Coggin & zur Hausen, 1979).

The hybridisation refers specifically to the formation of double strands of nucleic acid from 2 single strands; one in the clinical material and one from the radioactively labelled probe. Hybridisation is controlled by the rate of formation and stability of the hybrid, which in turn depends on the reaction temperature, salt (sodium ions) concentration, DNA concentration, fragment length and base composition, and pH (Hames & Higgins, 1985).

The experimental conditions are usually set so that the hybrids are as stable as possible. After the hybridisation step has occurred, any remaining unbound probe is washed away. There is a similar organisational pattern of the viral genome and localised areas of homology exist between the different types. Alignment of HPV DNA sequences reveals that they have similar genetic organisation of protein coding potential, recognised as open redaing frames (ORF's) (Broker, 1987). The degree of cross-reactivity depends upon the relatedness or homology of the DNA strands and the stringency under which the method is performed.

Stringency relates to the degree of discrimination obtained between imperfectly matched hybrids through the careful criteria chosen in the hybridisation and wash conditions. Mismatch in the hybrids is associated with instability and as conditions become more stringent only very close matches will survive. By manipulating the temperature and/or the salt concentration, the conditions of stringency can be chosen (Lorincz, 1987).

Nick translation is a method of preparing the radioactive probes for the hybridisation methods (Rigby et al, 1977). Initially, multiple nicks are introduced into an intact double strand of DNA by a method of controlled digestion with a DNA degrading enzyme. A second enzyme with a nucleotide polymerizing activity uses the 'nicked' DNA as a substrate to catalyze the substitution of radioactively labelled mononucleotides into one strand of the DNA (Cunningham & Mandy, 1987). The other strand of the DNA acts as a template to allow synthesis of a radioactive double stranded molecule that is a faithful copy of portions of the input DNA. The probes are usually labelled with isotopes of phosphorus, sulphur, iodine or hydrogen. This method has been simplified and superseded by the development of random primer synthesis (Feinberg & Vogelstein, 1983) and the manufacture of oligolabelling kits by commercial companies.

These probes are added to the nitrocellulose filters which contain the clinical specimen DNA (Meinkoth & Wohl, 1984). The cellular material is trapped in the nitrocellulose filter, which is denatured with an alkali solution, and the DNA is then baked into the filter. Extensive washings take place to remove all the unhybridised probe before the filters are placed on X-ray films for autoradiography. Nitrocellulose filters are brittle and allow only one probing, whilst the newer nylon filters can be re-used.

1.10.4 SOUTHERN BLOTTING HYBRIDISATION

This is a technique for transferring nucleic acid fragments from an agarose gel to nitrocellulose filters (Southern, 1975). It is the most specific and sensitive method of nucleic acid hybridisation and is regarded as the 'gold standard' for the detection of the individual HPV DNA genome (Lancaster & Norrild, 1989). Purified DNA is digested with restriction endonucleases and the DNA fragments are separated by electrophoresis

through an agarose gel (Sealey & Southern, 1982). The DNA fragments are denatured in situ and transferred by capillary action to the nitrocellulose filter. High molecular weight DNA fragments transfer poorly, so depurination of the DNA in situ (before denaturation and transfer) can increase the ultimate signal strength. The DNA in the gel is exposed to weak acid (which results in partial depurination), followed by a strong base (which hydrolyzes the phosphodiester backbone at the sites of depurination). The resulting fragments of DNA (approximately 1kb in length) can then be transferred rapidly from the gel wih high efficiency. The membranes are prehybridised with non-specific DNA to reduce any non-specific binding of the probe to the membrane (Denhardt, 1966). The main drawback of this procedure is due to its labour intensive nature and it may take up to two weeks to obtain a result.

1.10.5 FILTER IN-SITU HYBRIDISATION

This method was developed as a more simple and rapid method of detecting HPV DNA without the need for DNA extraction and purification (Wagner et al, 1984). Cytological samples are collected in a phosphate buffered saline solution and the cells drawn through a nitrocellulose membrane by suction. The cells, which are thus trapped, are lysed and denatured by alkali before undergoing hybridisation. This method has been used in epidemiological studies (de Villiers et al, 1987), but problems arise because the FISH method is prone to false positive results. A substantial amount of cellular debris and mucus may trap the probe, making assessment of the autoradiographs difficult. It is, therefore, important to use known positive and negative controls during the hybridisation process.

At the outset of the project, the FISH method was used as it was relatively quick and easy to perform. It was regarded as giving good results especially where high positive rates were expected. It has been used and

continues to be used for large scale studies (Wagner et al,1984; de Villiers et al, 1987; Campion et al, 1986; Reeves et al, 1987; Kjaer et al, 1990).

1.10.6 POLYMERASE CHAIN REACTION (PCR)

This method has been developed over the past few years and is a way of amplifying specific target DNA sequences using primer directed enzymes, namely Taq polymerase. The advantage of this enzyme is that it is thermostable (Erlich et al, 1988; Saiki et al, 1988). The sensitivity of the polymerase chain reaction (PCR) is 100,000 times that for Southern blotting, so that a single HPV DNA molecule can be detected in 100,000 cells. The problem with such sensitivity is the risk of even the minutest amount of contamination affecting the results. The specificity of the reaction depends on two oligonucleotide primers that flank the HPV DNA fragment due to be amplified. Denaturation and re-annealing of the primers to their complementary sequences are repeated sequentially using Taq polymerase. Each cycle doubles the amount of target DNA. A recent study by Young et al (1989) found HPV DNA types 11 and 16 in all 38 women with a cytological abnormality and in 7 out 10 women with normal cytology, and Tidy et al (1989) found a prevalence of HPV16 in over 80% of his women with normal cytology. In a larger study using PCR, Melchers et al (1989b) found that HPV was present in 56 out of 80 women (70%) with an abnormal smear. The frequency with which it was found increased as the severity of the cytological abnormality increased. However, in their control group, HPV could only be identified in 5% of cases using the same method. The value of PCR in prevalence studies, therefore, remains uncertain because of its extreme sensitivity and the problems of contamination.

1.11 CERVICAL INTRAEPITHELIAL NEOPLASIA

The adoption of the term Cervical Intraepithelial Neoplasia, abbreviated to CIN (Richart, 1967), has now superseded the previous terminology of dysplasia introduced by Reagan et al (1953). Premalignant disease of the cervix is confined to the area superficial to the basement membrane of the squamous epithelium. It has been recognised that between 45-70% of cases of carcinoma-in-situ/CIN3 may eventually progress to invasive cancer (Kottmeier, 1961; Fidler et al, 1968) and that spontaneous regression at this stage rarely occurs. Biopsy (Koss 1978) and size (Richart 1980) of the lesion may affect the natural history of the lesion whatever its grade of severity.

In CIN, the full thickness of the epithelium is occupied by neoplastic cells, though differentiation may be present in the upper part of the epithelium. CIN is usually graded in terms of the proportion of the epithelial thickness occupied by undifferentiated neoplastic cells of the basaloid type and the presence of abnormal mitotic figures (Buckley et al, 1982).

As a rule of thumb, in CIN 1, the cells throughout the epithelium show nuclear abnormalities, though the cells in the upper and middle thirds of the epithelium undergo cytoplasmic differentiation. The nuclei tend to have prominent nucleoli, are pleomorphic, irregular in outline, enlarged and hyperchromatic, with coarse granular or filamentous chromatin pattern. The cells in the lower third, or less, of the epithelium show no evidence of any cytoplasmic differentiation or ordered stratification; they lack clearly defined boundaries and have a high nucleocytoplasmic ratio with nuclear crowding. In comparison, the rest of the epithelium shows evidence of stratification, cytoplasmic maturation and decreasing nucleocytoplasmic ratios.

CIN 2 has similar features within the lower and middle thirds of the epithelium. In CIN 3, these features occupy two-thirds or more of the full thickness of the lining with abnormal mitotic figures frequently seen in the upper one third. Variants of CIN 3 do exist and may reflect their site of origin within the TZ from the endocervical region to the ectocervical region (Buckley et al, 1982). Problems can be posed by other epithelial features, namely atrophic squamous epithelium and immature squamous metaplasia.

CIN is generally asymptomatic and naked eye examination of the cervix may be unrewarding. Diagnosis stems from the use of exfoliative cytology as a screening method introduced by Papanicolaou (1949). The nuclear appearance of the dyskaryotic cell distinguishes it from the norm. The nucleus is large, hyperchromatic, with an irregular chromatin pattern showing granular condensation. The degree of cytoplasmic differentiation is important in reflecting the final assessment of the smear. In cells with no cytoplasmic differentiation, there is a consequent high nucleocytoplasmic ratio. The interpretation of the cervical smear depends upon an assessment of the relative numbers of different types of dyskaryotic cells present and it is usual to report the most severe histological lesion detected.

Agreement between the smear report and histological diagnosis has been reported to be as high as 80% (Husain et al, 1974). However, problems in reporting may be due to variable exfoliation, especially with a small lesion, reactive changes due to infection e.g. HPVI; reactive changes due to trauma, e.g., IUCD presence; the presence of immature metaplastic and

reserve cells, and finally, atrophy. This last feature may make the accuracy of reporting cytology from post-menopausal women especially difficult.

1.12 THE NATURAL HISTORY OF CIN

HPVI of the cervix, which histologically has no dysplastic cells present, appears to represent the earliest stage in the CIN continuum and could be regarded as the earliest cervical lesion with the potential for malignant change (Mitchell et al, 1986; Reid et al, 1984). Mitchell and her colleagues (1986) followed a group of 846 women with cytological evidence of HPVI alone. Thirty of these women developed CIN 3 over the next 6 years compared to an expected figure of 1.9 for a similar sized group in the normal population. This gives a Relative Risk (RR) of 15.6 for HPVI positive women developing CIN 3, compared to the general population. CIN is a readily treatable condition but knowledge of progression is very limited due to ethical problems in progression studies, especially when the end result is cancer.

Evidence for progression comes from several studies (Evans & Monaghan, 1985; Syrjanen et al, 1985). Evans and Monaghan demonstrated that 16% of their group with histologically proven cervical subclinical HPVI progressed to CIN 2-3, including one case of microinvasive cancer, within a 12 month period. Syrjanen and colleagues found a similar progression rate in their study of subclinical HPVI. Their group size was 343 compared to 51 in the Gateshead study. The regression rate was 25% in the Finnish study compared with 46% in the other one. Sixty-one percent of these lesions persisted, compared with only 38% in the smaller study of Evans.

The progressive potential of mild cervical atypia was studied by Campion et al, 1986. They found that in their study population of 100 patients, 26(%) progressed from CIN1 to CIN3 within a 2 year period; spontaneous regression occurred in 11(%) cases, though the disease recurred in 4 of this group. HPV16 was identified by filter in-situ hybridisation in 39(%) of cases. In the patients whose disease progressed, 22 (85%) were positive for HPV16 at the outset of the prospective follow-up. The criticism of this study is the lack of initial biopsy to confirm the diagnosis. Colposcopic diagnosis can be difficult (Barrasso et al, 1987) and from the descriptions quoted earlier, a histologic diagnosis cannot be made from colposcopic findings alone. Another criticism used is that a more severe grade of disease was present at the outset of the study. The problem is also aggravated by the fact that, should a biopsy have been taken, then the natural history of a lesion may be affected by removal of tumour load and/or induction of local immune mechanisms to facilitate regression of the lesion.

The most convincing evidence for progression of premalignant disease comes from the results of a long term follow-up of women with carcinoma-in-situ (CIS) in New Zealand (McIndoe et al, 1984). In the study, 948 women were closely monitored for a period of 5-28 years. All the women received some form of therapeutic procedure ranging from punch biopsy and cone biopsy to hysterectomy. In the larger group of 814 women who had a cytological return to normal, 1.5% (12) women developed invasive disease between 4-19 years later (median time of 9 years). In the other group of 131 women, whose cytology remained abnormal, 22% (29) developed invasive disease, with 69% (90) having persistent CIS. These final diagnoses were made at least one year after the initial diagnosis (range 1-19 years, with a median time of 6 years). The authors concluded that the

'study clearly demonstrates that CIS of the cervix had a significant invasive potential'. Most evidence suggests that progression occurs over a period of 3-10 years from CIN 3 to invasive disease (Richart & Barron, 1969), with a few cases of spontaneous regression. It is unfortunate that two terminologies have been used but the CIN classification has now superseded the dysplastic one, CIN3 and carcinoma-in-situ being synonymous (Richart, 1990).

The number of women with HPVI and minor grade CIN lesions is very large compared to those developing cervical cancer. This suggests that many early lesions have little or no malignant potential. Kinlen and Spriggs (1978) traced 52 women who had had abnormal smear results at least two years prior to the study and had been lost to the follow-up system for a number of reasons. Ten of these women (19%) had developed invasive disease, while 19 (37%) had no apparent abnormality, the rest of the women had evidence of premalignant disease of varying grades. Galvin et al (1955) found that the rate of progression depended on the severity of abnormality, with the milder abnormalities having a greater chance of regression. The fact that not all CIN lesions progress to malignancy even when HPV is present suggests that other factors may be important.

1.13 KOCH'S POSTULATES

With any infectious agent, it is pertinent to examine the Henle-Koch postulates (Brock, 1988) and assess whether these may be fulfilled. They are:

1. The organism must be found in every case of the disease.

2. The organism must be isolated and cultured repeatedly in a pure form in-vitro.

3. Infection of a healthy individual with the purified organism should cause an identical illness to that seen in the individual from whom the organism was isolated.

4. The organism can then be reisolated from that individual.

The problem with fulfilling these criteria arises from the fact that they were initially applied to bacterial infection. With viral diseases, difficulty arises in the isolation and culturing of viruses. It is a little misleading to try to apply the postulates to a potentially oncogenic virus for the following reasons:

1. It is optimistic to expect to find the oncogenic agent responsible for a tumour in every case, especially after millions of divisions and several further mutations.

2. At present there is no system available to culture the papillomavirus and it is impossible to isolate live particles in-vitro.

3. It would be unethical to expose someone to such a virus.

The equation of HPV plus cervix equals neoplastic disease may, therefore, be dependent on cofactors including sexual behaviour (age of first intercourse and number of partners), smoking, oral contraceptive pill exposure and history of other sexually transmitted diseases. Prospective follow-up of women who have a normal cervix and are positive for HPV16 DNA may shed light on some of these variables.

1.14 RISK FACTORS FOR CIN.

Many studies (Thomas, 1973; Meisels et al, 1977; Harris et al, 1980; Fasal et al, 1981; Buckley et al, 1981; Lyon et al, 1983; Trevathan et al, 1983; La Vecchia et al, 1986a & b; Rawls et al, 1986) have looked at the epidemiological features of preinvasive disease and found that they are similar to those of invasive disease. The main factors that appear to be important in the development of cervical neoplasia include:

1. sexual history - age of first intercourse (coitarche) and number of sexual partners;

2. partner's sexual history;

3. smoking;

4. contraceptive methods.

There are other features which have been considered in the epidemiology of cervical neoplasia and these include: obstetric history, partner's occupation, diet (Marshal et al, 1983; Brock et al, 1988) and genital infections other than HPVI. These features will be discussed with regard mainly to the development of preinvasive disease, with comparison to invasive disease where it seems appropriate. Definition of relative risk (RR) can be found in Section 2.13.

1.14.1 SEXUAL FACTORS

The evidence of an association between cervical cancer and sexual activity was reported as early as 1842 by Rigoni-Stern, who showed the increased frequency with which these tumours occurred in married women compared to nuns and single women, implying a possible protective effect of celibacy. More recently, epidemiological studies have shown that cervical cancer behaves in a similar manner to a sexually transmitted disease (Beral, 1974). Many studies have been published looking at the age of first intercourse, number of partners and age of first pregnancy in relation to the development of CIN and invasive cancer (Harris et al, 1980; Clarke et al, 1985; Reeves et al, 1985; La Vecchia et al, 1986b; Brinton & Fraumeni, 1986). Most of these studies agree that the epidemiological features of both preinvasive and invasive cancer of the cervix are similar. In Rotkin's study (1967), which looked at 416 women with invasive disease

and compared them with a matched control group of equal size, it was found that the age of first intercourse was the variable with the greatest discriminatory value. Twice as many patients as controls began coitus between the ages of 15-17 years, comparatively few began coitus after the age of 21 years and almost none as late as 27 years. He also found that parity and gravidity showed only a borderline difference between the two groups.

Meisels et al (1977) reviewed a much larger population: 84,540 women with no lesion, compared to 2017 women with mild/moderate dysplastic changes on the cervical smear test. These would probably be equivalent to CIN 1-2 lesions in today's nomenclature (CIN for histology and dyskaryosis for cytology). They found highly significant correlations between early onset of sexual activity and the occurrence of dysplasia and also between early age of first intercourse and oral contraceptive pill (OCP) use. The mean age for dysplastic lesions was 30.5 years, for CIN 3 36.8 years and for squamous carcinoma 49 years. This suggests a progression in severity of the lesion with time. These figures, as already discussed, have changed over the decade since their publication.

Harris et al (1980) looked at a group of 237 women with abnormal smears plus a control group of almost double the size. The criticism of the study was that they drew on gynaecological outpatients for their control group. This may not be a truly representative sample of the normal population. Nevertheless, they found that the risk of developing an increasingly severe CIN lesion was correlated with an earlier age of first intercourse. If the first intercourse was earlier than age 17 years, then the risk of dysplasia was increased by a factor of 2-3 compared to women whose coitarche was after 21 years of age. The risk of developing a cervical abnormality also increased with the number of sexual partners. Indeed, the

effect of coitarche disappeared after adjusting for the number of sexual partners, though the reverse was not true. Other studies have found a significantly increased relative risk for the number of sexual partners and age of first intercourse after adjusting for sexual variables in dysplasia (Clarke et al, 1985; Reeves et al, 1985 & La Vecchia et al, 1986b) and in invasive disease (Brinton et al, 1987; Reeves et al, 1985 & La Vecchia et al, 1986b).

A further study from Brinton et al (1987) looked at invasive cancer patients (418) and a large group of controls (704). It was found that there was no association between the age of menarche and menstrual irregularities in these groups, but that there was a significant risk of disease if the coitarche was before the age of 20 years. It was noted in this study that there was no apparent relationship between disease, use of tampons and genital hygiene.

Data regarding the age at first pregnancy is difficult to interpret as it may reflect the age of coitarche. Fasal et al (1981), looking at premalignant lesions in a large population (34,000+) showed no risk of disease when looking at the mean ages at first pregnancy. In a study by Thomas (1973) from Baltimore , there was a greater risk of CIN 3 in women who had had a pregnancy before the age of 20 years. Harris et al (1980) found no relationship between age of menarche or age at which the first pregnancy ended and dysplasia. The relative risks in all categories of dysplasia were lower with a late pregnancy than with an early one. They found no relationship to the number of pregnancies though nulliparous women were at lowest risk of dysplasia. Women with a pregnancy out of legal marriage had a relative risk (RR) of 2.6 for CIN 3 and women who had

had a termination of pregnancy had a significant (RR) of 2.8 for mild dysplasia only.

In Brinton's study (1987) on invasive disease, there appeared to be no increased risk with parity or gravidity, likewise there was no risk associated with mode of delivery, though multiple births seemed to increase the risk of disease significantly after 5 deliveries. In this study, early age of first birth was an important predictor of risk, but was found to reflect correlation with the number of births. After adjustment for parity, the effect of early age of first birth was no longer significant. It is possible that the age of first pregnancy reflects the age of first coitus, the non-use of contraception and social class.

Much has been written about the multiplicity of sexual partners and evidence suggests that, with an increased number of partners there is an increasing risk of cervical disease. This effect has been mentioned earlier (Harris et al, 1980; LaVecchia et al, 1986b). Schachter (1984) reported that the prevalence of CIN in young Israeli born women was approaching Western populations. Disease had previously been low in the Jewish population, suggesting that as their sexual mores were changing to a more permissive pattern, then this may explain the increased incidence of cervical lesions in these young women. This evidence lends further support to the idea of a venereal transmission of the disease. The association between increasing number of partners and disease suggests that some agent may be passed to the female at the time of intercourse, which causes or makes her cervix more susceptible to neoplasia.

However, it is salutory not to forget Gagnon's (1950) painstaking work looking through the medical record of nuns, which found 3 cases of

cervical cancer, though he did have some problems with the notes. Another study by Towne (1955) also found 6 cases of cervical carcinoma in apparently virginal women. Unfortunately, it is difficult to decide whether these tumours were adenocarcinoma or squamous in origin, but it would suggest that other factors may be responsible.

1.14.2 MALE FACTOR

It is easy to attribute cervix cancer entirely to female sexual behaviour, but it would seem pertinent to discuss the concept of the 'high risk male'. This term was proposed in 1976 (Singer et al) as someone whose female partners were at an increased risk of developing cervical neoplasia. In a well controlled study by Buckley et al (1981), which was a follow-up on the study of Harris et al (1980), husbands of women with CIN/cancer, who had reportedly had no other sexual partners, were interviewed. Despite the relatively small group (31 in all), compared to a similar sized control group, the study demonstrated an increased risk (RR) of 7.8 for disease in women whose partner had had other sexual partners (>16) and an earlier age of first intercourse. These men were also more likely to have had a venereal disease (RR 3.36), visited prostitutes (RR 2.97) and had extramarital affairs (RR3.32). These features were confirmed by Zunzunegui et al (1986), who showed in a Hispanic population in Los Angeles that a man increases his wife's risk of developing carcinoma by a factor of 5 if he has had more than 20 partners.

Skegg et al (1982) suggested that male sexual behaviour is important and discussed this in relation to Latin America, where the incidence of cervical carcinoma is high. This may be related to the accepted use of prostitutes to gain sexual experience as the women are expected to remain chaste until marriage. In a study by Alzate (1977) looking at the behaviour

of male medical students in Colombia, 91% had had coitus, 92% of this group admitted to using prostitutes and 65% had had their first sexual experience with a prostitute. It may be that the prostitutes are a reservoir for the mutagen which is then passed to the wives, causing a high incidence of cervical neoplasia.

Another significant fact suggesting a venereal aetiological agent comes from Smith et al (1980), who looked at 711 men who had died from carcinoma of the penis and showed a statistically significant excess ($p=0.002$) of cervical carcinoma amongst their partners, suggesting a common aetiology. In a much smaller study, Campion et al (1985) examined 25 women who had been the sole sexual consort for at least a year, of men with penile condylomata. Nine (36%) of these women had evidence of an abnormality on the cervix (HPVI/CIN) as judged by cytology, colposcopy and histology, whereas none of the age matched controls had any evidence of a cervical abnormality. The inference was that penile HPV infection places the woman partner at risk of cervical neoplasia.

1.14.3 SMOKING

Winkelstein (1977) proposed the hypothesis that smoking affects the risk of developing cervical neoplasia from the observations that:

1. cervical cancer incidence correlates with the distribution of other smoking related cancers;
2. cigarette smoking is associated with the development of squamous types of carcinoma;
3. certain studies seemed to support such a relationship.

A Swedish study illustrates the third observation. It involved recruiting 55,000 subjects, of which 27,732 were female. It was found that

there was a relative risk (RR) of 7.2 for uterine cervix cancer amongst women who smoked more than 15 cigarettes per day (Cederlof et al, 1975). Trevathan et al (1983) demonstrated a cumulative dose dependent effect, whereby the relative risks (RR) were shown to be 12.7 for CIN 3, decreasing to 4.2 for mild disease when cigarette smoking exceeded 12 pack years. (Pack year is defined as number of cigarettes smoked per day multiplied by the number of years smoked, the total being divided by 20.) The risk seemed greatest in women who had commenced smoking during their teenage years. La Vecchia et al (1986a), in their study looking at preinvasive as well as invasive disease, confirmed that there was an increased risk in developing disease in smokers, that this was related to the amount smoked and that the risk was increased in women who had started to smoke at an early age. They also found an apparent synergistic effect between smoking and the number of sexual partners, though this was not statistically significant.

These findings were confirmed by Nischan et al (1988), who found that the unadjusted relative risk of invasive cervical carcinoma in a group of 225 patients was significantly elevated for women who had ever smoked. They found the crude relative risk (RR) for invasive carcinoma in smokers to be 1.54, and in this series the risk depended significantly on the number of sexual partners.

The possibility that cigarette smoke constituents affect the cervix, either directly as mutagens or indirectly by affecting local immunity, is a topic of much interest (Winkelstein et al, 1984). It has been shown that nicotine and cotinine are found in the cervical mucus (Sasson et al, 1985; Hellberg et al, 1988). These may not be the carcinogenic agents but may be markers for other agents that also concentrate locally and have a more

deleterious effect. Langerhans' cells are the main antigen presenting cells in the cervical epithelium (Tay et al, 1987). There is a depletion of these cells in both HPVI of the cervix and CIN. Barton et al (1988) showed that women who smoked cigarettes have significantly reduced numbers of Langerhans' cells in both the normal and diseased cervix. This may provide the key to the role of smoking in the aetiology of cervical neoplasia; local immunosuppression may permit the persistence of viral infection and affect the regulation of neoplastic changes.

1.14.4 CONTRACEPTION USE

The relationship between oral contraceptive pill (OCs) use and the development of cervical neoplasia has been studied and reviewed extensively (Thomas, 1972; Harris et al, 1980; Swann & Pettiti, 1982; Clarke et al, 1985; Brinton et al, 1986; La Vecchia et al, 1986b; Vessey, 1986, & Beral et al, 1988). Problems arise in these studies because of difficulties in adequately controlling for possible confounding factors such as coitarche, number of sexual partners and smoking history. Because these variables are strongly related to CIN and also to OCs, it will be very difficult to detect a small to moderate direct effect of OCs. It must also be remembered that OCs use is associated with an increased incidence of eversion of the endocervix (Jordan, 1985) and so make abnormalities easier to detect.

Meisels et al (1977) found an excess of OCs users in the CIN group which was confirmed by Fasal et al (1981), who also found that the diaphragm had a protective effect on dysplasia. Harris et al (1980) agreed with these findings; they controlled for diaphragm use and still found that OC users had higher risks than women who had not used barrier methods of contraception, though the numbers in the study were small. In dysplastic lesions, Clarke et al (1985) found that the use of OCs increased the risk of

CIN when adjusted for coitarche and smoking history, but was no longer statistically significant when adjusted for number of partners.

A WHO study (1985) showed that there was a relative risk of 1.19 in women who had ever used OCs and the risk increased with the duration of use, giving a relative risk of 1.53 after 5 years. However, they did comment that the paper supported a causal interpretation, though qualified it by saying that this 'could be due to incomplete control for confounding sexual variables and other sources of bias'. This increased risk has been reported with long term usage >5 years (Brinton et al, 1986); >7 years (Ebeling et al, 1987); >10 years (Beral et al, 1988). Vessey et al (1983) in a prospective study of all grades of cervical neoplasia, showed that the incidence of disease rose from 0.9/1000 woman years in those with up to 2 years OC use, to 2.2 in those with more than 8 years use. It was particularly worrying that all the cases of invasive disease occurred amongst the OC users. However, Celentano et al (1987), in a study of 153 women with invasive disease and matched controls, found that the use of OCs, diaphragms and spermicides was more frequent in the controls than the cases. They concluded a protective effect of lifetime use of OCs as well as a possible beneficial use of spermicides. The protective effect of the diaphragm in all grades of cervical neoplasia has also been reported by Wright et al (1978).

1.14.5 SOCIAL CLASS

It has been accepted that cervical cancer is much more common in women of low social class (OPCS, 1981) and the risk among women in the lowest social class is about five times that amongst the highest socioeconomic class (Brinton, 1986). This may be due to differences in sexual behaviour, hygiene, cigarette smoking and contraceptive practices, although this has been challenged by Brown et al (1984).

Statistics on cervical cancer and social class are fraught with problems. In the OPCS (Office of Population and Census Surveys) reports for England and Wales, the social class breakdown for cancer of the cervix is difficult to interpret, mainly due to a lack of registrable information. Nevertheless, there seems to be an almost linear relationship between the standard mortality rate from cervix cancer and social class. This class difference may be changing with increasing social mobility and we may find it is more related to the woman's educational attainment.

In a report in 1983, Robinson highlighted the increase in standard mortality rates (SMR) in the textile towns in England. It had been observed in the 1950's (Stocks) that there was a high cervical mortality rate in these textile towns, but it was not until the 1970's that further interest in this aspect of the problem was shown. Robinson (1983) showed that the SMR was related to the partner's risk of exposure to carcinogens as a result of his job, e.g., dust, metals, chemicals, tar and machine oils. This risk was greater in these workers than in other members of the same social class doing jobs in which exposure was not evident. Beral (1974) almost ten years earlier, looked at men whose jobs were related to prolonged absence from home, which may make them more likely to have extramarital sex and developing a venereal disease and so place the female partner's cervix at an increased risk of exposure to a sexually transmitted agent.

1.14.6 OTHER INFECTIOUS AGENTS.

During the 1970's, interest concentrated on Herpes simplex virus (HSV) as an aetiological factor for cervical neoplasia. Seropositivity for HSV 2 in cancer of the cervix patients was as high as 90% and as low as 30% in some series. With the advent of molecular biology, HSV nucleic acid was not consistently demonstrated in cervical neoplastic lesions (Maitland,

1988). Vonka et al (1984) in Prague followed a large number of women (10,000) over a 10 year period, comparing serological status for HSV. They concluded that there was not an increased risk for the development of cervical neoplasia in the seropositive group.

Investigations into the relationship of other genital infection to neoplasia have shown varying associations. LaVecchia et al (1986) showed an association between Trichomonas vaginalis and candida infections and CIN, but not in invasive cancer. Schachter and colleagues (1982) found an increased risk of cervical neoplasia in women with antibodies to Chlamydia trachomatis. However, Brinton and her colleagues (1987) could find no significant association with any specific genital infection in their case-control study of invasive cancer. In a small case control study of CIN lesions (Guijon et al, 1985), there was no association of the lesion with any of the following genital infections: HSV2, CMV, Chlamydia trachomatis, Neisseria gonorrhoea, Gardnerella vaginalis, Trichomonas vaginalis, Ureaplasma urealyticum, Mycoplasma hominis, Candida albicans and other yeasts. Although these infections cannot be overlooked, their significance remains uncertain.

SECTION TWO

MATERIALS & METHODS

2.1 STUDY DESIGN

AIMS:

1. To determine the risk of CIN, especially CIN 3, in women with negative cervical cytology and a colposcopically normal cervix, who have been found to be positive for HPV 16 DNA on filter in-situ hybridisation (FISH).

2. To examine the relationship of smoking and oral contraceptive use to the prevalence of HPV infection and the modifying effects of these agents on the progression of HPV infection to CIN 3.

These first two aims of the study were achieved by screening women who came to their family planning clinic for cervical cytology. Cases were defined as women who were positive on DNA hybridisation but negative for any degree of dyskaryosis. These cases were examined colposcopically on a 6 monthly basis over a two year period from their initial screening. A control group was also recruited who were similar cytologically and colposcopically normal but were negative for HPV 16 DNA.

3. To compare the results of FISH on cells from cervical scrapes to those of Southern blot hybridisation on similarly obtained samples. In order to achieve this aim, cell scrape samples, deposited into a phosphated buffered saline solution, were divided into two equal aliquots. One half was used for the FISH method and the other half was used to extract the DNA from the cells for Southern blotting.

District Ethical Committee approval was obtained from the committee for Clinical Research in the Islington Area Health Authority ref: EC 87/14.

2.2 GENERAL SCREENING.

In order to obtain the population of women for the study, a general screening programme for HPV 16 was introduced within the Islington District.

The main aim of the screening plan was to include those women who were attending their Family Planning Clinic or local General Practitioner for a routine cervical smear test. Each woman was informed of the study and a letter was sent giving details of the study and describing the nature of colposcopy. If the woman accepted the invitation, she was given a brief questionnaire (see Appendix) which she completed giving details of name, address, date of birth, current smoking and contraceptive history. It was felt that any further information at the initial screening in a busy clinic would be difficult to obtain. The main exclusion criteria was a history of previous abnormal cervical cytology or therapy for cervical disease.

At the time of routine cytology, the cervix was exposed using either a Cuscoe's (bivalve) or a Sim's speculum. An Ayres's or Aylesbury wooden spatula was used to obtain cell scrapes from the cervix by sweeping the spatula through 360^{o}. The cytology slide was obtained initially (2.5) and the remaining cells on the spatula were washed in a sterile universal container holding a 10ml aliquot of phosphate buffered saline solution (PBS). A further scrape was taken with another spatula and this was washed in the same sample of PBS. Both spatulae were agitated vigorously in the solution in order to release the maximum number of cells. The container was labelled with the patient's name and stored in the refrigerator or freezer (if available), from which it was collected daily.

The specimens were then processed for filter in-situ hybridisation at Guy's Hospital Medical School in the Department of Microbiology. If the samples were unable to be placed on the nitrocellulose filters immediately, then they were stored at -20^{o}C.

The Family Planning Clinics were situated at various locations within the Islington District and included:

Finsbury Health Centre, Pine St, E.C.1.

Manor Gardens Health Centre, N7.

Hornsey Rise H.C., Hornsey Lane, N19.

Highbury Grange H.C., N5.

Riverplace H.C., Essex Rd, N1.

Barnsbury H.C., N1.

Goodinge H.C., N7.

To prevent bias, various clinics were used at differing times throughout the days of the week.

The G.P. Practices involved were:

The Highgate Group Practice, N6.

The Highbury Group Practice, N5.

Dr. J. Chomet, Crouch End, N4.

The cytology slides were read at the Royal Northern Hospital, Holloway Rd, N7, as part of the routine work load (2.5). Women with abnormal cytology were referred for colposcopy to either the Royal Northern Hospital Colposcopy Clinic or sent for review at the Finsbury H.C. Colposcopy Clinic (2.3). A number of these women with abnormal cytology attended private colposcopists or were lost to follow-up despite repeated tracing. These will be presented in the results section.

The case group included women who were cytologically negative but positive for HPV 16 on FISH and they were invited to attend for colposcopy at Finsbury H.C. Colposcopy Clinic (2.3) within six months of their original screening. The control group of a similar number of women, who were cytologically negative as well as negative for HPV 16 on FISH, were also invited for colposcopy. At their initial visit, a more detailed questionnaire was completed and further information and advice was given as required.

Each woman was informed of her results by post and a further appointment was sent for a follow-up visit 6 months later. It was planned to screen each woman every 6 months for a period of 2 years from their original screening. Attendance was not always feasible because of pregnancy, domestic, family and job commitments. In a number of cases, the woman became pregnant and so appropriate antenatal and postnatal follow-up was arranged.

2.3 COLPOSCOPY CLINIC

1. The Clinic

In order to examine our population, a colposcopy clinic was started at the Finsbury Health Centre, Pine Street, London, E.C.1. It was situated next to the Family Planning Clinic (FPC) with which it ran concurrently. It was considered that the patients would feel more relaxed in this setting than in a routine hospital colposcopy clinic and so compliance would be greater. Apart from the study population of patients with normal cytology, a service commitment was undertaken of patients with minor cytological abnormalities who had been screened in the FPCs within Islington. A number of women were seen as self-referrals and, if they fulfilled the appropriate criteria,were included in the study popultation.

2. Counselling and Examination

The initial clinic visit of each patient was divided into three main parts:

1. A full explanation of the project and the patient's involvement was given, together with details of what a colposcopy entailed. A detailed history was taken and recorded (questionnaire in appendix).

2. The woman was asked if she had any questions or worries, which were duly answered.

3. Colposopy was then performed, together with repeat cytology and a further sample for filter in-situ hybridisation. The findings were fully explained and the woman informed that her cytology and biopsy results, if taken, would be sent to her directly. Arrangements were made for follow-up appointments once these results were reviewed. At each subsequent visit, repeat cytology, colposcopy and sample collection for FISH were performed.

3. Records and Results

The information about each woman was recorded on individual record sheets, which consisted of three history sheets and one for examination. The initial questionnaire was completed by the woman before details of her gynaecological, sexual and smoking history were taken. An examination sheet was used for documentation of the colposcopy, with a tentative diagnosis added. Any colposcopic abnormality was biopsied and the final diagnosis was based on the histological report of the lesion. Examples of these sheets are included in the appendix.

4. Treatment of Abnormalities

If cervical intraepithelial neoplasia was reported on the histology report, arrangements were made for laser therapy to be undertaken at the Royal Northern Hospital, London, N.7. This treatment involved either laser vaporisation or laser cone biopsy, which was undertaken using a local anaesthetic. The guidelines of the RCOG were followed regarding suitability of an individual for local ablative therapy (Ninth Study Group, RCOG, 1981). If the patient was considered unsuitable for an outpatient procedure, arrangements were made for admission to have treatment under a general anaesthetic. The RCOG guidelines are:

1. The patient is seen and assessed by an expert colposcopist;

2. The expert colposcopist can see the entire lesion, i.e. see the squamocolumar junction;

3. The exert colposcopist must exclude invasive carcinoma by biopsy or biopsies;

4. The expert colposcopist must perform the laser vaporisation himself;

5. There must be good cytological and colpsocopical follow-up.

2.4 SAMPLE COLLECTION

Cytological scrapes (2.5) and microbiological specimens (2.6) were obtained before detailed colposcopic examination was performed. Colposcopic examination was performed using a Zeiss colposcope (Oberkochen, W.Germany) fitted with a green filter and a variety of lenses for magnifying the cervix from 6-40 times. The cervix was cleaned of any remaining mucus or debris before being painted with a 5% w/v solution of acetic acid. This was achieved initially by soaking a cotton wool ball (held by a pair of sponge holding forceps) in the acetic acid and washing the

cervix with it. This did cause discomfort in some patients. The main effects of the acetic acid are:

1. the coagulation of any mucus, which may then be removed;
2. the endocervical villi can be seen more easily;
3. atypical epithelium becomes white. (Recording of acetowhiteness is the most important part of the colposcopic examination.)

The effect of the acetic acid is transient, lasting only a few minutes, but can be maintained by using Q-tips to keep the cervix moistened with the acetic acid.

The inspection of the endocervical canal was aided by the use of Dejardins forceps, as it is important to identify the squamocolumnar junction and the upper limit of any abnormality. It was at this point that representative biopsies were taken using Eppendorfer biopsy forceps (2.7). Any bleeding from the biopsy site was stopped by applying silver nitrate sticks (AVOCA caustic applicator 75% silver nitrate) to this area. (The patient was warned that this might cause a certain amount of blackish discharge over the ensuing few days.) Aqueous Iodine BP (William and Son, Hitchin, UK) was then applied to the cervix. The iodine can cause artefacts which may make histological examination of any biopsy difficult to interpret, and so it was usually not applied until after a biopsy had been taken or there had been no obvious acetowhitening present. The iodine usually serves to delineate the extent of an abnormal area but cannot always distinguish between normal endocervical tissue, early metaplasia, or premalignant and malignant disease.

If an abnormality was identified, a pictorial record was made of each visit and a diagnosis suggested, following the guidelines suggested by Reid and Scalzi (1985), which are as follows:

FEATURES	SCORE		
	0	1	2
MARGIN	Indistinct	Regular lesions with smooth straight outline	Rolled peeling edges
COLOUR	Shiny, snow-white	Intermediate shade	Dull oyster white
VESSELS	Fine calibre Poorly formed patterns	Absent vessels	Definite punctation or mosaicism
IODINE UPTAKE	Positive uptake	Partial uptake	Negative staining of significant lesions

Scores 0-2 suggests HPVI/CIN 1, 3-5 suggests CIN 1/CIN 2, 6-8 suggests CIN 2/CIN 3. (See examination sheet in the appendix.)

2.5 CYTOLOGY

A Papanicolaou or cervical smear was taken at the time of routine screening and at each colposcopy visit. The woman was examined in the dorsal position in the presence of a nurse sister, who acted as assistant and chaperone. The cervix was visualised by a Cuscoe's speculum, which had been lubricated for the patient's comfort with a very small amount of K-Y jelly (Beecham, UK). The smear was performed by rotating a wooden Ayre's or Aylesbury spatula through 360^{o} around the cervical os under direct vision. The cells obtained from the transformation zone were initially spread thinly and evenly over a glass slide (Menzel-Glaser, West Germany),

which had been labelled with the patient's name in pencil. The cells were fixed with 95% v/v industrial methanol/acetic acid, which contained 5% v/v carbowax (DHSS Central Supplies, London, UK). The slides were allowed to dry in the air and were stored in plastic transport cases for safe carriage to the RNH cytology laboratory.

In some cases, where the squamocolumnar junction was not visualised, an endocervical brush was also used to sample cells from the canal.

The cytological slides were prepared by the cytotechnicians of the Royal Northern Hospital (RNH), using an automated staining system (Shandon Southern Products Ltd., Runcorn, Cheshire, UK) based on a modified Papanicolaou technique. The slides were read by the cytology staff of the RNH. The smears were reported as one of the following:

Inadequate specimen

Negative - Endocervical cells not seen (ECNS)

Negative

Squamous atypia not amounting to dyskaryosis

Borderline dyskaryosis

Mild dyskaryosis

Moderate dyskaryosis

Severe dyskaryosis

Changes suggestive of invasive carcinoma.

Findings suggestive of human papillomavirus infection (koilocytosis), candidal, trichomonal and actinomycotic infection were also included in the smear result. The criteria for the cytology reporting was based on Evans et al (1986). Morphological abnormalities of the nucleus comprise:

1. disproportionate nuclear enlargement;
2. irregularity in form and outline;
3. hyperchromasia;
4. irregular chromatin condensation;
5. abnormalities of the number, size and form of nucleoli;
6. multinucleation associated with any of the above.

Inflammation causes minor nuclear abnormalities, which are usually disproportionate nuclear enlargement with or without hyperchromasia. Dyskaryosis is used to describe nuclear abnormalities more numerous or more severe than those associated with inflammation alone. Irregularity of chromatin distribution is the most important change in nuclear morphology. Dyskaryotic cells are classified as mild, moderate and severe according to:

1. the diversity of abnormal nuclear characteristics listed and the degree of morphological abnormality;
2. cytoplasmic characteristics which include quantity, density, shape and staining quality.

2.6 BACTERIOLOGY

When the clinic was commenced, the first 130 women were screened routinely by taking a high vaginal swab (HVS) for routine culture and Gram staining for:

1. anaerobes
2. candida
3. trichomonas
4. gonorrhoea.

Endocervical swabs were taken for Herpes simplex virus in the first 25 cases and for Chlamydia in the first 130 cases. These tests were performed

on the women attending the clinic, regardless of the presence or absence of a cytological abnormality, to assess the incidence of vaginal infections within our clinic population. These swabs were usually performed before taking a cervical smear.

After this original screening, women who had either a symptomatic or asymptomatic discharge noted at the time of cervical smear taking, had an HVS taken. If there was any suspicion of a sexually transmitted disease (STD), arrangements were made for full screening and appropriate treatment at the Genito-Urinary Medicine clinic at RNH. The swab was taken from the vaginal discharge prior to the application of acetic acid, using a transport swab containing Amies transport medium (Exogen, Clydebank, Scotland) and forwarded to the Bacteriology Department at the Whittington Hospital.

The HVS from the clinic was plated out onto agar and horse blood agar plates by the microbiology technicians. They were cultured under both aerobic and anaerobic conditions at $37^{o}C$. The culture plates contained discs impregnated with Penicillin G or metronidazole to aid bacterial identification. The organisms cultured from these plates were identified by the microbiology staff using a combination of colony morphology, Gram stain and biochemical kits (API 20, France). Any culture that could not be positively identified in the laboratory was forwarded to the National Collection of Typed Cultures (Central Public Health Laboratory, Colindale, London, UK) for formal identification.

The swabs for Chlamydia trachomatis were taken using a nylon cytobrush (Medscand) and the sample was taken from the endocervical canal. These cells were then transferred to a welled slide and fixed with

acetone for direct monoclonal identification (Thomas et al, 1984). These slides were processed by Dr. B. J. Thomas of St.Mary's Hospital, London, using a direct monoclonal kit (Syva, UK).

The swabs for Herpes simplex virus isolation were placed in 1ml of viral transport medium containing nutrient broth, penicillin (1000IU/ml), streptomycin (1000IU/ml) and ampicillin (50mg/ml) and were processed immediately or stored at -70°C. A 0.2ml volume of the sample was inoculated into two tubes containing monolayers of human embryonic lung fibroblasts (MRC5, Flow Laboratories, Rickmansworth, England), which were incubated at 37°C. The monolayers were observed daily for cytopathic effect. A specimen was considered to be negative if a characteristic change had not occurred by 7 days. Monolayers exhibiting a cytopathic effect were harvested by scraping the cells into the medium and the suspensions were frozen at -70°C until used for virus typing.

If pathogenic bacteria were identified, appropriate treatment was arranged.

2.7 HISTOPATHOLOGY

At the time of colposcopy, representative biopsies of the most abnormal looking areas were taken using Eppendorfer biopsy forceps. These biopsies were immediately placed onto small squares of blotting paper to aid orientation and then placed into a 2ml tube of formalin calcium solution for preliminary fixation and transport. The formal calcium solution was made up of 1gm of calcium chloride in 100mls of distilled water, to which was added 10 mls of 40% formalin, followed by 1ml of ethylene glycol and 7% sucrose. The pH of this solution was maintained at 4.0.

These punch biopsies were prepared by the staff of the Histopathology Department at the Whittington Hospital. Fixation of the biopsy in the formal calcium solution lasted for up to 48 hours. The biopsies were orientated, placed on filter paper, then they were loaded onto a V.I.P. (Vacuum Infiltration Processor) Tissue Processor (automatic) and embedded in paraffin wax at 60°C. The biopsy was then sectioned on a base sledge microtome at a thickness of 4 microns. Each section was stained with Ehrlich's haemotoxylin and aqueous eosin. All the biopsies were read in conjunction with the same Consultant Histopathologist (Dr. D. Jenkins) during regular weekly session. This assessment was based on the criteria for CIN (Buckley et al, 1982) and for human papillomavirus infection (HPVI) without neoplasia (Dyson et al, 1984).

The HPVI changes without neoplasia are characterised by a perinuclear halo with some degree of nuclear abnormality, on staining there is a strong eosinophilic reaction but a relatively poor affinity for haemotoxylin. In all cases of CIN, the full thickness of the epithelium, whether on the surface or in the crypts, is occupied by neoplastic cells. In many cases, however, there is cytoplasmic differentiation in the upper part of the epithelium and hence CIN is usually graded in the term of the proportion of the epithelial thickness occupied by undifferentiated neoplastic cells of basaloid type.

CIN 1: the essential feature is that whilst the cells throughout the full thickness of the epithelium show nuclear abnormalities, the cells in the upper and middle thirds of the epithelium undergo cytoplasmic differentiation. At all levels of the epithelium the nuclei tend to have prominent nucleoli and be pleomorphic, of irregular outline, enlarged and hyperchromatic with a coarse granular or filamentous chromatin pattern.

The cells in the lower third, or less, of the epithelium show no evidence of cytoplasmic differentiation or of orderly stratification, lack clearly defined boundaries and have a high nucleocytoplasmic ratio with nuclear crowding, whilst those in the middle and upper thirds of the epithelium show, to a variable degree, evidence of stratification and of cytoplasmic maturation with a decreasing nucleocytoplasmic ratio.

CIN 2: the features are similar to those of CIN 1 except that undifferentiated non-stratified cells with pleomorphic nuclei and a high nucleocytoplasmic ratio extend beyond the lower third of the epithelium but not into the upper third. The cells in the upper third of the epithelium undergo a variable degree of stratification and of cytoplasmic differentiation.

CIN 3: undifferentiated, non-stratified basaloid cells with nuclear crowding, indistinct boundaries and a high nucleocytoplasmic ratio occupy more than two-thirds or the full thickness of the epithelium. The degree of nuclear pleomorphism is often greater that that seem in CIN 1 or 2. Mitotic figures are frequently seen, are commonly present in the upper one third of the epithelium and are often of abnormal form.

DNA-DNA HYBRIDISATION

2.8 FILTER IN-SITU HYBRIDISATION

This method was used for identification of HPV16 in the cervical scrapes that had been collected in a phosphate buffered saline solution at the time of routine cytology.

Millipore nitrocellulose filters (24mm in diameter with a pore size of 0.45μm diameter) were used to collect the cells from these samples. The

filters were labelled with a patient identification and visit number which were placed at diametrically opposite ends of the filter. The filter was then placed onto the metal grid of a vacuum manifold. The sample was poured onto the filter and the liquid was drawn through it by suction. This caused the cells to be trapped on the filter. The filters were then removed from the manifold and allowed to dry in the air. The metal grid was cleaned and dried between samples.

Two pieces of cartridge paper were cut and placed on the top of a larger piece of cling film. One was soaked with 0.5M Sodium Hydroxide solution, whilst a 0.5M Tris/1.5M Sodium Chloride solution was used on the other sheet. In order to lyse the cells and denature the DNA, the dry filters were placed on the NaOH sheet for between 3-5 minutes. The filters were then transferred to the other sheet for a similar time period to neutralise the effects of the NaOH. The filters were removed and placed on a fresh piece of cartridge paper and allowed to air-dry prior to baking. A small wallet was made out of the cartridge paper, and when they were thoroughly dry, the filters were placed in it with a dividing piece of cartridge paper between each one. These wallets were then baked in a vacuum oven at 80^{o}C for one hour. The wallets were then removed and stored in a vacuum sealed dessicator prior to hybridisation.

2.9 RANDOM PRIMER OLIGOLABELLING WITH LINEAR HPV16

1. Prehybridisation Washing

The filters were removed from the dessicator and cut in half with scissors, only one half being used from each patient visit, the remainder were returned to storage. A negative control filter was prepared by using one million 3T3 cells (a mouse fibroblast cell line) onto a nitrocellulose

filter and treated in a similar manner to the others, likewise a positive control was prepared using Caski cells. CaSki cell lines contain DNA sequences which hybridize intensely with HPV16 DNA probe, the cells containing greater than 500 copies per cell (Yee et al, 1985). Approximately 30 of these half filters with controls were placed in a polythene bag. These bags were made from 500 gauge clear polythene sheeting, being double sealed by the heat sealer on each side.

A prehybridisation washing solution was added to the filters made up of: 8mls. distilled water

1ml 20x SSC

1ml 100x Denhardts solution.

Any air bubbles were expelled from the bag before being sealed with the heat sealer (Hulme Martin polythene sealer, melting temperature for 2-3 seconds). This prehybridisation wash lasted at least 2 hours in a water bath at 68°C with the polythene bag being completely immersed in the water. The prehybridisation solution blocks non-specific binding sites in order to reduce background signals. The concentration of Denhardt's solution was part of the routine protocol of the laboratory as described in Hames and Higgins (Chapter 4, 1985) and at this concentration has been found to be optimal for reducing background signals.

2. Labelling of Linear HPV

This was performed using the Amersham International Random Primer kit. All the components of the kit are stored at -20°C. The labelling materials included:

Primer/BSA - described in section 2.12

Labelling buffer - described in section 2.12

Linear HPV 16 DNA

Radioactive dCTP - Deoxycytidine 5'-[Alpha P^{32}] Triphospate, approximately 3000 Curies/mmol

Enzyme - Klenow, as described in section 2.12.

The method used was that as described by the manufacturers. 100ng of linear HPV 16 DNA was placed in a screwcap eppendorf tube together with a volume of distilled water, the total volume being 30µl. Micropipettes were used throughout for measurement of the volumes. The eppendorf tube was placed in a boiling water bath for 10 minutes in order to denature the strands of HPV DNA. After removal from this bath, the tube was immediately placed on ice. 10µl of labelling buffer plus 5µl of primer/BSA were added to the contents of the eppendorf tube, followed by 30 microcuries of dCTP radioactively labelled with ^{32}P. This manoeuvre was performed behind a perspex screen, and appropriate precautions were taken for the use of radioactive material. 2µl of Klenow enzyme (a fragment of DNA polymerase 1) was then added, making the volume up to 50µl. The mixture within the tube was then placed in a small lead pot and incubated for at least one hour at 37°C (Klenow et al, 1971).

3. Checking Incorporation

Two fibreglass filters (Whatman glass microfibre filters GF/C, 2.5cm diameter) were used. 55µl of 'magic stop mix' was pipetted into an eppendorf tube, to which was added a minute amount of the radioactive mix. This was mixed thoroughly and 25µl of this solution was pipetted onto each filter. Once these were fully dried, one of the filters was placed on a small suction manifold and washed with 7% tricholoroacetic acid followed by IMS (Industrial methylated spirits).

After drying, each filter was placed in a scintillation vial together with 5mls of scintillation fluid (see 2.12). Each vial was then placed in a glass outer vial and then into the Beta counter. Two readings were obtained and the incorporation of the radioactive phosphorus was calculated:

$$\frac{\text{washed filter count}}{\text{unwashed count}} \times 100 = \text{Percentage incorporation}$$

If the incorporation of ^{32}P into the linear HPV DNA was greater than 30%, then the rest of the hybridisation technique could proceed.

4. Separation of unincorporated nucleotides

A G-50 spin column was used for this process, which was made using a 1ml plastic syringe. A small amount of siliconised glass wool was pressed down into it and then the rest of the syringe was filled with sephadex in STE. By spinning this in the centrifuge at 2000 revolutions per minute (rpm) for 4 minutes and refilling it with the sephadex, the sephadex column was prepared so that the level was at the 1ml mark.

The column was kept in a centrifuge tube with its narrow end in an eppendorf tube. The labelled material was loaded onto the top of this column with 50µl of STE. This column was then spun at 2000 rpm for 4 minutes in the centrifuge. In this way, the probe containing the radionucleotide is separated from the unincorporated nucleotides and collected.

0.8ml of 5mg/ml SSST DNA (single stranded salmon testes DNA) was pipetted into an eppendorf tube. The two eppendorf tubes one containing probe, the other, SSST-DNA, were both placed in the boiling water bath for

10 minutes, and afterwards placed in ice. The aim of this procedure was to denature the DNA strands. A hybridisation mix was made concurrently, consisting of:

1ml x20 SSC

1ml x100 Denhardts solution

0.1ml 20% SDS

7ml distilled water.

The probe and SSST-DNA were added to the above solution, after denaturation.

The prehybridisation fluid was then emptied from the bag containing the filters. The hybridisation mix was added to the filters and after expelling any air bubbles, it was double sealed in the usual way. This bag was then placed in a larger outer bag, which was also double sealed, to prevent leakage of any radioactive material. This was then placed in a waterbath at 68°C overnight, hybridisation being allowed to proceed for at least 12 hours (Tm - 10°C). Tm is the melting temperature, that is, the temperature at which the strands of a DNA duplex or an RNA-DAN hybrid are half dissociated or denatured (Hames & Higgins, 1985).

5. High stringency wash

One litre of wash solution was prepared using:

25mls x20SSC

5mls 20% SDS

970mls distilled water.

The filters were removed from the waterbath and the hybridisation mix discarded appropriately. The filters were placed in a small plastic box and washed with some of this wash solution twice at room temperature.

Each wash lasted for 1-2 minutes. The third wash mix was left in the box, which was then placed in the 68°C waterbath (Tm -10°C). The remainder of the wash solution (500ml) was poured into a clean bottle and also placed in the waterbath.

The washing was left for an hour, and the solution discarded. The prewarmed solution was then used for 2 further washings, again each one lasting 1-2 minutes. When the remainder of the solution had been drained, a small amount of 50% glycerol/x2 SSC was added. This solution prevents the filters becoming brittle when placed at -70°C for exposure to the X-ray film.

6. Autoradiographs

A clean, exposed X-ray plate (18cm x 24cm) was used, on which the filters were positioned with their straight edge to the right and adequately spaced. This was then covered with cling film, removing air bubbles and wrinkles as far as possible.

This was placed in an X-ray holder (Protex Cuthbert Andrews, 18cmx24cm) with an X-ray film (Fuji Photo Film Co. Ltd.), so that the filters were sandwiched between the intensifying screens. The holder was then placed in the -70°C refrigerator.

7. Developing the autoradiographs

The films were exposed for 1 day and then 7 days. Before development, the cassette was removed from the freezer and allowed to reach ambient temperature. In the darkroom, the film was removed and placed in Kodak LX X-ray film developer (1 part to 4.7 parts water) for 5 minutes, during which time the tray was gently agitated. The filter was then washed with tap water prior to fixing in Kodak FX 40 X-ray liquid fixer

(1part to 4 parts water) for 3 minutes. The film was then washed and allowed to dry.

A further X-ray film was inserted into the cassette for the 7 days exposure, the exposure again being at -70°C.

8. Interpretation

This was determined by reviewing the overnight and 7 day films (Illustration 1). A positive signal, i.e., an area of blackness on the X-ray film, would denote the presence of HPV 16 DNA. It was quite common for the appearance to suggest clumping of the cells on the filter. A negative result occurred when there was lack of any signal on the X-ray film. There was also an intermediate group, the plus-minus or equivocal group, where the signal on the film was mid-way between these two, i.e., not definitely positive and not unequivocally negative. The result may suggest the possible presence of viral DNA of HPV16 or some contaminant.

2.10 SOUTHERN BLOTTING

This was performed on a number of samples, to compare it with the filter in-situ method. The sample obtained from a cervical scrape was washed in 10 mls of PBS. This was divided into 2 aliquots, one for the FISH method and one for the Southern blotting method.

1. Extraction

The 5ml sample of PBS containing the cells were centrifuged at 2,500 rpm for 10 minutes. This action resulted in a small pellet at the bottom of the universal container. The supernatant was discarded and the pellet resuspended in 0.25ml of PBS to which 5mls of Buffer A was added together with 500µg (50µl) of Proteinase K (100µg per ml of final solution). This

mixture was incubated for approximately 3 hours at 37°C, and then transferred to a 50ml polypropylene tube before adding 5mls of phenol (i.e. an equal volume). The tube was spun at 2,000 rpm for 5 minutes at room temperature. The top layer of the mixture was kept, the bottom layer being pipetted away. This procedure was repeated with a phenol/chloroform mix (2.5mls of each) and then with 5mls of chloroform (the chloroform was 24 parts to 1 part of isoamyl alcohol). The aqueous phase was always kept as the DNA remains in this upper layer, whilst the debris of the cell proteins and membrane separate into the bottom layer. This procedure was performed in a fume cupboard.

RNAase was added to the remaining solution (5mls): 50µg for each millilitre of solution (total volume = 25µl). This was incubated for 15 minutes at 37°C. 500µg of Proteinase K was then added to the mixture with 250µl of 20% SDS. The resulting mixture was incubated for 30 minutes at 37°C. A further chloroform/phenol extraction was then repeated.

2. Dialysis

The aqueous layer was placed in dialysis tubing and dialysed against 2 changes of Buffer C at 4°C overnight. The dialysis took place overnight and then the buffer was changed in the morning and dialysis allowed for a further one hour. The OD_{270} was measured to compare the readings of used and unused Buffer C, aiming for the two readings to be equal. The purpose of the dialysis was to remove and further phenol contamination. Once the dialysis was complete, the fluid from the tubing was transferred to a corex tube and precipitated at -20°C with an equal volume of isopropanol (5mls) and 1/10 volume of ammonium acetate (0.5M). The tube was then centrifuged in a Sorvall centrifuge at 4°C for 30 minutes. The supernatent was discarded and 1ml of 70% ethanol was carefully added to the DNA to dry

it. This process was completed by placing the tube in a vacuum dessicator. The DNA was resuspended in 0.5ml of TE buffer and the amount of DNA quantified by measurement of the solution's OD at OD260 and OD280, the ratio determines the amount of contaminating protein.

One OD unit at 260nm = 50μg/ml of DNA.

3. Restriction Endonuclease Analysis

The amount of DNA used for this next part of the process depended on the amount of DNA obtained. Ideally, 10μg was used, which was precipitated by using an equal volume of isopropanol and 1/10 volume of 0.5M ammonium acetate and incubated at -70°C for 1 hour or -20°C overnight. This sample was then centrifuged for 15 minutes and the supernatant drained. Each sample then had 10μl of tRNA and 100μl 70% ethanol added to it before being spun for a further 2 minutes. The ethanol was drained, and any remainder removed by using the vacuum dessicator. The resulting pellet was then resuspended in 80μl of distilled water.

In order to check the restriction, controls were necessary. 10μg of brain DNA was used, in each of 2 eppendorf tubes. 100pcg of Plasmid pAT HPV6 was added to one sample and 100pcg of Plasmid pAT HPV16 added to the other. The volume of each of these specimens was made up to 80μl using distilled water. The DNA in each sample was cut with the restriction endonuclease BAM H1, 10 units per microgram of DNA (The concentration of BAM H1 was 10u/μl) in the presence of BAM H1 buffer. The tubes were then incubated for at least 2 hours at 37°C. The resulting DNA was precipitated using isopropanol and ammonium acetate, as described above. The samples were then centrifuged at 12,000 rpm for 15 minutes and the supernatent was then drained. 100μl of 70% ethanol was added to each sample and centrifuged for 2 minutes, the ethanol was pipetted away and

any remainder was removed by using the vacuum dessicator. The resulting DNA was then resuspended in 18µl of distilled water and 2µl of loading buffer.

Each sample was loaded onto a gel made up of 0.8% Agarose, made up in TAE, with 0.05µg of ethidium bromide per millilitre of gel. The gel dimensions were 10cm x 15cm. The gel was run at 20 volts for as long as it took to show any banding; this was usually overnight.

4. Depurination

The gel was was trimmed and placed in a plastic box on the top of a rocker. The gel was bathed in 500mls of 0.1M HCl and left off the shaker for 15 minutes. The gel was washed with distilled water and then denatured with 500mls of 1M NaCl/0.5M NaOH. This was agitated for 30-60 minutes. The gel was washed again and then neutralised using 500mls of TRIS/ NaCl. This process decreases the size of DNA molecules and eases transfer to the nitrocellulose filter.

5. Setting up the Southern Blot

The gel was cut off from the level of the wells. Two layers of 3MM filter paper were placed across a glass plate. The paper was presoaked in x20SSC and the ends of the paper were dipped into a tray containing x20SSC. The gel was placed on this paper. A piece of nitrocellulose paper was cut to same dimension as the gel and soaked in distilled water and then in x20 SSC. A small triangle was cut in the corner to aid orientation. The nitrocellulose filter was placed on top of the gel ensuring there were no air bubbles. Two further pieces of the blotting paper were cut to cover the filter, again being presoaked in x20SSC. Clingfilm was used to cover the gel and paper,

and about 8cm of paper towels were placed on top of the blotting paper. A glass plate was placed on top with a weight. This was left overnight for the DNA to transfer to the nitrocellulose filter paper.

The following morning, the apparatus was dismantled and the nitrocellulose filter was allowed to dry in the air, before being baked at 80°C in a vacuum oven for one hour.

6. Hybridisation

The filter can either be stored, or hybridised using the same method as in the FISH technique. The amount of linear HPV 16 used is 200ng, with 50 microcuries of dCTP. The autoradiographs were interpreted according to the characterisitc band patterns of the HPV16 probe that was used as a control.

2.11 PREPARATION OF LINEAR HPV16

HPV 16 is ligated into pAT153 plasmid at the BamH1 restriction site. This is a method of propagating the viral DNA, and it is necessary to cut the HPV 16 DNA out of the plasmid so that it can be used for hybridisation. Fragments of DNA from any source can be amplified more than a million-fold by inserting them into a plasmid or bacterial virus, and then growing them in bacterial (or yeast) cells. This process is known as DNA cloning. Plasmids are small circular molecules of double stranded DNA that occur naturally in both bacteria and yeast where they replicate as independent units as the host cell proliferates.

Thirty micrograms of pAT/16 was mixed with 150 units of Bam H1, the volume being made up to 100µl with distilled water. This mixture was incubated at 37°C for at least two and a half hours. A large 0.8% agarose gel

was prepared, using 1.48gm of agarose and 160mls of TAE. To this mixture, 16µl of ethidium bromide (concentration 5 µg/ml) was added, i.e., a 10,000 times dilution. The gel was placed in an electrophoresis tank, which was filled with TAE. Loading buffer was added to the pAT/16 mix (10µl) and this was loaded into the gel. A pAT marker (500ng) was also loaded. The gel was electrophoresed at 100 volts until the blue dye front ran to near the bottom of the gel or overnight at 20 volts.

A freeze thaw method was used to isolate the linear HPV16 DNA. The gel was placed onto the UV transilluminator and the band viewed. The band of linear HPV 16 DNA could be identified, as the pAT marker band corresponded to the pAT band from the cut plasmid. The gel piece containing the linear HPV 16 DNA was placed into eppendorf tubes and these were then inserted into a vacuum flask containing liquid nitrogen for approximately 2 minutes, until frozen. This was then thawed at 37°C and the process repeated twice. The tube and contents were then spun at 4000 revolutions for 15 minutes. The resulting supernatent was placed into a separate tube.

In a different eppendorf tube, a small hole was made in the bottom of the tube and a small amount of siliconised glass wool placed at the bottom. This tube was placed into another one and the gel was layered onto the glass wool in order to try and collect more DNA. The material was freeze-thawed once and then cetrifuged at 4000 revolutions per minute for 10 minutes. the supernatent was collected from the bottom tube.

All the supernatants were pooled and the DNA was precipitated by adding one tenth the volume of 0.5M ammonium acetate, 1 volume of isopropanol and 50µg of tRNA. This mixture was placed in the -70°C

refrigerator for 60 minutes. The resulting solution was spun for 20-25 minutes in order to obtain a small pellet of DNA. The supernatant was discarded and 100µl of 70% ethanol was used to rinse the pellet and remove any remaining salt. The ethanol was discarded and the pellet was dried using the vacuum dessicator for approximately 8 minutes. The pellet was then redissolved in 100µl of STE. A G-50 sephadex column was made as in the hybridisation section, which was washed through with 100µl of STE twice. The DNA in STE was loaded onto the column and collected at the bottom in an eppendorf tube after centrifuging for 4 minutes at 3,500 revolutions per minute.

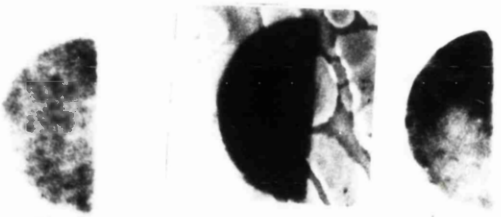
A small 0.8% agarose gel (60mls) was made and 5µl of the collected DNA in STE was run against a range of pAT markers: 50ng, 100ng, 200ng. 300ng and 400ng. This process uses about one twentieth of the DNA available. It important to evaluate the DNA concentration for future use by using the markers for calibration. The remaining DNA was reprecipitated as before and the pellet dried. The pellet was then resuspended in distilled water to a concentration of 50ng/µl. This probe was then ready for use in the hybridisation techniques already described.

EXAMPLES OF FISH RESULTS

NEGATIVE

OVERNIGHT EXPOSURE

7 DAY EXPOSURE



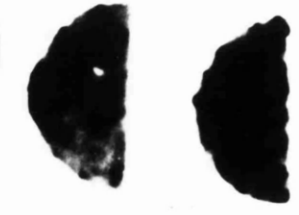
EQUIVOCAL

OVERNIGHT EXPOSURE

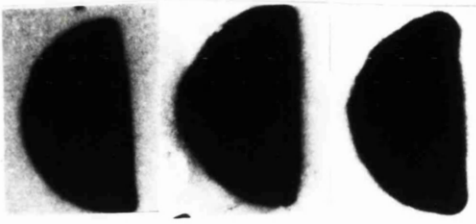
7 DAY EXPOSURE

POSITIVE

OVERNIGHT EXPOSURE



7 DAY EXPOSURE



2.12 SOLUTIONS.

The composition of the solutions used throughout the various techniques are as follows:

1. x100 Denhardts solution.

2g Ficoll 4000

2g Polyvinyl pyrrolidone

2g Bovine serum albumin (BSA)

Distilled water is used to make this volume up to 100 mls.

2. x20 Salt sodium citrate (SSC).

175.8g sodium chloride

88.2g sodium citrate

These are added to 1 litre of distilled water, the pH being adjusted to 7 using citric acid.

3. STE.

2.0ml 5M sodium chloride (S)

1.0ml 1M Tris pH 8 (T)

0.2ml 0.5M EDTA (E) Diaminoethanetetracetic acid disodium salt.

This is made up to 100mls with distilled water.

4. Stop Mix.

5.0ml 1M Tris

2.0ml 500mM EDTA

0.5ml 20% SDS (sodium dodecyl sulphate)

The volume is made up to 100 mls with distilled water.

5. TE.

1.0ml 1M Tris pH 8

0.2ml 500mM EDTA

The volume is made up to 100mls with distilled water.

6. Tris 1M solution.

121.1g Tris base (Trizma)

800mls distilled water.

The pH is adjusted with concentrated hydrochloric acid and the solution is then made up to 1 litre using more distilled water.

7. PBS (Phosphate buffered saline).

PBS was prepared using tablets of Dulbecco 'A' manufactured by Oxoid. One tablet was used per 100mls distilled water, once the tablets had been dissolved, the solution was autoclaved at 115^{o}C for 10 minutes. The PBS was then aliquoted into sterile universal containers, approximately 10 mls in each one. The pH was 7.3.

8. 50% Glycerol/x2 SSC solution.

This was prepared using equal amounts of glycerol and x2 SSC. The x2 SSC was prepared by diluting the x20 SSC with distilled water.

9. G-50 Sephadex.

12.5g of G-50 sephadex was placed in a 150ml bottle, which was then filled with STE and left overnight. During this time, the sephadex swells and any remaining STE was poured off before adding more STE to fill the bottle.

10. TAE Buffer E.

242g Trizma base (i.e. 2M)

57.1ml Glacial acetic acid

100ml 0.5M EDTA

The volume was made up to 1 litre with distilled water.

11. SSST-DNA.

This solution was made up to a concentration of 10mg/ml by dissolving DNA (Sigma Type III-sodium salt). This solution was stirred with a magnetic stirrer for at least 2-4 hours at room temperature. The DNA was then sheared by passing it repeatedly through an 18 gauge hypodermic needle until the viscosity was reduced. The DNA was boiled for 10 minutes before being stored at -20^{o}C.

12. Loading buffer.

This was prepared in 20 mls using 0.25% Bromophenol blue and 25% Ficoll 4000, with 1ml of Tris (pH 8). The action of the buffer being to draw the DNA down in the gel by its high molecular weight.

13. Tris/NaCl solution.

This was prepared using 180g of Trizma base and dissolving it in 800mls of distilled water. The pH of the solution was adjusted using concentrated HCl to pH 8. To this solution 20g of NaCl was added and the volume made up to 1 litre with distilled water. This solution was, therefore, 1.5M Tris/0.5M NaCl.

14. Buffer A.

10mM Tris-HCl pH

10mM EDTA pH 8

10mM NaCl

0.5% SDS

15. Buffer C.

1M Tris-HCl pH 8

200mM EDTA pH8

200mM NaCl

The volumes of Buffer A and C solutions being made varied from 3 to 5 litres at any one time and the amount of the above constituents was varied accordingly so that the concentration of each was as above.

16. BAM H1 incubation buffer (x10).

100mmol/l Tris-HCl pH 8.

50mmol/l magnesium chloride

1000mmol/l sodium chloride.

50µg/ml Bovine serum albumin.

17. Labelling buffer - Amersham International plc - contained dATP, dGTP and dTTP in a concentrated buffer solution containing Tris-HCl, pH 7.8, magnesium chloride and 2 mercaptoethanol. (RPN.1601Y 300µl and RPN.1601Z 600µl.)

18. Primer solution - Amersham International plc.

RPN.1600Y and RPN.1601Y 150µl

RPN.1600Z and RPN.1601Z 300µl

Random hexanucleotides in an aqueous solution containing nuclear-free BSA.

19. 20% SDS.

200g of SDS (sodium dodecyl sulphate) plus 800mls of distilled water, the pH was adjusted with hydrochloric acid unitl it was 7.2, and then the solution was made up to 1 litre in volume.

20. Scintillation Fluid.

Two parts toluene, 1part scintillant grade Triton X and 0.4% w/v diphenyloxazole.

21. Enzyme solution

(RPN 1600Y and RPN 1601Y: 60µl, RPN 1600Z and RPN 1601Z :120µl): 1 unit per µl DNA polymerase I 'Klenow' fragment (cloned) in 50mM potassium phosphate, pH 6.5 10mM 2-mercaptoethanol and 50% glycerol.

2.13 STATISTICAL METHODS

ARMITAGE TEST FOR TREND & GOODMAN TEST FOR ASSOCIATION

To test a hypothesis of trend in a 2 x k table (k>2), Armitage's (1955) trend test was used which has an approximate χ^2 distribution with one degree of freedom.

Let r be the number of rows and c the number of columns in a 2-way table. For general r and c (r or c >2), we often wish to test whether there is association down the rows and across the columns, i.e. a s more general test for trend. Following McCullagh and Nelder (1983), we can construct a log linear model to test for a trend down the diagonal of an r x c table (Goodman test for association).

Let n_{ij} be the expected number of observations in the cell (i,j). Under H_0, we would then have with Poisson errors:

$$H_0: \log n_{ij} = \alpha_i + \beta_j$$

If there is a trend we can expect γ to be significantly different from 0 in the model:

$$\log n_{ij} = \alpha_i + \beta_j + ij\gamma$$

Here we can use the likelihood ratio statistic for $\gamma = 0$ versus $\gamma = 0$ which is approximately distributed as χ^2 with 1 degree of freedom, as a suitable test for trend.

KRUSKAL-WALLIS TEST

This is a k-sample generalization of the Mann-Whitney-Wilcoxon test The test assumes that the data arise as K independent random samples from continuous distributions all having the same shape. The null hypothesis of no difference among the k population locations is tested against the alternative of at least one difference.

Under the null hypothesis, the distribution of the test statistic H can be approximated by a chi-square distribution with k-1 degrees of freedom. The approximation is reasonably accurate if no group have fewer than 5 observations. Large values of H suggest that there are some differences in location among the K populations. Some authors suggest an adjustment of the test statistic H when there are ties in the data.

CHI-SQUARED TESTS

The chi-square test is a test of whether the observed frequencies are significantly different to those expected form some specified theory of hypothesis.

$$\chi^2 = \Sigma\ (O\text{-}E)^2/E$$

O = observed; E=expected. Degrees of freedom = Number of cells minus number of ways in which observed and expected distributions are made to agree.

KAPPA STATISTICS

This is a statistic of association and the range of values for kappa suggest the degree of agreement. The range is from -1 to +1. If the value is +1 then the agreement is one for one. Armitage (1971) suggests that the following applies:

Values of >0.75, then this represents excellent agreement beyond chance. Values <0.40 may represent poor agreement. Values between 0.40 and 0.75 may be taken to represent fair to good agreement beyond the realms of chance.

LINEAR LOGISTIC REGRESSION

The linear logistic mode has been developed specifically where the dependent variable is binary, usually a disease outcome. It derives its name from the fact that the 'logit' transform of the disease probability in each risk category is expressed as a linear function of 'regression' variables. In symbols, if P denotes the disease risk, the logit transformed y is defined by:

$$y = \text{logit } P = \log (P/1-P)$$

or in terms of y:

$$P = \frac{\exp(y)}{1+\exp(y)}$$

and the regression is of the form $y= \alpha + \beta x$.

To test the adequacy of the generalised linear model after fitting by maximum likelihood, an approximate χ^2 test statistic is given by deviance. The approximation to the distribution of the deviance by χ^2 is unreliable if a high proportion of the observations are based on small values (overfitting).

BINOMIAL DISTRIBUTION

This distribution was used for the serial HPV16 results looking at the probability of the number of successes (r) out of the total number of trials (n). This was calculated using the following formula through Minitab.

$$\Pr [r \text{ successes in } n \text{ trials}] = \frac{n!}{(n-r)!\, r!} p^r (1-p)^{n-r}$$

ODDS RATIO

The odds ratios were used in the transitional modelling using a 2 x 2 table thus:

a	b	R_1
c	d	R_2
S_1	S_2	N

The Odds ratio $= \frac{a.d}{b.c}$

The χ^2 with 1 degree of freedom $= \frac{N(a.d - b.c)^2}{R_1R_2S_1S_2}$

The statistical packages that have been used for the above analyses are Minitab, GLIM (Generalised linear interactive modelling) and BMDP (Biomedical data processing).

RELATIVE RISK (RR)

RELATIVE RISK or RISK RATIO $=$ Incidence rate in exposed group / Incidence rate in non-exposed group

INCIDENCE $=$ number of new cases in a period of time / population at risk or previously minus disease

SECTION THREE

RESULTS

3.1 SCREENING RESULTS

TOTAL NUMBER OF WOMEN SCREENED	1412
MEAN AGE	30.3 years
MEDIAN AGE	28 years
STANDARD DEVIATION	8.5 years
RANGE	15-65 years.

The age range is shown in the histogram (Graph 1), which reveals that the bulk of the women were in the 15-45 year age range.

Table 1 **AGE RANGE OF THE SCREENED POPULATION**

AGE RANGE (Years)	NUMBER OF WOMEN
15-19	73
20-24	324
25-29	393
30-34	236
35-39	174
40-44	114
45-49	62
50-54	22
55+	14

The screened population comprised women attending for routine cervical cytological screening. The majority of the women were in the 20-45 year age group (Table 1 & Graph 1) which is to be expected from a population consisting mainly of women attending their local F.P.C. for screening. The older women were using the F.P.C. on a 'well-women' basis, preferring to have their cytology performed at the clinic rather than by their own G.P.

GRAPH 1
AGE DISTRIBUTION OF THE SCREENED POPULATION BY HPV16 STATUS

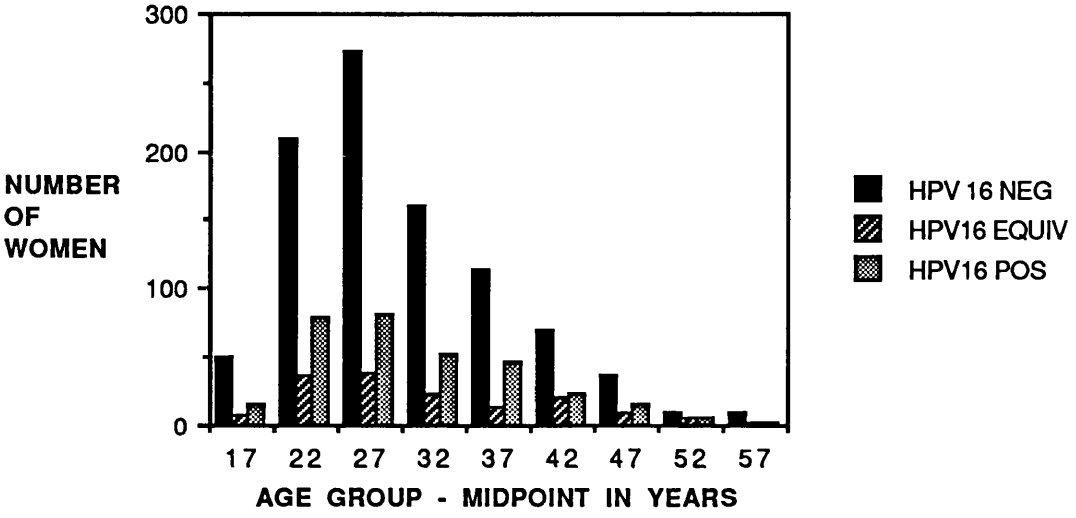


Table 2 CYTOLOGY RESULTS

RESULT	NUMBER	
NEGATIVE	1076	(76.2%)
SQUAMOUS ATYPIA (NOT AMOUNTING TO DYSKARYOSIS)	120	(8.5%)
BORDERLINE DYSKARYOSIS	61	(4.3%)
MILD DYSKARYOSIS	69	(4.9%)
MODERATE DYSKARYOSIS	64	(4.5%)
SEVERE DYSKARYOSIS	22	(1.6%)
TOTAL	**1412**	

These results have been simplified in some of the following analyses. On the cervical smear reporting forms, squamous atypia not amounting to dyskaryosis is classed as a negative result and borderline dyskaryosis is classed as mild dyskaryosis. Therefore, these cytology results may be simplified to:

Table 2.1

Result	Number	
Negative	1196	(84.7%)
Mild Dyskaryosis	130	(9.2%)
Moderate Dyskaryosis	64	(4.5%)
Severe Dyskaryosis	22	(1.6%)

The cytological abnormality rate appears high at 15.3% (Table 2), but may be a reflection of the age distribution of the patients and the fact that the population is drawn from an inner city area where there has been an increase in the cytological abnormality rate over the last 15-20 years (Hollingworth & Cuzick, In Press). Table 2.1 is included to emphasize that over half of these abnormalities (9.2%) were of a mild or borderline degree.

Table 3 **KRUSKAL-WALLIS TEST FOR EQUALITY OF AGES ALL CYTOLOGY**

Cytology group	No. Cases	Median Age	Ave Rank
Negative	1076	29.0 yrs	713.3
SATNAD	120	29.0 yrs	716.3
Borderline Dysk	61	28.0 yrs	722.7
Mild Dysk	69	27.0 yrs	650.0
Moderate Dysk	64	26.5 yrs	598.7
Severe Dysk	22	29.0 yrs	765.1
TOTAL	1412	28.0 yrs	706.5

χ^2 = 6.73 with 5 degrees of freedom (d.f.) p = 0.24 (adjusted for ties).
This result shows that there is no significant age difference between the different smear result groups. If these groups are condensed into just 4, there is still no significant difference with regard to age.

Table 4 KRUSKAL-WALLIS TEST FOR EQUALITY OF AGES
SUMMARY OF CYTOLOGY RESULTS

Smear Result	Number	Median Age	Av.Rank
Negative	1196	29.0 yrs	713.6
Mild Dysk	130	28.0 yrs	684.1
Moderate Dysk	64	26.5 yrs	598.7
Severe Dysk	22	29.0 yrs	765.1
TOTAL	1412	28.0 yrs	706.5

χ^2= 5.69 with 3 d.f. giving a p value of 0.13, again suggesting no significant age difference between the cytology groups.

Table 4.1 P-VALUES FOR PAIR WISE WILCOXON COMPARISONS FOR TABLE 4

	Normal	Mild	Moderate
Mild	0.44		
Moderate	0.028	0.18	
Severe	0.57	0.37	0.078

These tables show that for most of the screened population, there was no statistical difference in the ages of the women within the various groups. The exception was comparing the women with moderate dyskaryosis to the normal women. The former group of women had a significantly lower median age.

Table 5 CYTOLOGY VERSUS HPV16 RESULT BY FISH

Cytology Result	HPV 16 Result		
	Negative	**Equivocal**	**Positive**
Negative	709 (65.9%)	119 (11.0%)	248 (23.1%)
SATNAD	85 (70.8%)	14 (11.2%)	21 (17.5%)
Bord Dysk	38 (62.3%)	7 (11.5%)	16 (26.2%)
Mild Dysk	48 (69.6%)	6 (8.7%)	15(21.7%)
Moderate Dysk	39 (60.9%)	6 (9.4%)	19 (29.7%)
Severe Dysk	16 (72.7%)	4 (18.2%)	2 (9.1%)
TOTAL	935 (66.2%)	156 (11.1%)	321 (22.7%)

This can be simplified to (with the percentage value per row):

Table 5.1

Cytology Result	HPV 16 Result		
	Negative	**Equivocal**	**Positive**
Negative	794 (66.4%)	133 (11.1%)	269 (22.5%)
Mild Dysk	86 (66.2%)	13 (10.0%)	31 (23.8%)
Moderate Dysk	39 (60.9%)	6 (9.4%)	19 (29.7%)
Severe Dysk	16 (72.7%)	4 (18.2%)	2 (9.1%)

Table 5.2

Normal Cytology	794 (66.4%)	133 (11.1%)	269 (22.5%)
Abnormal Cytology	141 (65.3%)	23 (10.6%)	52 (24.1%)

Originally, the equivocal results were classed with the positive results but it has become increasingly apparent that these results should be kept in an equivocal category from the point of view of further analysis.

In assessing a trend towards cytological abnormality in relation to HPV16 status, only the positives and negatives have been included, due to the difficulty in knowing to which group the 'equivocal' readings should be assigned (Tables 5 & 5.1).

Armitage's Test for Trend on Table 5.1 gives a χ^2 value with 1 d.f., equal to 0.032, with p = 0.86. Using the standard χ^2 Test for heterogeneity then χ^2 = 3.64, with 3 d.f. giving a p value = 0.30. This suggests that there is no trend for the patients with an increasing cytological abnormality to be positive for HPV16 by the FISH method. Using the log linear model (Goodman Test for Association see page 65) so that the 'equivocal' category can be included gives a χ^2 with 1 d.f. equal to 0.042 (p = 0.84).

If normal and abnormal cytology are compared with the HPV16 status then the χ^2 value with 2 d.f., equals 0.27 or p = 0.87.

All these findings show that the HPV16 status by FISH is not related to either the presence or severity of a cytological abnormality.

Table 6 KRUSKAL-WALLIS TEST FOR AGE VERSUS HPV16 RESULT

	No.Obs.	Median Age	Ave Rank
Negative	935	28.0 yrs	698.5
Equivocal	156	28.0 yrs	743.0
Positive	321	29.0 yrs	712.2
TOTAL	1412	28.0 yrs	706.5

χ^2 = 1.68 with 2 d.f. giving p = 0.43 (adj. for ties), thus there is no significant difference in the age distribution within these groups.

Table 6.1 P-VALUES FOR PAIR WISE WILCOXON COMPARISONS IN TABLE 6

		HPV16 STATUS	
		NEGATIVE	EQUIVOCAL
HPV16 STATUS	EQUIVOCAL	0.21	
	POSITIVE	0.59	0.41

These results (Tables 6 & 6.1) show that there was no age difference between the HPV16 result group, either overall (Table 6) or comparing the individual groups against one another (Table 6.1). These results are at variance with those of Meanwell et al (1987), whose study suggested that positivity for HPV16 was age-related.

Table 7 SMOKING HISTORY AND CYTOLOGY RESULT

Smear Result	Non-smoker	Smoker	ExSmoker	NK
Negative	734 (87.1%)	449 (80.9%)	10	3
Mild Dysk	63 (7.5%)	67 (12.1%)	0	0
Moderate Dysk	35 (4.1%)	28 (5.0%)	1	0
Severe Dysk	11 (1.3%)	11 (2.0%)	0	0
TOTAL	843 (100%)	555 (100%)	11	3

Using the Armitage test for trend regarding history of smoking and increasing severity of smear result, χ^2 with 1 d.f. = 6.55, giving a value for p=0.011. This is just taking into account the current smokers and non-smokers. This significance is also present using the standard χ^2 test giving a value of 10.68 with 3 d.f., which produces a value for p = 0.014. This result suggests that there is a significant trend towards abnormal cytology in the smoking group.

The cigarette consumption ranged from 1-40 cigarettes per day in those 555 women who smoked, with the median being 15 per day (mean = 13.6, s.d. = 8).

Table 8 SMOKING HISTORY VERSUS HPV16 RESULT

HPV16 STATUS	Non-smoker	Smoker	Ex-smoker	NK
NEGATIVE	569 (67.5%)	357 (64.3%)	8	1
EQUIVOCAL	100 (11.9%)	54 (9.7%)	2	0
POSITIVE	174 (20.6%)	144 (26.0%)	1	2
TOTAL	843 (100%)	555 (100%)	11	3

Using the standard χ^2 test for heterogeneity with regard to smoking and HPV16 positivity (either positive or negative), the χ^2 with 2 d.f (using smokers ex-smokers and non-smokers) is 5.45, which gives a p value = 0.065. The approximation is poor because the numbers in the ex-smoker category are small. If one uses the χ^2 test with 1 d.f. for smokers and non-smokers (excluding the ex-smokers), the value is 4.46, giving a value for p = 0.035. This would suggest that smoking and HPV16 positivity are in some way associated.

Table 9 CURRENT CONTRACEPTION USE AGAINST CYTOLOGY

	Cytology Result			
Contraception Type	**Negative**	**Mild Dysk**	**ModDysk**	**Sev.Dysk**
(Row Percentages)				
None	211 (84.1%)	29 (11.5%)	9 (3.6%)	2 (0.8%)
OCP	443 (86%)	43 (8.3%)	23 (4.5%)	6 (1.2%)
Minipill	47 (88.7%)	3 (5.7%)	2 (3.8%)	1 (1.9%)
IUCD	185 (82.9%)	20 (9.0%)	12 (5.4%)	6 (2.7%)
Tubal Ligation	29 (80.6%)	4 (11.1%)	2 (5.6%)	1 (2.7%)
Sheath	110 (82.7%)	16 (12.0%)	5 (3.8%)	2 (1.5%)
Cap	166 (85.1%)	14 (7.2%)	11 (5.6%)	4 (2.1%)
Sponge	1 (100%)	0	0	0
Vasectomy	4 (80%)	1 (20%)	0	0

The majority of the screened population used either no contraception (17.8%), hormonal (40.2%), an intrauterine contraceptive device (15.8%) or barrier methods (23.2%) with the other methods accounting for 3% of the population. 80% of women had had some exposure

to the OCP, however short the duration (Table 10), though this had no significant effect on the degree of dyskaryosis.

Table 10 HISTORY OF OCP USAGE:

Never	269 (19.1%)
Ever	1138 (80.6%)
Not Known	5 (0.3%)

Table 11 HISTORY OF OCP USAGE VERSUS CYTOLOGY RESULT

	Never	Ever	NK.
Negative	225 (83.6%)	967 (85.0%)	4
Mild Dyskaryosis	22 (8.2%)	107 (9.4%)	1
Moderate Dyskaryosis	16 (6.0%)	48 (4.2%)	0
Severe Dyskaryosis	6 (2.2%)	16 (1.4%)	0
TOTAL	269 (100%)	1138 (100%)	5

Applying the Armitage test for trend in this situation, gives a χ^2 value with 1 d.f. of 1.33, $p = 0.25$. (This table shows column percentages.) A similar result is obtained using the Log linear model (Goodman Association test), $\chi^2 = 1.28$ ($p = 0.26$). These results show that OCP usage had no significant effect on the degree of dyskaryosis. This is not entirely unexpected as any effect would be more likely to be exposure-time related.

Table 12 HPV16 STATUS VERSUS HISTORY OF OCP USAGE

HPV16 Status	Never	Ever	NK
Negative	175 (65.1%)	757 (66.5%)	3
Equivocal	36 (13.4%)	120 (10.5%)	0
Positive	58 (21.5%)	261 (23.0%)	2

Using a standard χ^2 test for heterogeneity with regard to history of OCP use and HPV 16 status (excluding the 'equivocal' group), χ^2 with 1 d.f. is 0.056, ($p = 0.81$). This suggests that there is no association between OCP usage and HPV16 status.

Table 13 KRUSKAL-WALLIS TEST FOR OCP USAGE TIME COMPARED TO CYTOLOGY RESULT

(This includes all the population unless the period of usage is unknown)

Cytology	No.Obs	Median time	Av.Rank
Negative	1184	31.0 mths	701.7
Mild Dysk	129	24.0 mths	703.8
Moderate Dysk	64	24.0 mths	633.0
Severe Dysk	21	60.0 mths	754.0
TOTAL	1398	31.0 mths	699.5

[Mean exposure time = 44.3 mths, Range 0-240 mths.]

χ^2 = 2.19 with 3 d.f., giving a p = 0.53 (adj. for ties). This value suggests that there is no difference in exposure time to the OCP between the various groups of cytological result. This value is not changed by omitting the never users from each group, though the median time of OCP use in the severe dyskaryosis/ever-users is 84.0 months compared to 45 months in negative cytology/ever-users, 48 months in mild dyskaryosis/ever-users and 40 months in moderate dyskaryosis/ever-users.

Table 13.1 **P-VALUES FOR PAIR WISE WILCOXON COMPARISONS FOR TABLE 13**

		CYTOLOGY RESULT		
		NEGATIVE	MILD	MODERATE
CYTOLOGY RESULT	MILD	0.99		
	MODERATE	0.15	0.21	
	SEVERE	0.39	0.46	0.095

These results (Tables 13 & 13.1) show that there is no significant difference in OCP exposure time and the severity of the cytological abnormality. These results are analysed both overall (Table 13) and then between the different groupings (Table 13.1).

Table 14 TIME OF OCP EXPOSURE IN THE SCREENED POPULATION

	No. of Women
Never	269 (19.2%)
1-11 mths	153 (10.9%)
12-23 mths	152 (10.9%)
24-47 mths	258 (18.4%)
48-95 mths	350 (25.0%)
96+ mths	219 (15.6%)

This table includes only the women where the OCP exposure time was known.

These groupings of OCP usage time were those used for the logistic regression analysis. These groupings are used in keeping with the findings of Vessey et al (1983) of a RR of 2.2 for CIN in women with more than 8 years OCP usage. The majority of the OCP 'ever-users' (72.7%) have had more than 2 years exposure time.

Table 15 TIME OF OCP EXPOSURE COMPARED TO CYTOLOGY RESULT

Time of OCP use	Cytology Result			
	Negative	Mild	Moderate	Severe
Never	225 (83.6%)	22 (8.2%)	16 (6.0%)	6 (2.2%)
1-11 mths	129 (84.3%)	17 (11.1%)	5 (3.3%)	2 (1.3%)
12-23 mths	133 (87.5%)	11 (7.2%)	8 (5.3%)	0
24-47 mths	219 (84.9%)	25 (9.7%)	13 (5.0%)	1(0.4%)
48-95 mths	291 (83.1%)	32 (9.1%)	20 (5.7%)	7 (2.0%)
96 + mths	189 (86.3%)	22 (10.0%)	3 (1.4%)	5 (2.3%)

Using the log linear model (Goodman Association Test), χ^2 = 0.19, giving a value for p = 0.67. This result shows that there was no association between severity of cytology result and the OCP exposure time.

Table 16 **KRUSKAL-WALLIS TEST COMPARING TIME OF OCP USAGE & HPV16 STATUS**

Status	No.Obs	Median Time	Av.Rank
Negative	926	30.0 mths	693.6
Equivocal	155	27.0 mths	684.0
Positive	317	36.0 mths	724.2
TOTAL	1398	31.0 mths	699.5

This table includes the women who have a known exposure time to the OCP. The value for χ^2 is 1.63 with 2 d.f. resulting in p = 0.44 (adj. for ties).

Table 16.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS FOR TABLE 16

		HPV16 STATUS	
		NEGATIVE	EQUIVOCAL
	EQUIVOCAL	0.73	
HPV16 STATUS	POSITIVE	0.22	0.28

These two tables show that there was no relationship between OCP usage time and HPV16 status either overall (Table 16) or by comparing the individual groups (Table 16.1).

LOGISTIC REGRESSION ANALYSIS.

This was performed with the cytology result being the dependent variable i.e., either normal or abnormal. The possible cofactors that were used included smoking status, HPV16 status (positive or negative), history of oral contraceptive pill usage and the age of the patient. The ages were grouped into 15-24 years, 25-34 years, 35-44 years, 45-54 years and 55 plus. This resulted in 1243 cases being used, 1052 with negative cytology and 191 cases with abnormal cytology.

The only significant cofactor associated with an increased risk of the woman having an abnormal cytology result was smoking. The improvement in the χ^2 value was 6.25 with 2 d.f. giving a value of $p = 0.044$. This value was even more obvious when the 'equivocal' HPV16 status women were included ($p = 0.0042$). There were no other features from the histories available that seemed to have any significant effect on the development of abnormal cytology.

3.2 ABNORMAL CYTOLOGY DATA

TOTAL NUMBER OF WOMEN WITH ABNORMAL CYTOLOGY	216
MEAN AGE	29.4 years
MEDIAN AGE	28 years
RANGE	15-52 yrs
ABNORMALITY RATE	15.3%

Table 17 AGE DISTRIBUTION OF THE ABNORMAL CYTOLOGY GROUP

AGE RANGE (Years)	NUMBER OF WOMEN (plus percentage of group)
15-19	12 (70.6%)
20-24	57 (26.0%)
25-29	62 (16.1%)
30-34	35 (11.1%)
35-39	20 (13.4%)
40-44	18 (22.2%)
45-49	10 (26.3%)
50-54	2 (9.5%)

Table 17 and Graph 2 show the age distribution of the women with cytological abnormalities, and it can be seen that the majority of these women are in the 20-39 years age range.

GRAPH 2 - AGE DISTRIBUTION OF WOMEN & CYTOLOGY RESULT

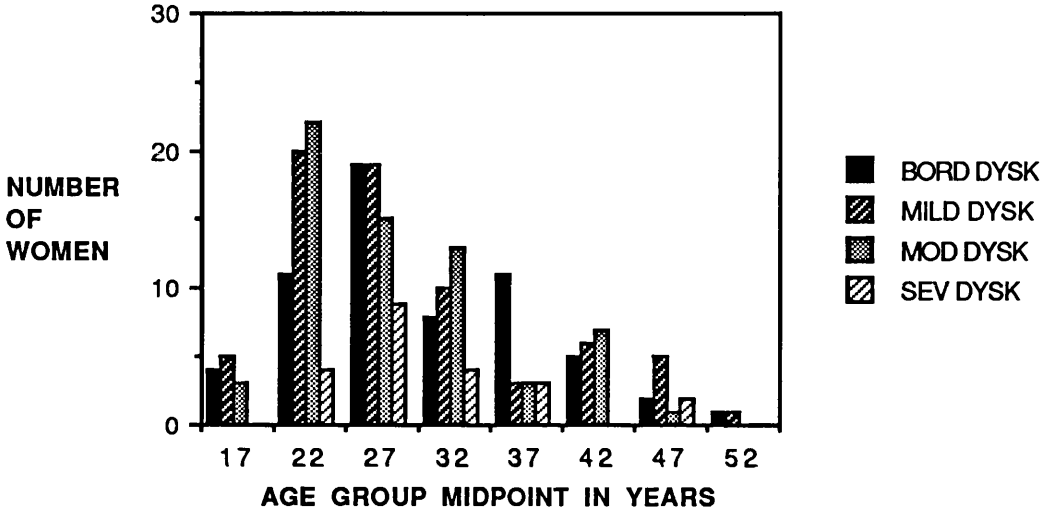


Table 18 CYTOLOGY RESULT WITH HPV16 STATUS

	HPV16 STATUS BY FISH		
	Negative	Equivocal	Positive
BORDERLINE DYSK	38 (62.3%)	7 (11.5%)	16 (26.2%)
MILD DYSK	48 (69.6%)	6 (8.7%)	15 (21.7%)
MODERATE DYSK	39 (60.9%)	6 (9.4%)	19 (29.7%)
SEVERE DYSK	16 (72.7%)	4 (18.2%)	2 (9.1%)

This table is a summary of the results already shown in the screening data section, including row percentages (Table 5).

Table 19 SUMMARY OF THE OUTCOME OF THESE PATIENTS

NORMAL COLPOSCOPY	53 (24.5%)
HPVI	52 (24.1%)
CIN 1	36 (16.7%)
CIN 2	19 (8.8%)
CIN 3	29 (13.4%)
LOST TO FOLLOW-UP	27 (12.5%)

The outcome results (Table 19) are the histopathological diagnoses after colposcopic biopsy but are not complete in that 12.5% of the women were lost to follow-up due to persistent non-attendance for colposcopy or repeat cytology, or they had left the area, or could not be traced. Almost a quarter of these results (24.5%) were falsely positive, especially those with borderline or mild dyskaryosis.

Table 20 **CYTOLOGY RESULTS AGAINST OUTCOME.**

	SMEAR RESULT			
OUTCOME	**BORDERLINE**	**MILD**	**MODERATE**	**SEVERE**
NORMAL	27 (44.2%)	17 (24.6%)	7 (10.9%)	2 (9.1%)
HPVI	10 (16.4%)	18 (26.1%)	22 (34.4%)	2 (9.1%)
CIN 1	10 (16.4%)	14 (20.3%)	10 (15.6%)	2 (9.1%)
CIN2	4 (6.6%)	3 (4.4%)	9 (14.1%)	3 (13.6%)
CIN3	3 (4.9%)	5 (7.3%)	9 (14.1%)	12(54.6%)
NOT KNOWN	7 (11.5%)	12 (17.4%)	7 (10.9%)	1 (4.5%)
TOTAL	61 (100%)	69 (100%)	64 (100%)	22 (100%)

As can be seen from Table 20, CIN 3 correlates with a severely dyskaryotic smear in over 50% of cases.

Table 21 HPV 16 STATUS COMPARED TO OUTCOME

OUTCOME	HPV16 STATUS NEGATIVE	EQUIVOCAL	POSITIVE
NORMAL	40 (75.5%)	7 (13.2%)	6 (11.3%)
HPVI	29 (55.8%)	5 (9.6%)	18 (34.6%)
CIN 1	27 (75%)	3 (8.3%)	6 (16.7%)
CIN 2	12 (63.2%)	2 (10.5%)	5 (26.3%)
CIN 3	19 (65.5%)	4 (13.8%)	6 (20.7%)
NK	16	2	9

This table includes row percentages, i.e., the HPV status with respect to the outcome. Using Armitage's test for trend in the severity of disease compared to HPV16 status, no significance could be found. If the level of disease is as above then:

χ^2 for trend with 1 d.f. = 0.36, giving a p value of 0.59.

If the disease state is divided into normal, minor grade (HPVI +/or CIN1) and major grade (CIN 2-3), then:

χ^2 for trend with 1 d.f. = 2.20, giving a p value of 0.14.

Using the log linear model (Goodman Association test) using each grade of HPV16 status, the χ^2 value with 1 d.f. is 0.33 (p = 0.57).

The HPV16 status did not show any significant relationship to the severity of the histological lesion, unlike the findings of McCance et al (1985), who found HPV16 DNA with increasing frequency as the histological grade of disease became more severe.

Table 22 KRUSKAL-WALLIS TEST FOR AGE AND HPV16 STATUS

HPV16 STATUS	No.Obs	MEDIAN AGE	AV.RANK
Negative	143	27.0 years	106.8
Equivocal	23	28.0 years	104.0
Positive	50	29.0 years	113.6
TOTAL	216	28.0 years	108.5

χ^2 = 0.44 with 1 d.f., giving a value for p = 0.51 (adj. for ties), this is omitting the 'equivocal' group.

Table 22.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS FOR TABLE 22

	HPV16 Status	
	Negative	Equivocal
Negative	0.95	
HPV16 Status		
Positive	0.51	0.73

There was no relationship, in this subpopulation, between HPV16 and increasing age.

Table 23 KRUSKAL-WALLIS TEST FOR AGE AND CYTOLOGY RESULT

RESULT	No.Obs.	MEDIAN AGE	AV.RANK
BORDERLINE DYSK	61	28.0 years	117.9
MILD DYSK	69	27.0 years	104.0
MODERATE DYSK	64	27.0 years	99.1
SEVERE DYSK	22	29.0 years	124.0
TOTAL	216	28.0 years	108.5

χ^2 = 4.57 with 3 d.f. giving a value for p = 0.21(adj. for ties).

Table 23.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS FOR TABLE 23

		CYTOLOGY RESULT		
		Borderline	Mild	Moderate
CYTOLOGY RESULT	Mild	0.22		
	Moderate	0.097	0.66	
	Severe	0.79	0.18	0.96

It has previously been demonstrated that cytological and histological abnormalities are age related (Hakama, 1986). This relationship could not be demonstrated here and may be a reflection of the high number of abnormalities in the younger groups. Tables 22-23 differ from Tables 3-5 by only including patients with a cytological abnormality and comparing the individual cytological abnormality groups, without the influence of the patients with negative cytology.

Table 24 KRUSKAL-WALLIS TEST FOR AGE AGAINST OUTCOME

OUTCOME	No.Obs	MEDIAN AGE	AV.RANK
NORMAL	53	27.0 years	96.4
HPVI	52	27.0 years	91.8
CIN 1	36	27.5 years	91.1
CIN 2	19	27.0 years	86.1
CIN 3	29	30.0 years	108.9
TOTAL	189	27.0 years	95.0

χ^2= 2.78 with 4 d.f., giving a value for p = 0.60 (adj. for ties).

Table 24.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS FOR TABLE 24

		OUTCOME			
		Normal	HPVI	CIN 1	CIN 2
	HPVI	0.75			
	CIN 1	0.66	0.95		
OUTCOME					
	CIN 2	0.43	0.75	0.93	
	CIN 3	0.39	0.19	0.18	0.11

These two tables (24 & 24.1) show that there was no apparent relationship between age and the colposcopic findings.

Table 25 OUTCOME VERSUS SMOKING HISTORY

	NON-SMOKER	SMOKER	EXSMOKER
OUTCOME			
NORMAL	28 (52.8%)	25 (47.2%)	0
HPVI	22 (42.3%)	27 (51.9%)	3 (5.8%)
CIN 1	16 (44.4%)	19 (52.8%)	1 (2.8%)
CIN 2	12 (63.2%)	7 (36.8%)	0
CIN 3	9 (31.0%)	19 (65.5%)	1 (3.5%)
NK	17	10	0

The percentage values are for the rows, i.e., the percentage of smokers to non-smokers within each outcome group. If the Armitage trend test is applied to the outcome and smoking status, there is no significant difference between the two groups using all 5 categories or breaking the data into disease versus normal cervix, normal, minor and major grade of lesion, and also normal, HPVI and any degree of CIN. The Trend test gives a χ^2 value of 1.16 for trend with 1 d.f., which gives a significance value of p=0.28.

Unlike the association between smoking and cytology outcome (Table 7), in this subpopulation there was no significant relationship between smoking and grade of disease (Table 25). However, it must be remembered that the exposure time was unknown, and this may be more significant for the grade of abnormality.

Table 26 HISTORY OF OCP USAGE AND OUTCOME

	NEVER USER	EVER USER	NK
OUTCOME			
NORMAL	13 (24.5%)	40 (75.5%)	0
HPVI	10 (19.2%)	42 (80.8%)	0
CIN 1	7 (19.4%)	29 (80.6%)	0
CIN 2	5 (26.3%)	14 (73.3%)	0
CIN 3	3 (10.3%)	26 (89.7%)	0
NOT KNOWN	6	21	1

Applying Armitage's test for trend does not reveal any significant trend towards disease between OCP 'ever-users' and 'never-users'. If the test

is applied using the 5 grades from normal to CIN 3, the χ^2 value for trend equals 1.30 with 1 d.f., and p = 0.25. This is not improved by grouping the grade of disease into normal and any disease or normal, minor grade and major grade disease. Likewise with the Log linear model (Goodman test for Association), the χ^2 value with 1 d.f. is 0.72 (p = 0.40).

Table 27 **KRUSKAL-WALLIS TEST FOR OCP USAGE TIME IN EVER USERS**

OUTCOME	No.Obs.	MEDIAN TIME	AV.RANK
NORMAL	53	36.0 months	93.0
HPVI	52	24.0 months	89.7
CIN 1	36	24.0 months	96.1
CIN 2	19	24.0 months	86.6
CIN 3	29	54.0 months	112.3
OVERALL	189	36.0 months	95.0

χ^2 = 3.95 with 4 d.f. giving a p value = 0.41 (adj. for ties). This table uses the cases where the time on the OCP was accurately known. Amongst these 189 cases the range of time on the OCP was 0-208 months, with a mean time of 42.1 months and a median of 36 months (s.d. 41.09).

Table 27.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS OF OCP USAGE TIME

	OUTCOME			
	Normal	HPVI	CIN 1	CIN 2
HPVI	0.77			
CIN 1	0.78	0.62		
OUTCOME				
CIN2	0.66	0.85	0.55	
CIN 3	0.14	0.067	0.25	0.11

Table 28 CYTOLOGY RESULT WITH REFERENCE TO OCP USAGE

SMEAR (Row %)	NEVER USER	EVER USER	NK
BORDERLINE	10 (16.7%)	50 (83.3%)	0
MILD	12 (17.2%)	57 (81.4%)	1 (1.4%)
MODERATE	16 (25.0%)	48 (75.0%)	0
SEVERE	6 (27.3%)	16 (72.7%)	0

χ^2 for trend with 1 d.f. = 2.06 giving a value for p of 0.15.

Table 29 TIME OF OCP EXPOSURE COMPARED TO COLPOSCOPIC OUTCOME

TOCP	Normal	HPVI	CIN1	CIN2	CIN3
Never	13 (34.2%)	10 (26.3%)	7 (18.4%)	5 (13.2%)	3 (7.9%)
0-11 mths	7 (30.4%)	6 (26.1%)	5 (21.7%)	3 (13.0%)	2 (8.7%)
12-23 mths	2 (15.4%)	5 (38.4%)	2 (15.4%)	0	4 (30.8%)
24-47 mths	9 (23.7%)	13 (34.2%)	7 (18.4%)	5 (13.2%)	4 (10.5%)
48-95 mths	15 (28.85%)	15 (28.85%)	8 (15.4%)	4 (7.7%)	10(19.2%)
96+	7 (28.0%)	3 (12.0%)	7 (28.0%)	2 (8.0%)	6 (24.0%)

This table shows the row percentages. Using the log linear model (Goodman test for association), the value for χ^2 is 1.82 ($p = 0.18$).

Table 30 HPV16 STATUS WITH REFERENCE TO OCP USAGE

HPV16 STATUS	NEVER	EVER	NK
Negative	34	109	1
Equivocal	4	19	0
Positive	6	44	0

Using a standard χ^2 test for heterogeneity excluding the 'equivocal' group, χ^2 = 2.89, (p = 0.089), this is not improved by including the 'equivocal' group as positives. Using the Goodman test for association, so that all three categories of HPV16 status can be included, the χ^2 value for 1 d.f. is 3.25 giving p = 0.071.

No association could be found between OCP usage and grade of cervical lesion either as ever or never users (Table 26) or if the OCP exposure time was categorised as in Table 29. The median time of OCP usage was greatest in the CIN 3 group (Table 27) though this was not statistically significant either overall or comparing the individual outcome groups (Table 27.1). There was no association between OCP usage and HPV16 status (Table 30) even though protection against unwanted pregnancy may have been expected to affect sexual mores and increased the risk of acquiring genital infections.

LOGISTIC REGRESSION ANALYSIS

NORMAL VERSUS DISEASE

This was applied to the above data using histology as the dependent variable. The two categories for histology were normal or any abnormality. The smear result was excluded from the analysis as were the cases where the outcome was not known and where the period of OCP usage was not accurately known. The 'equivocal' group in the HPV16 status were also excluded for the purpose of this analysis in the first instance. The categorical values are for smoking, HPV16 status, OCP usage and age. The ages of the population have been grouped into 10 year groups starting at age 15 years.

Of the 216 cases in this group, 27 were excluded because their outcome was not known. A further 21 cases were excluded because the HPV16 result was equivocal.

Number of false positive smears included in analysis 46

Number of women with disease 122

FACTOR	LEVELS	No.	COEFFICIENT	Improvement	
				χ^2	p value
HPV16	Negative	127	0.00	4.85	0.028
	Positive	41	0.49		

. In the logistic regression analysis, any abnormal colposcopy was used as a dependent variable. The only significant covariate was HPV16 status with a χ^2 of 4.85 which gives a significance of p = 0.028.

However, if logistic regression is applied to normal colposcopy compared to the presence of CIN only, (again excluding the 'equivocal' group and those where the OCP usage period is not known), no term is found to be significant. This is also the case when those cases with HPVI of the cervix are compared to those with evidence of CIN. In the latter case the p value for improvement in χ^2 = 3.40 which gives a p value of 0.065, above the level of significance.

If the normal cases are compared with the HPVI only group, the only significant finding from this regression was an improvement of the χ^2 value to 8.03 and a value for p = 0.005 for the significance of HPV16 status.

It would be inappropriate to compare these results with the original screening population results because the false negative cytology rate in the original screened population (Section 3.1) is unknown.

3.3 STUDY POPULATION

Women, who were cytologically negative and positive for HPV16 by the FISH method, were invited for colposcopy. A similar number of women who were cytologically normal but negative for HPV16 were also given the same invitation. The following section describes the population of women who accepted this offer. Originally, the women who had a 'equivocal' result on the autoradiograph were classified into the positive group. In this section, the women will be divided into three categories, namely negative, equivocal and positive for HPV16.

The tables that have been included may seem over inclusive but the relevance of them is pertinent to the logistic regression analysis on these findings from the first colposcopy clinic visit.

Table 31 NUMBER OF WOMEN WHO WERE COLPOSCOPED

	FISH RESULT FOR HPV16		
	NEGATIVE	EQUIVOCAL	POSITIVE
NUMBER OF WOMEN	132	77	217
		TOTAL = 426	

It was difficult to recruit a control group for a potentially uncomfortable procedure, especially when they had been classed normal by routine cytology. In the light of the subsequent serial FISH results (Section 3.9), it is probably more appropriate to regard the eventual patient outcome as a cohort population, with HPV16 status as one of the covariates, rather than an age matched population.

Table 32 **AGE RANGE**

MEAN AGE	31.3 years
MEDIAN AGE	29 years
STANDARD DEVIATION	8.4 years
RANGE	17-65 years.

Table 33 **KRUSKAL WALLIS TEST ON THE MEDIAN AGES DEPENDING ON THE HPV16 STATUS**

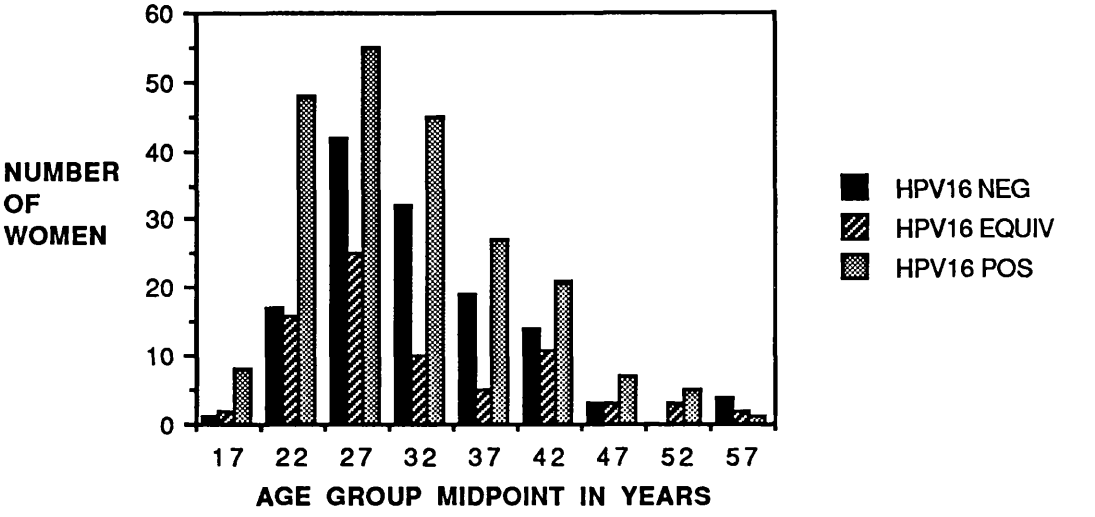
HPV16 STATUS	NUMBER	MEDIAN	AV RANK
NEGATIVE	132	30.0 years	226.4
EQUIVOCAL	77	28.0 years	211.0
POSITIVE	217	29.0 years	206.6
TOTAL	426	29.0 years	213.5

This gives a χ^2 value with 2 d.f. of 2.17 with p = 0.34 (adjusted for ties).

Table 33.1 **P VALUES FOR PAIR WISE WILCOXON COMPARISONS OF TABLE 33**

	HPV16 Status	
	Negative	Equivocal
Equivocal	0.32	
HPV16 Status		
Positive	0.15	0.73

GRAPH 3
AGE DISTRIBUTION OF THE COLPOSCOPY POPULATION BY HPV16 STATUS



The age distribution (Tale 32, Graph 3) was very similar to that of the original screened population with a median age of 29 years. The Kruskal-Wallis test (Table 33) and the Wilcoxon test (Table 33.1) did not reveal any significant difference in ages between the groups.

CONTRACEPTION

This section summarises the current contraceptive methods used by the women in the colposcoped population, the subsequent tables dealing with the oral contraceptive pill use by these women.

Table 34 CURRENT CONTRACEPTION

	HPV 16 STATUS		
	NEGATIVE	EQUIVOCAL	POSITIVE
NONE	26 (20%)	11 (14%)	42 (19%)
HORMONAL	46 (35%)	22 (29%)	85 (39%)
IUCD	18 (14%)	15 (19%)	26 (12%)
BARRIER	36 (27%)	27 (35%)	58 (27%)
STERILISATION	6 (4%)	2 (3%)	6 (3%)
TOTAL	132 (100%)	77 (100%)	217 (100%)

The majority of the colposcoped group were using either hormonal contraception, the IUCD or Barrier methods, as may have been expected in patients originating from screening in the Family Planning Clinics.

Table 35 ORAL CONTRACEPTIVE PILL USE

HPV16 STATUS	NEVER USED	EVER USED	TOTAL
NEGATIVE	11(8.3%)	121 (91.7%)	132 (100%)
EQUIVOCAL	18 (23.4%)	59 (76.6%)	77 (100%)
POSITIVE	32 (14.7%)	185 (85.3%)	217 (100%)

Table 36 KRUSKAL-WALLIS TEST ON TIME OF ORAL CONTRACEPTIVE PILL USAGE (INCLUDES NEVER USERS)

HPV16 STATUS	NUMBER	MEDIAN TIME	AV RANK
NEGATIVE	132	60.0 months	241.5
EQUIVOCAL	77	36.0 months	194.5
POSITIVE	217	42.0 months	203.5
TOTAL	426	48.0 months	213.5

χ^2 with 2 d.f. is 10.24 which gives p = 0.006 (adjusted for ties)

Table 36.1 **P VALUES FOR PAIR WISE WILCOXON COMPARISONS OF TABLE 36**

		HPV16 Status	
		Negative	**Equivocal**
HPV16 Status	**Equivocal**	0.0094	
	Positive	0.0062	0.59

It is interesting to note that there were, percentage-wise, less OCP 'never-users' in the HPV16 negative group compared to the other two groups (Table 35). It is also statistically significant that the OCP time usage was longer within this HPV16 negative group compared to the other two (Table 36). This was demonstrated by the Kruskal-Wallis test comparing the groups overall and including the 'never-users'. This difference was further highlighted by the Wilcoxon test comparing the individual groups. The difference in OCP usage time in the equivocal and positive groups was not significantly different, but it was markedly different in the negative group compared with the other two groups.

Table 37 KRUSKAL-WALLIS TEST ON MEDIAN COITARCHE

HPV 16 STATUS	NUMBER	MEDIAN AGE	AV. RANK
NEGATIVE	132	18.0 yrs	204.7
EQUIVOCAL	77	18.0yrs	214.3
POSITIVE	217	18.0 yrs	218.6
TOTAL	426	18.0yrs	213.4

χ^2 value with 2 d.f. is 1.07 which gives p = 0.58 (adj. for ties)

Table 37.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS OF TABLE 37

	HPV16 Status	
	Negative	Equivocal
Equivocal	0.55	
HPV16 Status		
Positive	0.29	0.77

Table 38 KRUSKAL-WALLIS TEST ON THE MEDIAN NUMBER OF SEXUAL PARTNERS (N.O.P.)

HPV 16 STATUS	NUMBER	MEDIAN N.O.P	AV. RANK
NEGATIVE	132	3	218
EQUIVOCAL	77	3	201.8
POSITIVE	217	4	215.6
TOTAL	426	3.0	214

χ^2 value with 2 d.f. is 0.96 (adjusted for ties) giving p = 0.62

Table 38.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS OF TABLE 38

	HPV16 Status	
	Negative	Equivocal
Equivocal	0.34	
HPV16 Status		
Positive	0.34	1.00

On reviewing sexual behaviour, there was no significant difference in the age of first intercourse between the three groups either overall (Table 37) or comparing the individual groups (Table 37.1). Likewise, there was no significant difference in the number of sexual partners (Tables 38 & 38.1). The same statistical tests were used for the menarche and the cycle length and again there was no significant difference between the various groups. The tables containing this information have not been included.

Table 39 HISTORY OF GENITAL INFECTION

HPV16 STATUS

39.1 ANY INFECTION (Excluding Candida)

	NEGATIVE	EQUIVOCAL	POSITIVE
NO	107 (81.1%)	57 (74.0%)	158 (72.8%)
YES	25 (18.9%)	20 (26.0%)	59 (27.2%)

χ^2 with 1 d.f. for trend =2.86, p = 0.091

χ^2 with 1 d.f. for heterogeneity = 0.29, p = 0.59.

39.2 HISTORY OF CANDIDA ALBICANS

NO	71 (53.8%)	42 (54.5%)	145 (66.8%)
YES	61 (46.2%)	35 (45.5%)	72 (33.2%)

χ^2 with 1 d.f. for trend = 6.40, p = 0.011

χ^2 with 1 d.f. for heterogeneity = 0.87, p = 0.35

HPV16 STATUS

39.3 HISTORY OF NON-SPECIFIC URETHRITIS (CHLAMYDIA)

	NEGATIVE	EQUIVOCAL	POSITIVE
NO	126 (95.5%)	73 (94.8%)	208 (95.9%)
YES	6 (4.5%)	4 (5.2%)	9 (4.1%)

χ^2 with 1 d.f. for trend = 0.044, p = 0.83

χ^2 with 1 d.f. for heterogeneity = 0.11 p = 0.74

39.4 HISTORY OF TRICHOMONAS VAGINALIS

NO	124 (93.9%)	74 (96.1%)	202 (93.1%)
YES	8 (6.1%)	3 (3.9%)	15 (6.9%)

χ^2 with 1 d.f. for trend = 0.17, p = 0.68

χ^2 with 1 d.f. for heterogeneity = 0.73, p = 0.39

39.5 HISTORY OF GENITAL WARTS

NO	125 (94.7%)	70 (90.9%)	199 (91.7%)
YES	7 (5.3%)	7 (9.1%)	18 (8.3%)

χ^2 with 1 d.f. for trend = 0.92, p = 0.34

χ^2 with 1 d.f. for heterogeneity = 0.47, p = 0.49

HPV16 STATUS

39.6 HISTORY OF GENITAL HERPES

	NEGATIVE	EQUIVOCAL	POSITIVE
NO	125 (94.7%)	75 (97.4%)	213 (98.2%)
YES	7 (5.3%)	2 (2.6%)	4 (1.8%)

χ^2 with 1 d.f. for trend = 3.19, p.= 0.074

χ^2 with 1 d.f. for heterogeneity = 0.20, p = 0.65

39.7 HISTORY OF NEISSERIA GONORRHOEA

NO	130 (98.5%)	72 (93.5%)	211(97.2%)
YES	2 (1.5%)	5 (6.5%)	6 (2.8%)

χ^2 with 1d.f. for trend = 0.20, p = 0.65

χ^2 with 1 d.f. for heterogeneity = 3.99, p = 0.046

As HPVI is considered to be one of the commonest sexually transmitted diseases (Campion, 1987), it seemed pertinent to look for a history of any other genital infections. Candida is included, though it is not necessarily a genital infection with the same STD implications as the others in this table. The above table (39.2) shows a trend towards a history of Candida in the HPV16 negative group. The only other venereal infection which showed any statistical value was a history of Gonorrhoea which was more common in the equivocal group (Table 39.7).

PREGNANCY HISTORY

Table 40 TOTAL NUMBER OF PREGANCIES AND HPV16 STATUS

	HPV16 STATUS		
GRAVIDITY	NEGATIVE	EQUIVOCAL	POSITIVE
0	53 (40.1%)	27 (35.1%)	96 (44.2%)
1	30 (22.7%)	17 (22.1%)	47 (21.7%)
2	27 (20.5%)	16 (20.8%)	36 (16.6%)
3	14 (10.6%)	8 (10.4%)	24 (11.1%)
4+	8 (6.1%)	9 (11.6%)	14 (6.4%)
TOTAL	132 (100%)	77 (100%)	217 (100%)

Using the Goodman test of association, χ^2 with 1 d.f. = 0.34, giving p = 0.56.

Table 41 NUMBER OF TERMINATIONS OF PREGNANCY PER WOMAN AND HPV16 STATUS

No/Pt.	NEGATIVE	EQUIVOCAL	POSITIVE
0	97 (73.5%)	54 (70.1%)	137 (63.1%)
1	29 (22.0%)	18 (23.4%)	61 (28.1%)
2	6 (4.5%)	3 (3.9%)	17 (7.9%)
3+	0	2 (2.6%)	2 (0.9%)
TOTAL	132 (100%)	77 (100%)	217 (100%)

Using the Goodman test of association, χ^2 with 1 d.f. = 5.05, giving p = 0.024.

Table 42 NUMBER OF CHILDREN PER WOMAN AGAINST HPV16 STATUS

PARITY	NEGATIVE	EQUIVOCAL	POSITIVE
0	69 (52.3%)	42 (54.5%)	141 (65%)
1	26 (19.7%)	15 (19.5%)	22 (10.1%)
2	24 (18.2%)	12 (15.6%)	42 (19.4%)
3	11 (8.3%)	5 (6.5%)	9 (4.1%)
4+	2 (1.5%)	3 (3.9%)	3 (1.4%)
TOTAL	132 (100%)	77 (100%)	217 (100%)

Using the Goodman test of association, χ^2 with 1 d.f. = 3.26, giving p = 0.071.

Table 43 KRUSKAL WALLIS ON AGE OF FIRST PREGNANCY (EXCLUDING THE NULLIPS) AND HPV16 STATUS

HPV16 STATUS	No.	MEDIAN AGE	AV.RANK
NEGATIVE	81	23.0 years	131.0
EQUIVOCAL	48	23.0 years	135.3
POSITIVE	120	22.0 years	116.9
TOTAL	249	22.0 years	125.0

This gives a χ^2 value with 2 d.f. of 3.07, p = 0.22 (adj. for ties).

Table 43.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS IN TABLE 43

	HPV16 Status	
	Negative	**Equivocal**
Equivocal	0.63	
HPV16 Status		
Positive	0.12	0.076

On review of the three groups' pregnancy history (Tables 40-43), the only significant finding is the association between number of pregnancy terminations and HPV16 positivity (Table 41). The percentage of women undergoing at least 1 abortion was greater in the HPV16 positive group (36.9%) compared to less than 30% in the other 2 groups. There may be a number of reasons for this difference, the most likely is related to unprotected intercourse as opposed to contraception failure. This may be related to the significant differences between the OCP usage in the HPV16 negative group compared to the others (Tables 36 & 36.1). However, the number of terminations is more likely to reflect sexual behaviour which would be linked with an increased risk of acquiring the HPV16 virus. Although the cervix undergoes a great deal of trauma in labour, it does not affect the HPV16 status and as will be seen from the logistic regression analysis (Section 3.5) does not appear to place the cervix at an increased susceptibility to disease. The age of first pregnancy, regardless of its outcome, does not seem to be associated with HPV16 positivity.

SMOKING HISTORY

Table 44 SMOKING HISTORY AND HPV16 STATUS

	HPV 16 STATUS		
	NEGATIVE	EQUIVOCAL	POSITIVE
NO	52 (39.4%)	43 (55.8%)	92 (42.4%)
YES	77 (58.3%)	34 (44.2%)	121 (55.8%)
EX or N/K	3 (2.3%)	0	4 (1.8%)
TOTAL	132 (100%)	77 (100%)	217 (100%)

Using a χ^2 test with 2 d.f. $\chi^2 = 5.071$ giving $p = 0.079$.

Despite the association of smoking and cytology (Table 7), and HPV16 status (Table 8), there was no obvious association between smoking and HPV16 status within the colposcoped group. This suggests that the groups are equally matched for smoking history,

A measure of cigarette smoking exposure is termed 'pack years'. This figure is derived by multiplying the number of cigarettes smoked per day by the number of years the person has smoked and dividing the product by 20.

This next section summarises the pack year findings within the population of all the women who were colposcoped.

Table 45 CIGARETTE CONSUMPTION IN PACK YEARS

	ALL WOMEN	EVER SMOKERS
POPULATION SIZE	426	236
MEDIAN CONSUMPTION	2.00	8.00
MEAN CONSUMPTION	5.96	10.75
STANDARD DEVIATION	9.35	10.31

[RANGE: 0 - 56.0 pack years]

Table 45.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS IN TABLE 45

	HPV16 Status	
	Negative	Equivocal
Equivocal	0.20	
HPV16 Status		
Positive	0.48	0.42

There was no significant difference between the various HPV16 status groups using the Kruskal-Wallis test on the median cigarette consumption in the HPV16 status group. If the non-smokers were included the χ^2 value with 2 d.f. was 1.62, p = 0.44, if the non-smokers were excluded then the χ^2 value with 2 d.f. was 3.50, p = 0.17. The Wilcoxon comparison (Table 45.1) shows that there was no significant difference in cigarette consumption between the 3 groups of HPV16 status.

COLPOSCOPIC FINDINGS

Table 46 **REPEAT CYTOLOGY IN THE STUDY POPULATION**

RESULT	**HPV16 STATUS**		
	NEGATIVE	**EQUIVOCAL**	**POSITIVE**
NEGATIVE	124 (93.9%)	72 (93.5%)	198 (91.3%)
MILD DYSK	7 (5.3%)	4 (5.2%)	11 (5.1%)
MODERATE DYSK	0	0	4 (1.8%)
SEVERE DYSK	1 (0.8%)	1 (1.3%)	4 (1.8%)
TOTAL	132 (100%)	77 (100%)	217 (100%)

Using the Goodman test for association, χ^2 with 1 d.f. is 2.06, giving p = 0.15, which shows no significant difference between HPV16 status and the repeat cytology result.

Table 47 COLPOSCOPIC FINDINGS (INCLUDES BIOPSY RESULTS) AND HPV16 STATUS

	HPV16 STATUS		
RESULT	**NEGATIVE**	**EQUIVOCAL**	**POSITIVE**
NORMAL	117 (88.6%)	53 (68.8%)	164 (75.6%)
HPVI alone	11 (8.4%)	17 (22.1%)	29 (13.4%)
CIN 1	2 (1.5%)	4 (5.2%)	16 (7.4%)
CIN 2	0	0	4 (1.8%)
CIN 3	2 (1.5%)	3 (3.9%)	4 (1.8%)
TOTAL	132 (100%)	77 (100%)	217 (100%)

With the Goodman test of association, the χ^2 value with 1 d.f. was 6.67 giving a p value = 0.01.

The colposcopic findings showed that 7.5% of women had an abnormal cervical smear when repeated, mostly in the mildy dyskaryotic category (Table 46). However, on colposcopy, 21.5% of the women had some degree of abnormality (Table 47). The bulk (85.9%) of these lesions were minor (HPVI +/- CIN 1). The Goodman's test of association showed a significant relationship (p = 0.01) between HPV16 status and the abnormal colposcopic findings.

Table 48 FISH RESULTS ON INITIAL SCREENING AND AT TIME OF FIRST COLPOSCOPY

		ORIGINAL HPV16 STATUS		
		NEGATIVE	EQUIVOCAL	POSITIVE
HPV 16 AT 1ST F.U. VISIT	NEG	102 (80.3%)	50 (65.8%)	134 (62.9%)
	EQUIVOCAL	14 (11.0%)	15 (19.7%)	39 (18.3%)
	POS	11 (8.7%)	11 (14.5%)	40 (18.8%)
	N/K	5	1	4
	TOTAL	132	77	217

[The unknown results are due to the problems in the processing for various reasons. F.U. = Follow-up; N/K = Result not known]

If kappa statistics are applied to Table 48 to measure the agreement between screening visit and first follow-up visit, then the kappa value using all the known results gives k = 0.088. If this is divided into those women with normal colposcopy only then k = 0.10. In women with any grade of disease then k = 0.015. Similarly poor values for levels of agreement are obtained if the results are divided into those women with HPVI only (k = -0.007), those with any grade of CIN (k = 0.05), and dividing

the group into women with a minor grade lesion i.e. HPVI/CIN1 (k = -0.002) or a major grade lesion i.e., CIN 2-3 (k = 0.12). However, the Goodman test for association using the known results gives a χ^2 value with 1 d.f. of 10.73 with p = 0.0011, suggesting some association between the two subsequent results These results fit the findings of the serial FISH results which are discussed more fully in Section 3.9.

Table 48.1 KAPPA RESULTS AND TIME INTERVAL BETWEEN VISITS

The time interval between these two visits was reviewed, and the kappa value derived for each group:

TIME BETWEEN VISITS	No OF PATIENTS	KAPPA VALUE
1-3 months	31	0.17
4-6 months	265	0.062
7-9 months	80	0.060
10+ months	41	-0.049

Most of the cases who comprised the last two categories included women who were originally seen in the colposcopy clinic as self-referrals and fulfilled the criteria for entry into the study. It would appear that the level of agreement between the two results deteriorates with the increase in time between the visits.

3.4 PREVALENCE OF CERVICAL INFECTIONS IN THE CLINIC POPULATION

At the inception of the colposcopy clinic, all the women attending had routine swabs taken as described in section 2.6, in order to assess the prevalence of any asymptomatic genital infections. The number of cytologically and colpscopically normal women who were screened was small compared to those attending for a cytological abnormality. Forty-nine infections were detected in 47 women and they are summarised in this table.

Table 49 HIGH VAGINAL & CERVICAL SWAB RESULTS

		COLPOSCOPIC FINDINGS	
		NORMAL	ABNORMAL
		n= 36	n = 94
1.	C. albicans	3 (8.3%)	12 (12.8%)
2.	Chlamydia trachomatis	1 (2.8%)	8 (8.5%) *
3.	Neisseria gonorrhoea	0	1 (1.1%)
4.	Trichomonas vaginalis	1 (2.8%)	2 (2.1%)
5.	Anaerobes	2 (6.6%)	15 (15.9%)
6	β Haemolytic streptococci	1 (2.8%)	1 (1.1%)
7.	Herpes simplex virus	1 +	1 +

+ Only 5 normal women and 20 women with cervical abnormalities were screened for HSV.

* Two of these patient with Chlamydia trachomatis also had anaerobes detected on a cervical swab.

Candida is a low grade opportunistic infection, often present without causing any symptoms. It may be aggravated by the administration of broad spectrum antibiotics, immunosuppressives or steroids (Emens, 1983). The number of candidal infections was 50% greater for those with abnormal colposcopy (12.8%) compared to the normal group (8.3%). This level of 8.3% within the normal colposcopy group is greater than the 4.3% (12 out of 280) of women attending a screening clinic which was recently reported by Ceddia et al (1990). However, these screened results (Table 49) are nowhere near the 27.4% found by Carne and Dockerty (1990) in women attending an STD clinic with genital warts. Indeed, the results in Table 49 probably reflect the nature of candidiasis that, in many cases, it may be transient and asymptomatic and diagnosed only by this opportunistic screening.

Chlamydia, which is potentially more damaging than candida, is an obligate intracellular parasite possessing many bacterial properties. Chlamydia has been isolated in 0.8-3% of normal cervices (Hilton et al, 1974; Schacter et al, 1975; Fish et al, 1987) and, though our normal group is small, the figure of 2.8% is in keeping with these previous findings. It is interesting that Chlamydia has been identified in 3 times the percentage of the women with abnormal cytology, though this level is relatviely low compared to the 17% for cervical dysplasia and the 25% of those with invasive disease in a study from Finland (Paavonen et al, 1979). The significance of these findings will be dealt with in the discussion of the logistic regression of Section 3.5.

The low level of Gonorrhoea is probably reflected by the fact that the disease, unlike Chlamydia, is usually symptomatic and has decreased in incidence over the past 20-30 years due to national control programmes

(Handsfield, 1990). A similar level of 0.6% (1 out of 168) was found by Carne and Dockerty (1990) in their recent study.

The level of Trichomonas was equal in both groups and probably of no significance except that it can sometimes make cytological interpretation difficult. The results for HSV are difficult to comment upon as there was a paucity of results for technical reasons. The number of asymptomatic anaerobic infections in the abnormal group is over twice the level for the colposcopically normal women, The significance of this finding may well just reflect the wide variety of species that can be responsible for this type of infection. The level of anaerobic and β Haemolytic streptococcal infections was much less than the findings of Goldacre et al (1979) who screened women between the ages of 16 and 47 years attending a Family Planning Clinic. The levels in the normal women for anaerobes was 14.2% and for β Haemolytic streptococci was 5.3%, the population size being 1498.

3.5 LOGISTIC REGRESSION ANALYSIS OF THE STUDY POPULATION

Logistic regression analysis was performed on the above data, using histological status as the dependent variable and looking at the following factors:

History of oral contraceptive pill use: ever or never.

Time of OCP usage, initially this was considered as a continuous variable and then using the following intervals: 0; 1-11 months; 12-23 months; 24-47 months; 48-95 months and over 96 months.

Age at first intercourse was used as a categorical variable, with the groupings being: less than or equal to 15 years of age; 16-17 years; 18-19 years; 20 years and over.

Number of sexual partners was divided into 1, 2-4, 5-9 and 10 or more.

Number of terminations was divided into three groups zero, 1, 2 or more.

Smoking history was divided into ever or never with the pack years being calculated in the usual way of multiplying the number of years smoked by the number smoked per day and dividing the product by twenty.

The age of first pregnancy and first birth were divided into less than or equal to 15 years, 16-20 years, 21-25 years, 26-30 years, 31 years and over. The number of pregnancies were also divided into zero, 1, 2-3, and 4 and over.

The age groupings that were used were the same as those for the logistic regression analysis of the screening and abnormal data.

The other categoricals which were used included history of any genital infections, candida, Non-Specific urethritis (Chlamydia), Trichomonas, genital warts, Herpes simplex, and gonorrhoea.

HPV16 status was included as negative, equivocal and positive.

When the coefficient is zero, the covariate is the factor that is taken as the baseline group. The data was incomplete in 9 normal patients and 1

case of CIN 3, therefore 417 cases are used for the logistic regression analysis.

The data has been analysed in various ways:

1. Normal colposcopy versus histologically proven disease by biopsy.
2. Normal colposcopy versus evidence of HPVI by colposcopic biopsy.
3. Normal colposcopy versus CIN (1-3) by colposcopic biopsy.
4. HPVI alone versus any grade of CIN confirmed by biopsy.
5. Normal colposcopy versus major grade disease (CIN 2/3).
6. Normal colposcopy versus minor grade disease (HPVI, CIN1), confirmed by biopsy.
7. Minor grade disease versus major grade disease.

3.5.1 NORMAL VERSUS DISEASE

Number of normal cases included in the analysis	326 cases
Number of cases with disease	91 cases
Data incomplete	9 cases

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
HPV	Negative	130	0.00	14.34 (2 d.f.)	0.001
	Equivocal	75	0.71		
	Positive	212	0.15		
AGE	15-24	111	0.00	18.29 (4 d.f.)	0.001
	25-34	200	3.68		
	35-44	81	2.99		
	45-54	19	-5.70		
	55+	6	-4.50		
SMOKING	Never	187	0.00	7.54 (1 d.f.)	0.006
	Ever	230	0.35		

Gonorrhoea with 1 d.f. was just outside statistical significance with an improvement of χ^2 to 3.58, p = 0.059.

In this analysis of normal against disease, the latter group includes HPVI and CIN. Overall, HPV16 status is important in comparing the 2 groups and reflects the association (p = 0.01) in Table 47. A history of smoking is also significant and the model also gives weight to those in the younger age groups.

3.5.2 NORMAL VERSUS HPVI ALONE

Number of Normals included in the analysis	326
Number of cases of HPVI alone	57

FACTOR	LEVELS	NO.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
HPV	Negative	126	0.00	9.08 (2 d.f)	0.011
	Equivocal	68	0.86		
	Positive	189	-0.03		
AGE	15-24	98	0.00	11.55	0.021
	25-34	181	3.88		
	35-44	79	3.51		
	45-54	19	-5.66		
	55+	6	-5.20		
H/O WARTS	No	357	0.00	5.48	0.019
	Yes	26	-1.00		

FACTOR	LEVELS	NO.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
SMOKING	Never	175	0.00	5.88	0.015
	Ever	208	0.38		

Gonorrhoea again does not reach significance the improvement in the value of p = 0.088.

In comparing the normal cervix and those with evidence of HPVI alone, the model is again weighted towards the younger age group, which is not unexpected with a peak incidence of HPVI of the cervix in the 20-30 year age group (Campion, 1987). A history of smoking also places a significant risk for these women to develop HPVI. The HPV16 status is significant in comparing these two groups but there is equal weight in the positive and negative group, the significant weighting is in the equivocal group. This could be explained if the equivocal result was due to cross-hybridisation with other HPV types (e.g. HPV 6 & 11) which have been detected in low grade lesions with greater frequency than HPV16 (Smotkin, 1990). A history of genital warts does not appear to be of significance in women developing HPVI lesions on their cervix in this analysis, but the difference in group size of patients with and without a history of genital warts probably explains this discrepancy.

3.5.3 NORMAL VERSUS CIN (ANY GRADE)

Number of cases of CIN 1-3	34
Number of cases of normals	326

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
HPV	Negative	119	0.00	9.03 (2 d.f.)	0.011
	Equivocal	58	0.61		
	Positive	183	0.44		
AGE	15-24	97	0.00	12.31 (4 d.f.)	0.015
	25-34	168	3.70		
	35-44	70	2.09		
	45-54	19	-5.43		
	55+	6	-4.15		
SMOKING	Never	167	0.00	3.87 (1 d.f.)	0.049
	Ever	193	0.83		
GONORRHOEA	No	352	0.00	3.90 (1 d.f.)	0.048
	Yes	8	0.86		

A history of NSU did not quite reach significance with an improved χ^2 value of 3.57 with 1 d.f., $p = 0.06$.

In comparing the normal cervix and the presence of any degree of CIN, the significant correlations include HPV16 status and smoking. The model again is weighted towards the younger age groups. A significant correlation with a history of gonorrhoea reflects the venereal nature of CIN, though the numbers are small in one of the comparison groups.

3.5.4 HPVI ALONE VERSUS CIN OF ANY GRADE

Number of cases of CIN	34
Number of cases of HPVI alone	57

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
H/O WARTS	No	86	0.00	4.04 (1 d.f.)	0.045
	Yes	5	5.24		
H/O NSU	No	88	0.00	6.74 (1 d.f.)	0.009
	Yes	3	-8.92		

The age factor did not reach significance in this group with an improved χ^2 value of 5.25 with 2 d.f., p = 0.072.

The venereal nature of cervical disease, as commented in Section 3.5.3, is further confirmed when comparing HPVI alone and any grade of CIN. This confirms the finding of Walker et al (1983), who found that women with a history of genital warts were at an increased risk of having concurrent CIN. A history of NSU would appear to provide the women with

protection from developing CIN. Although these findings are interesting and lend weight to the venereal origin of CIN, the numbers with a history of warts (5 out of 91) or NSU (3 out of 91) are small and so caution is necessary in trying to find any deeper interpretation of these results.

3.5.5 NORMAL VERSUS MAJOR GRADE DISEASE (CIN2-3)

Number of cases of CIN 2-3	12
Number of normal cases	326

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
GONORRHOEA	No	331	0.00	5.62 (1 d.f.)	0.018
	Yes	7	1.29		
PACK YEARS	Continuous	338	-0.16	4.85 (1 d.f.)	0.028

The Pack year range was 0-56, with a mean of 5.56 pack years from 161 never smokers and 177 ever smokers.

A history of NSU was just outside the level of significance with and improved chi-square value of 3.36 at 1 d.f. giving p = 0.067

A comparison of the normal cases and the presence of major grade disease showed significance for a history of gonorrhoea. The result as regards cigarette consumption in pack years is confusing because an increasing consumption would appear to add protection to the cervix. However, this effect is obviously age-linked and if the analysis is repeated

controlling for age, the effect of pack years disappears. The small number of cases with CIN 2-3, 12 compared to almost 30 times that number of controls, accounts for problems with this analysis and the apparent lack of other significant findings.

3.5.6 NORMAL COLPOSCOPY VERSUS MINOR GRADE LESIONS (HPVI & CIN1)

Cases of minor grade disease	79
Cases of normal colposcopy	326

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
HPV	Negative	128	0.00	12.92 (2 d.f.)	0.002
	Equivocal	72	0.74		
	Positive	205	0.14		
AGE	15-24	105	0.00	15.84 (4 d.f.)	0.003
	25-34	194	3.67		
	35-44	81	3.11		
	45-54	19	-5.67		
	55+	6	-4.51		
SMOKING	Never	181	0.00	8.29 (1 d.f.)	0.004
	Ever	224	0.39		

No other factors were of any significance.

Analysis of normal colposcopy against minor grade lesions (HPVI and CIN 1) shows the usual 3 covariates of: HPV16 status; a history of 'ever' smoking; the younger age groups, as being significant.

3.5.7 MINOR GRADE VERSUS MAJOR GRADE DISEASE

Number of cases of major grade disease (CIN2-3)	12
Number of cases of minor grade disease (HPVI/CIN1)	79

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
PACK YEARS	Continuous	91	-0.20	7.77 (1 d.f.)	0.005

The pack year range was 0-52.0 with a mean value of 6.66 pack years for this population which comprised 32 never smokers and 59 ever smokers.

There was no other significant difference between the two groups.

A comparison of minor versus major grade lesions would again imply that increased cigarette consumption protects the cervix from developing a major grade lesion. This effect is not removed by controlling for age but probably reflects the widely varying median and mean values for cigarette consumption in pack years. [Minor Grade: Mean = 7.42 pack years (py), with a Median of 5.0 py and a range of 0 - 52.0; Major Grade: Mean = 1.92 py, with a Median of 1.0 py and a range of 0 - 8.0 py.] Caution must be applied in placing too much emphasis on this result due to the relatively small numbers in the major grade group.

3.6 COMPARISON OF FILTER IN SITU HYBRIDISATION & SOUTHERN BLOTTING HYBRIDISATION

In order to compare the performance of filter in-situ hybridisation and Southern blotting, a number of samples of cervical cell scrapes were divided into 2 aliquots. One aliquot was used for the FISH method as described in section 2.8 and 2.9, the other one underwent DNA extraction and subsequent Southern blotting hybridisation as in section 2.10. The results of these procedures are summarised in this section.

Table 50 AMOUNT OF DNA EXTRACTED FOR SOUTHERN BLOTTING

SAMPLE NUMBER	= 165
MEAN AMOUNT OF DNA EXTRACTED	= 9.24 μg
MEDIAN AMOUNT	= 5.90μg
RANGE	= 0.1-49.8μg
[Standard Deviation = 10.27μg]	

This table is purely descriptive, but the overall distribution is given in Table 52.1 and demonstrated in Graph 4. [The sample in the group where the midpoint is 32.5μg, includes all those samples where 30μg or more DNA was extracted.]

Table 51 COLPOSCOPIC FINDINGS

NORMAL COLPOSCOPY	= 102	(61.8%)
HPVI CONFIRMED BY BIOPSY	= 38	(23.0%)
CIN CONFIRMED BY BIOPSY	= 25	(15.2%)

Table 52 AMOUNT OF DNA EXTRACTED PER GROUP

NORMAL mean = 9.05µg, median = 6.05µg (Range 0.13-40.95).

HPVI mean = 10.10µg, median = 5.1µg (Range 0.10-49.75).

CIN mean = 8.69µg, median = 6.20µg (Range 0.23-34.1).

Table 52.1 RANGE OF THE AMOUNT OF DNA EXTRACTED (Graphs 4 & 5)

AMOUNT OF DNA (µg)	NUMBER OF SAMPLES			
	ALL	NORMAL	HPVI	CIN
0.01 - 5.00	74 (44.8%)	43 (42.2%)	19 (50%)	12 (48%)
5.01 - 10.00	44 (26.7%)	32 (31.4%)	8 (21.1%)	4 (16%)
10.01 - 15.00	11 (6.7%)	3 (2.9%)	4 (10.5%)	4 (16%)
15.01 - 20.00	15 (9.1%)	10 (9.8%)	2 (5.3%)	3 (12%)
20.01 - 25.00	5 (3.0%)	4 (3.9%)	0	1 (4%)
25.01 - 30.00	5 (3.0%)	4 (3.9%)	1 (2.6%)	0
> 30.01	11 (6.7%)	6 (5.9%)	4 (10.5%)	1 (4%)
TOTAL	165	102	38	25

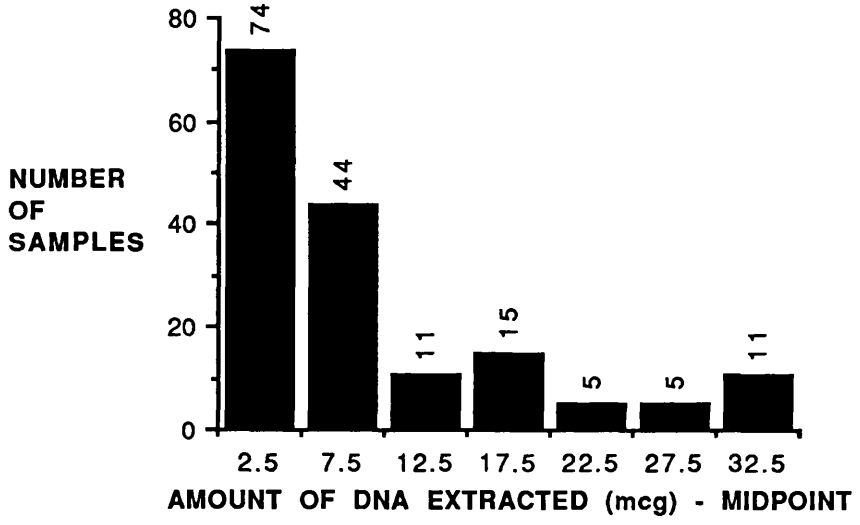
Graph 5 shows the distribution of extracted DNA broken down into the individual colposcopic groups. In the majority of cases (71.5%), less than 10μg of DNA was extracted and this is further highlighted in Table 50.2 and Graph 6. The significance of this low yield of DNA lies in the fact that 10μg of DNA was used for Southern blotting where it was available. As a result, 71.5% of cases had suboptimal levels of DNA for the Southern method and 44.8% had less than 5μg. Table 52 shows the mean and median amounts of DNA obtained within each colposcopic group.

Table 52.2 DISTRIBUTION OF SAMPLES WHERE THE AMOUNT OF EXTRACTED DNA WAS LESS THAN 10μg. (Graph 6)

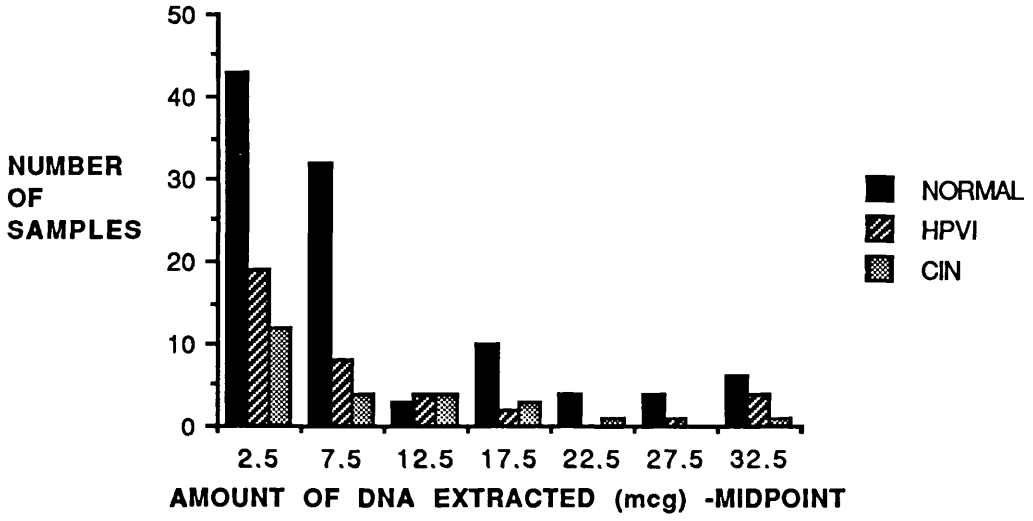
AMOUNT OF DNA (μg)		NUMBER OF SAMPLES			
		ALL	NORMAL	HPVI	CIN
0.01 - 1.00	(1)	26 (15.7%)	15 (14.7%)	7 (18.4%)	4 (16%)
1.01 - 2.00	(2)	14 (8.5%)	11 (10.8%)	2 (5.3%)	1 (4%)
2.01 - 3.00	(3)	13 (7.9%)	6 (5.9%)	5 (13.2%)	2 (8%)
3.01 - 4.00	(4)	10 (6.1%)	5 (4.9%)	2 (5.3%)	3 (12%)
4.01 - 5.00	(5)	11 (6.6%)	6 (5.9%)	3 (7.9%)	2 (8%)
5.01 - 6.00	(6)	10 (6.1%)	7 (6.9%)	3 (7.9%)	0
6.01 - 7.00	(7)	13 (7.9%)	10 (9.8%)	1 (2.6%)	2 (8%)
7.01 - 8.00	(8)	6 (3.6%)	4 (3.9%)	1 (2.6%)	1 (4%)
8.01 - 9.00	(9)	5 (3.0%)	5 (4.9%)	0	0
9.01 - 10.00	(10)	10 (6.1%)	6 (5.9%)	3 (7.9%)	1 (4%)
	TOTAL	118 (71.5%)	75 (73.5%)	27 (71.1%)	16 (64.0%)

The percentages are of the totals in Table 52.1.

GRAPH 4 - HISTOGRAM OF TOTAL AMOUNT OF DNA EXTRACTED



GRAPH 5 - AMOUNT OF DNA EXTRACTED PER COLPOSCOPIC GROUP



GRAPH 6 - DISTRIBUTION OF SAMPLES WHERE EXTRACTION WAS LESS THAN 10 mcg

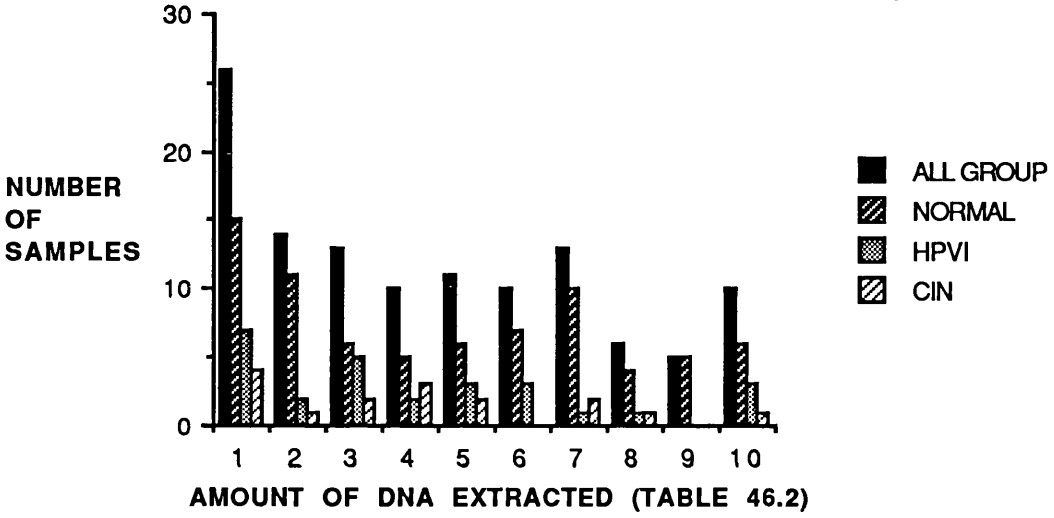


Table 53 KRUSKAL WALLIS TEST COMPARING MEDIAN AMOUNT OF DNA EXTRACTED IN THE DIFFERENT COLPOSCOPIC GROUPS.

	No.Obs	Median amount of extracted DNA	Av.Rank
	102	6.10μg	83.7
	38	5.10μg	80.2
	25	6.20μg	84.4
TOTAL	165	5.90μg	83.0

χ^2 with 2 d.f.= 0.17, which gives p = 0.92, suggesting that there is no significant difference in the amount of DNA extracted within the three groups.

Table 53.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS OF TABLE 53.

	Colposcopic Group	
	Normal	HPVI
HPVI	0.73	
Colposcopic Group		
CIN	0.98	0.66

Using these two methods of comparison (Table 53 & 53.1), there was no statistical difference between the amount of extracted DNA in these groups both overall and comparing group by group.

Table 54 FISH RESULTS COMPARED TO SOUTHERN BLOTTING

	SOUTHERN BLOT FOR HPV16		
FISH RESULT FOR HPV16	**NEGATIVE**	**EQUIVOCAL**	**POSITIVE**
NEGATIVE	**87**	1	**29**
EQUIVOCAL	17	1	5
POSITIVE	**8**	0	**17**

To measure the level of agreement between the two methods, Kappa statistics have been used (see Section 2.13). The values have been calculated just using the positives and the negatives in each category, i.e. omitting the equivocal groups. In this group using all the results regardless of colposcopic findings, **Kappa = 0.32.**

[If the equivocal group is reclassified as Positive, then the Kappa value is 0.21, if the same group is classified as negative then, this value is 0.30.]

This level of agreement (K=0.32) suggest that the agreement between Southern blotting and FISH is greater than would be expected by random chance but not good enough to suggest really good agreement and the level one would like to see if there is good correlation between the tests.

Table 55 KRUSKAL-WALLIS TEST ON THE MEDIAN AMOUNT OF DNA EXTRACTED WITHIN EACH GROUP

[S F]	No.Obs	Median Amount	Av.Rank
[- -]	87	5.18µg	67.0
[- +]	8	4.24µg	57.2
[+ -]	29	5.90µg	72.7
[+ +]	17	9.15µg	95.1
TOTAL	141	6.05µg	71.0

χ^2 = 7.74 with 3 d.f. giving p = 0.052 (adj. for ties). This figure is altered if the figures for the results that agree and disagree are compared.

[S = Southern result, F = FISH result)

104 agree	6.08µg	71.6
37 disagree	5.80µg	69.4

χ^2 = 0.08 with 1 d.f. giving p = 0.78.

Table 55.1 P VALUES FOR PAIR WISE WILCOXON COMPARISONS OF TABLE 55

		Results [S F]		
		[- -]	[- +]	[+ -]
	[- +]	0.57		
Results [S F]	[+ -]	0.51	0.29	
	[+ +]	0.01	0.029	0.62

Comparing the median amount of extracted DNA overall depending on the Southern and FISH result, the differences between the groups do not

quite reach a level of significance. However, in Table 55.1, it can be seen that there is a marked difference in the amount of material in the groups where both results were positive and both were negative.

Table 56 COMPARISON OF AMOUNTS OF DNA EXTRACTED RELATIVE TO THE SOUTHERN BLOT RESULT

These comparisons are made using the Kruskal Wallis test to compare the median amount of DNA extracted in μg in the various results groups.

Table 56.1 A COMPARISON OF ALL THE SOUTHERN BLOT RESULTS.

	No.Obs	Median Amount (μg)	Av.Rank
Negative	112	4.94	76.4
Positive	51	7.33	94.4
TOTAL	163	5.90	82.0

χ^2 = 5.11 with 1 d.f., giving p = 0.024 [If the equivocal groups are included which accounts for 2 results in the Southern blotting group, the χ^2 value is 5.12 with 2 d.f. p = 0.78.]

If the results are divided into groupings depending on the amount of DNA extracted then the following results are observed.

Table 56.2 RANGE OF THE AMOUNT OF DNA EXTRACTED

AMOUNT OF DNA EXTRACTED	NUMBER OF SAMPLES
0.1-2.5μg	48
2.6-3.0μg	26
5.1-7.5μg	28
7.6-10.0μg	16
10.1+μg	47

Table 56.3 SOUTHERN BLOT RESULTS COMPARED TO THE AMOUNT OF DNA EXTRACTED

AMOUNT OF DNA	NEGATIVE	EQUIVOCAL	POSITIVE
0.1-2.5μg	38 (80.9%)	[1]	9 (19.1%)
2.6-5.0μg	19 (73.1%)	0	7 (16.9%)
5.1-7.5μg	17 (60.7%)	0	11 (40.7%)
7.6-10.0μg	10 (62.5%)	0	6 (37.5%)
10.1+μg	28 (60.9%)	[1]	18 (39.1%)

Using the Goodman test for association, χ^2 with 1 d.f. is 4.84 giving a value for p = 0.028. Using the Armitage test for trend excluding the equivocal group, a similar value is obtained (χ^2 with 1 d.f. = 4.84, p = 0.028).

Table 56.1 looks at the amount of DNA extracted with relation to the Southern blot result and using the Kruskal-Wallis test on the median

amounts of DNA, it would appear that a positive result is associated with a significantly greater amount of extracted DNA ($p = 0.024$). The number of samples with specific weights is shown in Table 56.2, and then tabled against the Southern blot results (Table 56.3). Applying both the Armitage test for trend and the Goodman test for association, a significance of $p = 0.028$ is obtained. This implies that positivity by Southern blotting is related to the increasing amounts of DNA at these suboptimal levels.

Table 57 **FISH RESULTS RELATIVE TO THE AMOUNT OF DNA EXTRACTED FOR THE SOUTHERN BLOT**

AMOUNT OF DNA	NEGATIVE	EQUIVOCAL	POSITIVE
0.1-2.5μg	36 (75%)	9 (18.75%)	3 (6.25%)
2.6-5.0μg	19 (73.1%)	4 (15.4%)	4 (11.5%)
5.1-7.5μg	20 (71.4%)	4 (14.3%)	4 (14.3%)
7.6-10.0μg	9 (56.3%)	1 (6.2%)	6 (37.5%)
10.1+μg	33 (70.2%)	5 (10.6%)	9 (19.1%)

Using the Goodman test for association, χ^2 with 1 d.f. is 2.56 giving $p=0.11$. Interestingly, if this test is repeated using the DNA values for less than 10μg, the χ^2 with 1 d.f. is 4.20, with $p = 0.040$.

It could be expected that the amount of DNA present of the filter is equivalent to the amount used for the Southern blot as the original sample was divided into two equal aliquots. If one extrapolates the amount of DNA extracted for the Southern blot method and compares it with the FISH

results as above (Table 57), then the FISH positivity also seems to be related to the increasing amount of DNA when the amount is less than 10μg, as shown by the Goodman test for association (p = 0.04).

Tables 58 COMPARISON OF THE AMOUNT OF DNA EXTRACTED AND THE SOUTHERN BLOT RESULT WITHIN EACH COLPOSCOPY GROUP

Table 58.1 COMPARISON WITHIN THE NORMAL GROUP

	No.Obs	Median Amount (μg)	Av.Rank
Negative	67	5.18	46.7
Positive	33	7.45	58.2
TOTAL	100	6.05	50.2

χ^2 = 3.43 with 1 d.f. which gives p = 0.064.

[2 Equivocal results are not included.]

Table 58.2 COMPARISON WITHIN THE HPVI GROUP

	No.Obs	Median Amount (μg)	Av.Rank
Negative	30	5.10	19.2
Positive	8	5.05	20.5
TOTAL	38	5.10	19.5

χ^2 = 0.08 with 1 d.f. giving p = 0.78.

Table 58.3 COMPARISON WITHIN THE CIN GROUP

	No.Obs	Median Amount (μg)	Av.Rank
Negative	15	3.40	11.3
Positive	10	10.32	15.6
TOTAL	25	6.20	13.0

χ^2 with 1 d.f. = 2.08, giving p = 0.15.

Table 58 compares the amount of DNA extracted amongst the individual colposcopic groups (also displayed in Graph 5). Although the results in Table 56.1 show a significant difference between positive and negative results for the amounts of extracted DNA, this significance is not maintained when the amounts of DNA extracted are separated into the 3 colposcopic groups. It is interesting to note that the median amounts of extracted DNA in the CIN group differs widely (Table 58.3), though this is not statistically significant. The reason for this discrepancy is due to the small size of the group.

COMPARISON OF THE SOUTHERN BLOT AND FISH RESULTS WITHIN THE DIFFERENT COLPOSCOPIC GROUPS

This section shows the level of agreement between the FISH method and the Southern blotting methods for identifying HPV16 within the different colposcopic groups. The tables show the number of samples tested with their respective results, and the median amount of DNA extracted within each group. The kappa value as a measure of agreement is given excluding the equivocal group. The values in parenthesis refer to the kappa value for the equivocal group being included as positive and also negative. The median amount (μg/ml) of DNA extracted per group is given in the individual tables. The Kruskal Wallis test has been used to compare these median values and the chi-square value refers to this comparison.

Table 59 COMPARISON OF FISH AND SOUTHERN BLOT RESULTS IN THE COLPOSCOPICALLY NORMAL GROUP

		SOUTHERN BLOT		
		NEGATIVE	EQUIVOCAL	POSITIVE
FISH RESULTS	NEGATIVE	54 [5.5μg]	1	22 [6.4μg]
	EQUIV	7 [3.5μg]	1	4 [10.9μg]
	POSITIVE	7 [6.9μg]	0	7 [9.1μg]

Kappa = 0.17 [if Equivocal was negative k=0.15; if positive k=0.15]

χ^2 with 3 d.f on the median amounts of extracted DNA = 4.45, p = 0.22 (Equivocals excluded)

Table 60 COMPARISON OF FISH AND SOUTHERN BLOT RESULTS IN THOSE WOMEN WITH HPVI ON COLPOSCOPIC BIOPSY

		SOUTHERN BLOT	
		NEGATIVE	POSITIVE
FISH RESULT	NEGATIVE	**24** [5.5μg]	**3** [2.7μg]
	EQUIV	6 [4.4μg]	0
	POSITIVE	**0**	**5** [9.2μg]

Kappa = 0.71 [if Equivocal was negative k=0.73; if positive k=0.37]

χ^2 with 2 d.f. on the median amount of DNA = 3.24 giving p=0.20 (Equivocal group excluded).

Table 61 COMPARISON OF FISH AND SOUTHERN BLOT RESULTS IN THOSE WOMEN WITH CIN ON COLPOSCOPIC BIOPSY

		SOUTHERN BLOT	
		NEGATIVE	POSITIVE
FISH RESULT	NEGATIVE	**9** [3.4μg]	**4** [10.4μg]
	EQUIV	4 [5.6μg]	1 [1.1μg]
	POSITIVE	**2** [1.98μg]	**5** [13.3μg]

Kappa = 0.38 [if Equivocal is negative k=0.39; if positive k=0.19]

χ^2 with 3 d.f. on the median amount of extracted DNA per group (excluding the Equivocal) = 3.68, p = 0.30.

Table 62 COMPARISON OF FISH AND SOUTHERN BLOT RESULTS IN THOSE WOMEN WITH ANY GRADE OF ABNORMALITY

		SOUTHEN BLOT RESULT		
		NEGATIVE	EQUIVOCAL	POSITIVE
FISH RESULT	NEGATIVE	**33** [4.95μg]	0	**7** [3.2μg]
	EQUIV	10 [4.95μg]	0	1 [1.05μg]
	POSITIVE	**2** [1.98μg]		**10** [11.25μg]

Kappa = 0.58 [if Equivocal is negative k=0.57; if positive k=0.32]

χ^2 value with 3 d.f. on the median amount of extracted DNA is 5.04, p=0.17 (adj. for ties)

If one compares the median amount of extracted DNA in all of these three groups (i.e. normal colposcopy, HPVI only and CIN), where the FISH/Southern result agrees and disagrees, there is no significant difference in the amounts of DNA extracted between these groups (Table 55).

The FISH and Southern results in the three different colposcopic groups have been compared (Tables 59-61). There is no significant difference in the median amounts of extracted DNA in each group (i.e., the

4 cells -,-; +,-; -,+; +,+). However, in all the false negative FISH cases the median amount of extracted DNA is less than when both the FISH and Southern results are both positive (Normal 6.4μg against 9.1μg; HPVI 2.7μg against 9.2μg; CIN 10.4μg against 13.3μg). Although 2.7μg of extracted DNA is 3 times less than the 9.2μg in the HPVI group (Table 60), this result does not reach statistical significance. In the Normal group (Table 59), a Kappa value of 0.17 is not very encouraging for agreement between the two methodologies. In the HPVI alone group (Table 60), the Kappa value of 0.71 shows much better agreement and the level of result one would expect to see if the correlation between the two methods is good. The result for the HPVI group suffers from the false negative FISH results. The Kappa value for any degree of CIN (Table 61) is 0.38, suggesting relatively poor agreement. This level of agreement is improved (kappa = 0.58) when looking at the results from any abnormality regardless of severity (Table 62).

Table 63 COMPARISON OF THE DEGREE OF FISH POSITIVITY & SOUTHERN BLOT RESULT

The FISH results were reappraised and marked according to the positivity of the signal on the autoradiographs for 24 hour and 7 day exposure. This table represents the findings. The value for the FISH result is classified from negative to 4 plus.

TABLE 63

		FISH RESULT					
		NEGATIVE	P/M	1+	2+	3+	4+
SOUTHERN RESULT	NEGATIVE	87	17	7	1	0	0
	EQUIVOCAL	1	1	0	0	0	0
	POSITIVE	29	5	9	4	3	1

Using the Goodman test of association, $\chi^2 = 17.61$ with 1 d.f. which gives a value for $p < 0.001$. Though there were some discrepancies, with an increasingly positive signal by the FISH method, the association with the Southern blot result is enhanced.

Table 64 SUMMARY OF COLPOSCOPIC FINDINGS AND FISH RESULTS

FISH RESULTS	NORMAL	HPVI	CIN
NEGATIVE	77 (75.5%)	27 (71.1%)	13 (52%)
EQUIVOCAL	12 (11.8%)	6 (15.8%)	5 (20%)
POSITIVE	13 (12.7%)	5 (13.1%)	7 (28%)
TOTAL	102 (100%)	38 (100%)	25 (100%)

Using the Goodman test for association on Table 64, the χ^2 value with 1 d.f. is 4.30 which gives p = 0.038, suggesting some association between HPV16 status by FISH and the presence of disease.

Table 65 **SUMMARY OF COLPOSCOPIC FINDINGS AND SOUTHERN BLOT RESULT**

SOUTHERN BLOT	NORMAL	HPVI	CIN
NEGATIVE	67 (65.7%)	30 (78.9%)	15 (60%)
EQUIVOCAL	2 (1.9%)	0	0
POSITIVE	33 (32.4%)	8 (21.1%)	10 (40%)
TOTAL	102 (100%)	38 (100%)	25 (100%)

Using the Goodman test for association, χ^2 with 1 d.f. = 0.011, p = 0.92.

It is interesting to review Tables 64 and 65. The positivity rate for HPV16 DNA is higher in the Southern group than in the Fish group, regardless of the colposcopic findings. With either methods, the level of positivity for HPV16 in the CIN group is lower than in some series (Durst et al, 1983; McCance et al, 1985; Schneider et al, 1985), though the histological grade of the lesion has not been tabled. In the HPVI alone group, although the level of positivity was only 21%, it must be emphasized that no other viral typing was performed.

The positivity rate for HPV16 in the colposcopically normal cervix was also higher by Southern blotting compared to FISH. The 22.5% positivity rate in the cytologically negative population (Table 5.1) could, therefore, be interpreted as an underestimate.

3.7 DILUTIONAL EXPERIMENTS

1. FILTER IN SITU HYBRIDISATION METHOD

This was performed to assess the sensitivity of this method. Cells containing a high copy number of HPV16 DNA (50-100 copies per cell) and cells containing a low copy number (5-10 copies per cell) were used. The concentration of the cells within a small volume was measured by counting the cells using a haemocytometer and extrapolating that figure to the volume from which the sample was taken. By a serial dilution of a measured aliquot of suspension containing the cells, an approximate amount of cells was obtained. These were then treated in the usual way once they had been trapped onto the nitrocellulose filter.

Table 66 CELL CONTENT PER FILTER

High copy	No. cells per filter	Low copy	No. cells per filter
H_0	$1.45.10^6$	L_0	10^7
H_1	$7.25.10^5$	L_1	10^6
H_2	$7.25.10^4$	L_2	10^5
H_3	$7.25.10^3$	L_3	10^4
H_4	$7.25.10^2$	L_4	10^3
H_5	$7.25.10$	L_5	10^2

The filters were hybridised with linear HPV16 DNA labelled with ^{32}P and exposed for 24 hours and then 7 days. The photograph (Illustration 2) shows the results of this experiment.

L = Filters with low copy number cells
H = Filters with high copy number cells.

The number of cells drawn through the filter by suction in the FISH method is shown in Table 66. It is reasonable to expect a random distribution of cells on each filter. Therefore, the number is halved in each case to give the number of cell on each half filter. In the series of filters using high copy number cells, the signal on H_2 with 36,250 cells ($7.25.10^4$ divided by 2) is easily picked up after 7 days exposure and that on the H_3 filter with ten time less number cells, gives a weak signal (Illustration 2). This may be improved with increased exposure. However, using low copy number cells per filter the L_1 signal only appears after 7 days when 500,000 low copy cells per half filter are present. Therefore, if the cell yield from the cervical scrape is also low, false negative results as compared to the Southern blotting results will be obtained.

2. RECONSTRUCTION EXPERIMENT

This experiment was performed to assess the sensitivity of the Southern blot method. The following amounts of Linear HPV16 DNA were digested along with 10µg of brain DNA using BAM H1 for 4 hours at 37°C:

1pg, 10pg, 50pg, 100pg, 150pg, 200pg.

After digestion, the DNA was precipitated and loaded onto an agarose gel (0.8%). The gel was run overnight at 20 volts. The gel was depurinated for 15 minutes, denatured for 60 minutes and then neutralised for a further 60 minutes. This was then set up as a Southern blot. Linear HPV16 DNA was also used in the same increments as above. The nitrocellulose filter was air dried and baked at 80°C in a vacuum oven for 1 hour.

RESULTS

1, 10, 50, 100, 150, 200pg of HPV16 were digested:

1. with Brain DNA and BAM H1 = Reconstruction autoradiograph
2. with BAM H1 whilst being separated from PAT16 (i.e. linear)

Both were run on gels and probed with an HPV16 probe.

These results show the method to be sensitive to extremely small amounts of viral DNA, whether in the pure linear form or when added to other DNA. In the autoradiographs (Illustration 3), 1pg of linear HPV16 DNA gave a characteristic band after 7 days, though the 10pg was much stronger. In the reconstruction experiment, 10pg gives a band on the autoradiograph, as does 1pg though it is much weaker. This level of sensitivity is to be expected from a method generally regarded as the 'gold standard' for HPV detection.

ILLUSTRATION 2

SERIAL DILUTIONS.

24 HOUR EXPOSURE

7 DAY EXPOSURE

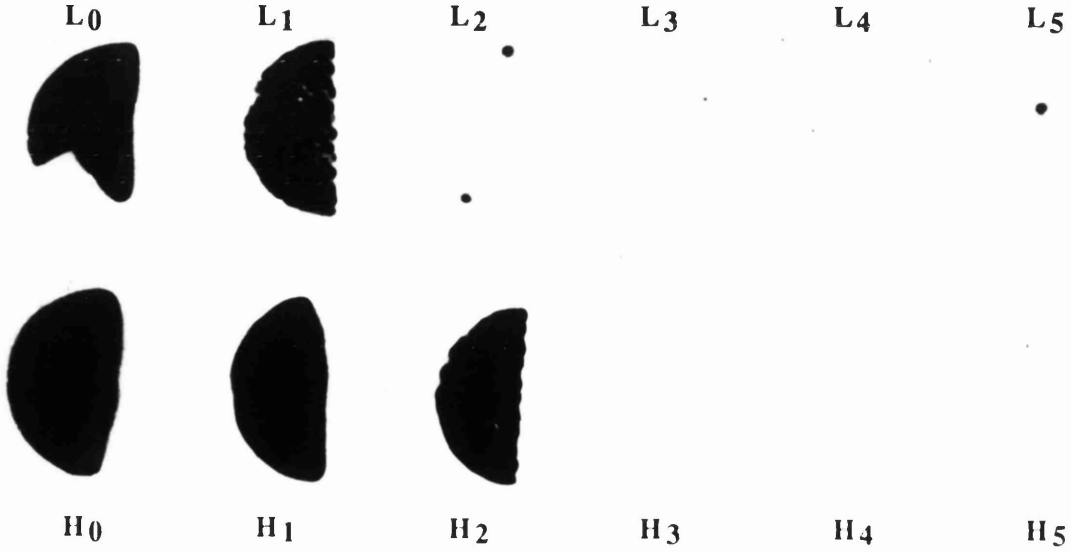
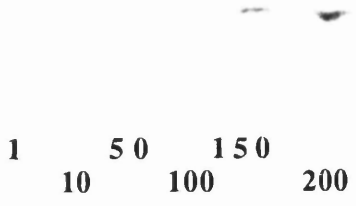


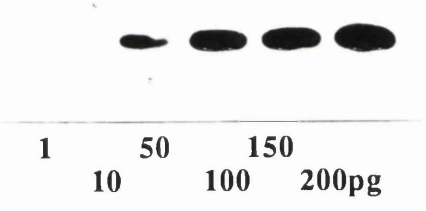
ILLUSTRATION 3

OVERNIGHT EXPOSURE

RECONSTRUCTION



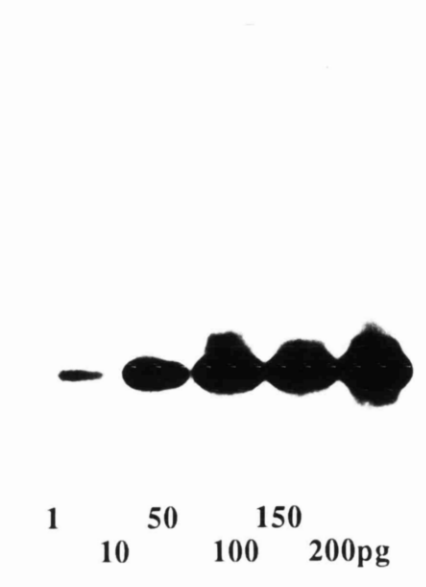
LINEAR HPV16



7 DAY EXPOSURE

RECONSTRUCTION

LINEAR HPV16



3.8 SUB GROUP OF PATIENTS USED IN SERIAL HPV16 RESULTS

During the course of the study looking at women with a colposcopically normal cervix, two further groups of women were reviewed prospectively. One group included the women found to have histological HPVI changes alone confirmed by biopsy from the original study group plus similar cases who were referred to our clinic with a cytological abnormality. The other group included women who had been referred to the clinic and found to have a false positive cervical smear result. These women were reviewed on a 6 monthly basis by repeat cytology, colposcopy and FISH from a cervical scrape. These groups of women are included at this point as their serial FISH results will be used for analysis in Section 3.9.

A brief summary of the cases is presented.

Number of cases	207
Mean age	29.01 years
Median age	26 years

Range 16-63 years with a standard deviation of 8.8 years.

Table 67 REFERRAL CYTOLOGY & FINDINGS

	HPVI ALONE	FALSE POSITIVE SMEAR
BORDERLINE DYSKARYOSIS	39	36
MILD DYSKARYOSIS	69	22
MODERATE DYSKAROSIS	27	11
SEVERE DYSKARYOSIS	2	1
TOTAL	137 (66.2%)	70 (33.8%)

Table 68 ORIGINAL FISH FINDINGS FOR HPV16

	NEGATIVE	EQUIVOCAL	POSITIVE
HPVI ALONE	87 (63.5%)	20 (14.6%)	30 (21.9%)
FALSE POSITIVES	52 (74.3%)	9 (12.85%)	9 (12.85%)
TOTAL	139 (67.1%)	29 (14.0%)	39 (18.9%)

There was no significant difference in the median ages between the different HPV16 status groups using the Kruskal Wallis test. However, the age of the women with the moderately dyskaryotic smear group was significantly lower than in the other groups and using the Kruskal Wallis test on the median ages compared with the cytology result gave a value of 11.86 for χ^2 with 1 d.f. $p = 0.003$.

If the kappa value, for agreement between the first 2 filter in-situ hybridisation results for each subject, is calculated for this cohort a value of 0.16 is obtained. If this group (Table 66) and the colposcopy patients (Table 31) from the original screening are included together, the kappa value for agreement between the first two results is 0.32.

3.9 SERIAL HPV16 RESULTS USING THE FISH METHOD

These results are taken from patients already in the study group plus those described in section 3.8. Table 69 shows the number of observed (O) positive results amongst the patients who attended the Colposopy Clinic for a similar number of visits. The equivocal results have been classed for the purposes of this table as negative. The values in brackets (E) are the expected number of positives, assuming that they were from a binomial distribution for the group of patients attending the same number of colposcopy visits. (Those patients with only one visit or seven visits have been excluded due to the small numbers in each group.)

Table 69 NUMBER OF OBSERVED & EXPECTED POSITIVE RESULTS FOR HPV16 ACCORDING TO THE NUMBER OF COLPOSCOPY VISITS

NO. CLINIC VISITS	OBSERVED (EXPECTED) POSITIVES					
	0	1	2	3	4	5
2	26 (25.9)	24 (24.4)	6 (5.7)	-	-	-
3	52 (46.3)	27 (37.0)	12 (9.8)	3 (0.9)	-	-
4	72 (68.3)	53 (59.9)	24 (19.7)	2 (2.9)	0(0.2)	-
5	95 (78.6)	51 (74.9)	29(28.5)	11 (5.4)	1 (0.5)	1(0.02)
6	40 (30.2)	27 (42.5)	29 (24.9)	10 (7.8)	1 (1.4)	0(0.1)

The number of positive results (Expected value E) in each group was calculated from the overall Observed value (O) using Binomial statistics thus:

p = 0.32 for 2 visits (56 patients; 36 positives for 112 visits);

p = 0.21 for 3 visits (94 patients; 60 positives for 282 visits);

p = 0.18 for 4 visits (151 patients; 107 positives for 604 visits);

p = 0.16 for 5 visits (188 patients; 151 positives for 940 visits);

p = 0.19 for 6 visits (107 patients; 119 positives for 642 visits).

The χ^2 value can be calculated by $\chi^2 = \Sigma\ (O-E)^2/E$, where O is the observed value and E is the expected one.

Table 69.1 **GOODNESS OF FIT FOR THE BINOMIAL DISTRIBUTION IN TABLE 69.**

Number of visits	χ^2 value	p value
2	0.013 (1 d.f.)	0.91
3	5.13 (1 d.f.)	0.024
4	1.44 (1 d.f.)	0.23
5	19.53 (2 d.f.)	0.0001
6	9.81 (2 d.f.)	0.0074

Table 70 NUMBER OF OBSERVED & EXPECTED POSITIVE RESULTS FOR HPV16 ACCORDING TO THE NUMBER OF SUBSEQUENT RESULTS [EXCLUDING THE INITIAL RESULT]

These values in Table 69 may be misleading because of the preponderance of positives from the initial screening results. The following table omits the initial screening result.

NO.CLINIC VISITS	OBSERVED (EXPECTED) POSITIVE				
	0	1	2	3	4
2	69 (67.7)	23 (25.8)	4 (2.5)	-	-
3	81 (80.8)	40 (39.5)	6 (6.4)	0 (0.3)	-
4	130 (135.2)	50 (54.1)	15 (8.1)	2 (0.5)	1 (0.01)
5	56 (54.6)	42 (44.4)	17 (14.5)	1 (2.4)	0 (0.2)

The probability of getting a positive result (Expected value E) at each visit was calculated from the Observed value (O) thus:

p = 0.16 for 2 visits (96 patients; 31 positives for 192 visits);

p = 0.14 for 3 visits (127 patients; 52 positives for 381 visits);

p = 0.091 for 4 visits (198 patients; 90 positives for 792 visits);

p = 0.14 for 5 visits (116 patients; 79 positives for 580 visits).

Table 70.1 GOODNESS OF FIT FOR THE BINOMIAL DISTRIBUTION IN TABLE 70

Number of visits	χ^2 value	p value
2	0.085 (1 d.f.)	0.77
3	0.080 (1 d.f.)	0.77
4	10.75 (1 d.f.)	0.001
5	0.21 (1 d.f.)	0.65

In comparing the Southern blot and FISH results, the agreement, on the whole, has been less than satisfactory. Therefore, it would seem obvious to ask if the serial FISH results were of a purely random occurrence. Table 69 deals with the observed and expected number of positives depending on how many times the patient had attended the clinic. In situations where the numbers of visits was small (less than 5), then the figures are added to the previous visit number from the point of view of calculating the chi-square value. This is reflected in the degrees of freedom in Table 69.1. The results show that in the cases where there were 3, 5 or 6 visits, then the results were not random events as reflected by the significant probability values

(Table 69.1). These results are mainly due to the consistently higher numbers of observed positive results in women with a full series of visits.

The results are not as clear cut in Tables 70 & 70.1, where the initial FISH result is removed because of the obvious selection of the patients into the study. The binomial distribution, in this case, is only rejected in 1 case where there were 4 successive visits. This suggests that once the initial result was excluded, [because of the predominance of positive results] then the distribution of further positive results was of a more random nature.

TRANSITIONAL MODELLING

The following tables show the results of two successive visits and the number of times a negative HPV16 result was followed by either a positive or negative result at the subsequent visit, and likewise for a positive HPV16 result. The equivocal results have been classed for the purposes of these tables as negative, and the values in parentheses are the proportion of the total number of results. The odds ratios and χ^2 values have been calculated using the formulae described in section 2.13.

The following tables (71.1 & 71.2) look at 2 consecutive results and the odds ratio reflects the probability of a subsequent result being positive if the previous result was positive. The odds ratio of 3.08 in Table 71.1 means that the patient has a 3 fold increased chance of having a positive FISH result if the previous result was positive. The χ^2 value is also highly significant. This means that the results are unlikely to occur by chance. However, if the initial visit result is excluded, the odds ratio is reduced to 1.57 (Table 71.2) which means that there is slightly increased risk of a next

result being positive if the former one was positive. The corrected χ^2 value of 3.48 is just outside the level of significance ($p = 0.06$), suggesting in this case the serial results are more randomly distributed.

Table 71.1 TRANSITION MODELLING USING ALL HPV16 RESULTS INCLUDING THOSE FROM THE INITIAL SCREENING DATA

		SUBSEQUENT VISIT	
	HPV16 NEGATIVE	HPV16 POSITIVE	
PREVIOUS VISIT			
HPV16 NEGATIVE	1434 (0.71)	144 (0.072)	1578
HPV16 POSITIVE	330 (0.16)	102 (0.051)	432
TOTAL	1764	246	2010

Odds ratio = 3.08

Corrected $\chi^2 = 64.92$ $p < 0.0001$

Table 71.2 TRANSITION MODELLING USING HPV16 RESULTS

EXCLUDING THOSE FROM THE INITIAL SCREENING DATA

		SUBSEQUENT VISIT	
	HPV16 NEGATIVE	HPV16 POSITIVE	
PREVIOUS VISIT			
HPV16 NEGATIVE	1089	121	1210
	(0.77)	(0.086)	
HPV16 POSITIVE	168	29	197
	(0.12)	(0.021)	
TOTAL	1257	150	1407

Odds ratio = 1.57

Corrected $\chi^2 = 3.48$ $p = 0.062$

Table 72 HPV16 POSITIVITY RATES AMONGST THE STUDY POPULATION

This table deals with the positivity rates for HPV16 at the separate visits of the study population.

ORIGINAL STATUS	POSITIVITY RATES			
	SCREEN	VISIT 1	VISIT 2	VISIT 3	VISIT 4
NEGATIVE	0%	5.3%	12.7%	5.9%	3.8%
EQUIVOCAL	0%	14.5%	4.7%	11.1%	13.3%
POSITIVE	100%	25.1%	17.9%	14.9%	13.3%

Table 73 SUBSEQUENT HPV16 RESULTS IN THE WOMEN OF THE COLPOSCOPY STUDY POPULATION.

ORIGINAL HPV16 STATUS	PERCENTAGE RESULTS OF AT LEAST ONE SUBSEQUENT:	
	EQUIVOCAL	POSITIVE
NEGATIVE (132)	22.7%	21.2%
EQUIVOCAL (77)	35.1%	24.7%
POSITIVE (217)	38.9%	45.2%

Table 72 shows the positivity rates at successive visits amongst the women of the study population. The positivity rate by FISH would appear to be transient and gradually falls in the positive group. The positivity rate in the other two groups is also variable. Table 73 shows the percentage of women in each of the initial groupings who have at least one subsequent positive and at least one subsequent equivocal result. The percentages are higher in the initially positive group, but it will be noted that less than 50% have a further positive result.

Interpretation of this data is not easy. Originally, it had been assumed that, once a woman had been identified as positive for HPV16 DNA, she would remain positive. The results may reflect that HPV16 positivity is transient or that it fluctuates, the variables which may affect this fact could be myriad. One reason for the problem with the results may be due to a variation in the amounts of DNA obtained at each visit. The comparison of Southern blotting and FISH has shown that there is a trend towards increasing positivity with the increasing amounts of extracted DNA (Tables 56.3 & 57). Differing amounts of DNA may be obtained because exfoliation rates for the cervical squamous cells varies though a comparison of the HPV16 results and cycle day did not show any relationship. There could be some variation in the amount of cellular material obtained due to differing operator performance (i.e., how vigorous I was when taking the repeat smears). If HPV16 causes cervical lesions, and these women have normal cervices, then the amount of virus present may be very small and account for the changing positivity by a method that is relatively insensitive (See Section 3.7). Screening at 6 monthly intervals may be too frequent for consistent results. However, the most likely explanation is that FISH may well be unsuitable and lack sensitivity in the normal cervix.

3.10 PROGRESSION OF DISEASE RESULTS

The patients were followed over a 2 year period. The following table shows the outcome of those patients at the end of this follow-up period. The CIN diagnoses were from colposcopically directed biopsies.

Table 74 PROGRESSION OF DISEASE IN THE COLPOSCOPICALLY NORMAL WOMEN

	ORIGINAL HPV16 STATUS		
DISEASE STATE	NEGATIVE	EQUIVOCAL	POSITIVE
NORMAL	111	49	154
TRANSIENT HPVI	1	3	5
PERSISTENT HPVI	0	1	1
CIN 1	3	0	2
CIN 2	2	0	2

The total numbers developing CIN within each group are in summary:

HPV16 NEGATIVE 5 out of 117 (4.2%)

HPV16 EQUIVOCAL	0 out of 53
HPV16 POSITIVE	4 out of 161 (2.5%)

On reviewing the women who were colposcopically normal but had a Southern blotting analysis performed on a cervical scrape specimen (see Table 65), only one woman developed any evidence of CIN. The follow-up period was for at least 24 months. The patient developed CIN 1 after 28 months follow-up and was seen as one of the control group and the Southern blot result was negative. No further analysis has been performed.

Table 75 **SUMMARY OF THE NORMAL CASES DEVELOPING CIN**

75.1 **CIN 1**

ORIGINAL HPV16 STATUS	TIME TO DEVELOP LESIONS	SERIAL HPV16 RESULTS				
POSITIVE	6 mths	Pos	Pos			
NEGATIVE	6 mths	Neg	Pos			
POSITIVE	25 mths	Pos	Pos	Pos	Neg	Neg
NEGATIVE	28 mths	Neg	Neg	Neg	Eq	Neg
NEGATIVE	12 mths	Neg	Pos	Neg		

75.2 **CIN 2**

NEGATIVE	30 mths	Neg	Pos	Neg	Neg	
POSITIVE	30 mths	Pos	Eq	Eq	Neg	
NEGATIVE	28 mths	Neg	Neg	Eq	Pos	Neg
POSITIVE	29 mths	Pos	Neg	Neg	Neg	Neg

Table 75 summarises the serial HPV16 results of the women who developed abnormalities, plus the time periods for the various lesions to develop. In most cases, the time for a lesion to develop was greater than 2 years from the original screening. The duration of the follow-up period ranged from 24-36 months. It is, therefore, reasonable to argue that the follow-up time was too short to expect many more lesions to develop in this population.

LOGISTIC REGRESSION ANALYSIS

Logistic regression analysis has been performed on the cases from the original study group who have developed CIN from normal compared to those who have remained normal. This analysis has been performed using the categories as described in section 3.5. This following section gives a brief summary of these two groups. The women who developed HPVI either transient or persistent have been excluded.

Table 76 **FEATURES OF THE WOMEN DEVELOPING CIN COMPARED TO THOSE REMAINING NORMAL**

	NORMAL (N = 314)	CIN GROUP (N = 9)
HISTORY OF OCP USAGE	268	9
MEDIAN OCP USAGE	48.0 months	42.0 months
MEDIAN COITARCHE	18.0 years	17.0 years

		NORMAL	CIN
MEDIAN NUMBER OF SEXUAL PARTNERS		3.0	3.0
HISTORY OF GENITAL INFECTION (excluding Candida)		73	3
H/O CANDIDA		128	
H/O NSU		15	1
H/0 TRICHOMONAS		19	0
H/O GENITAL WARTS		26	1
H/O GENITAL HERPES		11	0
H/O GONORRHOEA		6	1
NUMBER OF ABORTIONS	0	214	6
	1	77	3
	2+	23	0
NUMBER OF CHILDREN	0	185	3
(PARITY)	1	49	3
	2	59	2
	3+	21	1

NUMBER OF PREGNANCIES	0	130	3
	1	72	1
	2	59	3
	3	32	2
	4+	21	0
MEDIAN AGE FIRST PREGNANCY		22.5 (N = 184)	20.0 (N = 6)
MEDIAN AGE FOR FIRST BIRTH		23.0 (N = 129)	20.0 (N = 6)
HISTORY OF SMOKING - NEVER		148	3
- EVER		166	6
MEDIAN PACK YEARS		1.0	2.0
MEDIAN AGE		30.0 years	29.0 years

Table 76 summarises the characteristics of the normal population who were followed over a minimum of 2 years. No further information could be given in the way of statistical analysis as the numbers in the CIN group are too small to use in standard chi-square tests.

3.10.1 NORMAL CASES DEVELOPING CIN

Number of Normal cases	314
Number developing CIN	9
[Cases not included in the analysis	11]

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
COITARCHE	<15 years	24	0.00	8.29 (3 d.f.)	0.040
	16-17 yrs	104	2.51		
	18-19 yrs	111	1.50		
	20 + years	84	-7.10		

It must be stressed that the number of cases here is very small and the χ^2 approximations to the likelihood Ratio statistics is no longer valid. All the p values should be treated with caution in view of the numbers and any significant evaluation is, consequently, problematic.

However, it is interesting to use the Armitage test for trend with coitarche in the groupings as described and comparing the normal cases against those which progressed to disease, then:

χ^2 with 1 d.f. = 6.91, p = 0.0086.

Likewise using the Goodman test for association, a similar result is obtained: χ^2 with 1 d.f. = 7.04, p = 0.0080.

This finding of early age of first intercourse has been found in other studies (Rotkin, 1967; Harris et al, 1980; LaVecchia et al, 1986b), but it is important not to place too much emphasis on this analysis as the number of patients in the CIN group is so small.

Table 77 **FOLLOW-UP RESULTS FROM THOSE WOMEN WITH HPVI ALONE WITHIN THE ORIGINAL STUDY GROUP**

	ORIGINAL HPV16 STATUS		
DISEASE STATE	**NEGATIVE**	**EQUIVOCAL**	**POSITIVE**
NORMAL OR PERSISTENT HPVI	10	15	26
CIN 1	0	2	2
CIN 2	1	0	1

This table has been included for completeness. Only 6 (10.5%) developed CIN and only 2 (3.5%) of the cases were CIN 2. It is difficult to know how much store to place on these findings as there can be wide inter-reporting variation with HPVI and CIN 1. It could be argued that biopsy of the initial lesion altered its natural history which, in the absence of

cytological abnormality, may be different from other reported studies (Evans & Monaghan, 1985; Syrjanen et al, 1985; Campion et al, 1986).

3.10.2 HPVI CASES DEVELOPING CIN

If the HPVI group from the original study group is considered (see Table 77) then, the following results are obtained.

Number of cases progressing to CIN	6
Number of cases regressing to normal	50
[Cases not included in the analysis	1]

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
Number of children	1: Nil	37	0.00	10.21 (3 d.f.)	0.017
	2: 1	7	-8.44		
	3: 2	9	2.64		
	4: 3-4	3	-8.34		

These results suggest that having 2 children places the woman with HPVI at an increased risk of developing CIN, whilst having one child or three or more children would appear to protect the women in this group from CIN. Caution must, therefore, be taken in the interpretation of this analysis in view of the small numbers.

Using the Armitage trend test χ^2 with 1 d.f. = 4.021, giving a value of p = 0.044, however using the Goodman test for association the level for χ^2

with 1 d.f. is 3.67, with p = 0.055, just outside the level of significance. These results reflect the statistical difficulty in comparing small numbers.

No other factor reached any degree of significance.

3.10.3 CASES DEVELOPING CIN FROM THE ORIGINAL COLPOSCOPY POPULATION

If these two groups are combined for analysis then the following results are obtained:

Number of cases developing CIN	14
Number of normal cases	361
Cases not included in analysis	4

FACTOR	LEVEL	No.	COEFFICIENT	IMPROVEMENT OF χ^2	p value
Gravidity	0	155	0.00	8.72 (3 d.f.)	0.033
	1	84	-1.60		
	2-3	113	-0.44		
	4+	23	-45.03		
Number of children	0	222	0.00	8.02 (3.d.f.)	0.046
	1	58	2.22		
	2-3	90	2.29		
	4 +	5	30.94		

These results are confusing and suggest that on the one hand, women who have had 4 or more pregnancies would appear to have some

protection against developing CIN, whilst on the other hand those who have had 4 or more children are at an increased risk of developing CIN. This represents a clear case of overfitting (see Section 2.13) due to the small numbers within the various groupings and also within one of the comparison groups. These findings are tabled:

Table 78 **GRAVIDITY IN NORMALS AND THOSE DEVELOPING CIN**

GRAVIDITY	NORMAL	CIN
0	151 (97.4%)	4 (2.6%)
1	83 (98.8%)	1 (1.2%)
2-3	104 (92.0%)	9 (8.0%)
4+	20 (100%)	0 (0%)

(ROW PERCENTAGES)

TABLE 79 **PARITY IN NORMALS AND THOSE DEVELOPING CIN**

NO.CHILDREN	NORMAL	CIN
0	218 (98.2%)	4 (1.8%)
1	55 (94.8%)	3 (5.2%)
2-3	84 (93.3%)	6 (6.7%)
4+	4 (80%)	1 (20%)

(ROW PERCENTAGES)

Using the Armitage test for trend, only the parity had any significance for χ^2 with 1 d.f. = 6.97, giving p = 0.0083, and likewise with the Goodman test of association, χ^2 with 1 d.f. is 6.28, p = 0.012. No significance

could be found using the number of pregnancy categories, with both the Armitage and Goodman tests giving a value for p=0.18, though χ^2 with 2 d.f. for heterogeneity was 6.97 (p = 0.0083). This can be seen clearly in Tables 78 and 79, where there is a linear trend in the latter but not in the former.

No other factors reached any level of significance.

SECTION FOUR

DISCUSSION

DISCUSSION

There has been considerable laboratory data published to suggest an association between HPV16 and cervical neoplasia. The question: 'Does the presence of HPV16 in the normal cervix place that woman at an increased risk of developing CIN?' provided the background to this project. The FISH method was used to detect the presence of HPV16 because of its suitability in screening large numbers of cervical scrapes. Sections 3.1 and 3.2 deal with the epidemiological findings of the screened population with regard to the HPV16 status by FISH, OCP history, smoking and cytology results.

4.1 SCREENED POPULATION

There was a relatively high cytological abnormality rate (15%) within the screened population (Table 2.1). These figures should be judged in the light of the Health Trends report (Roberts, 1982) which revealed that there had been an increase in the number of positive smears (from 4.7 to 6.8 per 1000) between 1970 and 1980, in England and Wales, especially in the 30-34 year age group (4.5 to 11.4 per 1000). The rate of positive smears in Roberts' report, was greater in those screened in the Family Planning Clinic than by the General Practitioners, though these rates were nowhere near the abnormality rate in my screened population. Wolfendale and her colleagues questioned whether we were 'in for an epidemic' of cytological abnormalities in 1983 when the abnormality rate had more than doubled from 5.8 to 12.9 per 1000 smears in an unscreened population between 1965 and 1979. Indeed, Chomet (1987) found that 12% of her population of 1254 women, screened in the same geographical area as the study in this thesis, had a cytological abnormality, though only 6.3% were dyskaryotic and the remainder were inflammatory.

The HPV16 positivity rate by FISH of 22.5% (Tables 5 & 5.1) in the patients with normal cytology is much higher than the 2.2% (5 out of 229) in the Scheider et al series (1985) who used a similar FISH technique and the 10% in the much larger population of de Villiers et al (1987). The authors of the latter study suggested that this rate may be an underestimate of the true prevalence. However, using the Southern blotting method on biopsy material from normal cervices, Meanwell et al (1987) found 35% of his population had evidence of HPV16 DNA present, though the total group size was only 35. With the advent of PCR, Young et al (1989) found that 7 out of 10 women with normal cytology had evidence of HPV11 or 16 present in their cervical scrapes, though a lower and perhaps more realistic figure of 15% (33 out of 221 normal women) was reported by Bavin et al (1990) using the same method.

It is difficult to interpret this prevalence rate of 22.5% for a variety of reasons. It can be seen from the serial HPV16 results (Section 3.9) that positivity is transient in many cases. This may be due to differing amounts of cellular material obtained at the time of the cervical scrape, which in itself may be dependent on the exfoliation rates of the cells. The interpretation is, therefore, that either HPV16 positivity is really a transient phenomenon or just reflects the inherent problems with the FISH method. These problems will be discussed later when comparing the FISH and Southern blotting methods. It is, however, pertinent to review Table 65 which showed that the Southern blot for HPV16 DNA was positive in 32.4% of the women with a colposcopically normal cervix. Our findings in this study would agree with de Villiers et al (1987) that the FISH methodology underestimates the true prevalence in the population with a normal cervix.

The HPV16 positivity rate of 24% (Table 5.2) in the abnormal cytology group is lower than might be expected if HPV16 is associated with disease (McCance et al, 1985). This figure may reflect a number of features in the group of women with abnormal cytology. The cytology results do not necessarily reflect the true nature of the lesion as can be seen from the high false positive cytological abnormality rate. The figures only include HPV16 status, so there is no indication of the prevalence of other HPV types causing the rest of the abnormalities.

Using the crude analysis of cytology result only against HPV16 DNA results (Tables 5, 5.1, 5.2), there was no trend or association between the presence of wart virus and increasing cytological severity. This can be quickly assessed by reviewing the row percentages within each group of Table 5 and 5.1. There was no age difference between the HPV16 result groups (Table 6) unlike the Meanwell et al study (1987) which suggested that HPV16 positivity was age related.

The percentage of abnormal smears was greater amongst the smokers than the non-smokers (Table 7) and this is reflected in the significant trend ($p = 0.014$) towards an increased severity of abnormality in those who smoke. In retrospect, it may have proved interesting to record an estimate of total cigarette consumption (pack years) but this was not done. These findings confirm the observations of others that cigarette smoking is related to the earliest stage of disease (Trevathan et al, 1983; LaVecchia et al, 1986a) and lends support to the hypothesis that cigarette smoking may exert an immunosuppressive effect on the cervical epithelium (Barton et al, 1989). The theory that smoking and HPV16 DNA presence affect the cervix may also be strengthened by the association of HPV16 DNA and smoking ($p=0.035$) in Table 8.

A logistic regression analysis was performed using the presence or absence of abnormal cytology as the dependent variable and showed the only significant factor to be smoking ($p = 0.044$). The smear result is obviously a difficult endpoint to use as it does not necessarily equate with the true prevalence or severity of disease within any population. The analysis must be interpreted in the light of these drawbacks, but it would appear that women who were 'ever-smokers' had an increased tendency to develop abnormal cytology compared to the 'never-smokers' within this screened population. Oral contraceptive pill usage, using either 'ever' or 'never' as a function of exposure, does not appear to exert any significant influence on the presence or absence of normal cytology or HPV16 status in this original screened population. It would, therefore, appear from these screening results that the prevalence of HPV16 as detected by FISH has a significant association with cigarette smoking though this is not the case for OCP use.

The highest percentage of abnormalities occurred in the 15-24 year olds (29%), followed by 13.9% in the 25-34 year age group, 11.3% for the 35-44 year olds and 8% in the women over 45 years old. These findings (Table 17) are at odds with the findings of Roberts (1982) who found the highest abnormality rate in the 30-34 year age group (11.4 per 1000 smears), and Chomet (1987) who found the highest abnormality rate of 14% in the 25-34 year old age group, but they are consistent with more recent findings of Wolfendale et al (1987) who found the maximum abnormality rate occurred between 20-29 years of age. The age distribution of abnormalities in this thesis is identical to the recent finding of a greatly increased cytological abnormality rate in the 15-24 year age group within the same inner city area of London as this study (Hollingworth & Cuzick, In Press). The high

cytological abnormality rate reflects the skew in age distribution of the screened population.

The findings in Table 19 agree with the findings of Chomet (1987), who had a 20% false positive cytology rate in her patients with mild dyskaryosis; it is higher than the 11% false positive rate of Schwartz and Brecht (1984) though not as high as the study of Jones et al (1990). In the latter study, women who had had one mildly dyskaryotic smear at least 2 years earlier (mean time = 34 months) were reviewed. Part of this population (>200 women) had been kept under review by cytology alone. When these women were colposcoped, more than 40% had a cervix with a normal appearance. This would suggest that the mildly dyskaryotic smear was either falsely positive or that the original abnormality was of a transient nature.

4.2 COLPOSCOPY POPULATION

All the women who had a positive or equivocal result with FISH were invited to attend for colposcopy together with an equal number of women who were negative for HPV16 by FISH. It is, perhaps, understandable that women who were otherwise normal because of a normal cytology result should not subject themselves to colposcopy which can be both uncomfortable and undignified. It is difficult to know how much the study and control groups were 'self-selective' and whether they really do reflect the normal population. Despite these problems, the women in the study appeared to be a representative cross section sample of all women with negative cytology.

The false negative cytology rate (Table 47) appears high but needs to be taken in the context of grade of disease. Only 3.1% of the colposcopy

population (13 out of 426) had evidence of a major grade lesion (CIN 2-3). These figures can be compared to the study of Tawa et al (1988) comparing cytology and cervicography. In a population of 3271 women, 39 (1.2%) had abnormal cytology, and almost 10 times that number (373) had a suspicious cervicograph. Only 81 (2.5%) of the total population, however, had evidence of dysplasia confirmed by colposcopic biopsy, which would suggest a false negative cytology rate in the order of 1.3%. In Giles et al's study (1988) a smaller population of 200 asymptomatic women in a General Practice were colposcoped. The cytology abnormality rate was 5%, but the true abnormality rate using colposcopy was 11%. In both studies, the majority of lesions were minor grade. It would appear from these studies that many minor grade lesions are missed by cytological screening. This fact has been confirmed by the findings in Table 47 and lesions missed by cytology have been picked up by HPV16 screening with FISH. It would, therefore, appear that screening the population by FISH may have some limited value. The significance of these minor lesions with no history of abnormal cytology, however, remains unknown at present.

The logistic regression analysis has been performed on the data from the study population (Section 3.3). It must be remembered when assessing this data that these results are from a group of women with normal cytology, and so the comparison is between women with true negative cytology and those with false negative results. This analysis may not necessarily reflect the epidemiological features of women who present with an abnormal smear and are found to have similar grade lesions. The results of these analyses must also be treated with caution when the numbers are small as then the chi-square approximation to the likelihood ratio statistics is no longer valid.

Traditionally, CIN has been regarded as a continuum of disease progressing through its various grades to invasion. Richart (1990) has recently suggested that it is probably more appropriate to think in terms of low grade CIN (CIN 1) and high grade CIN (CIN 2-3). The analysis (Section 3.5) reflects both ways of assessing the disease state.

In the colposcopy population, HPV16 status and a history of smoking were significantly associated with the presence of any grade of lesion when compared with the colposcopically normal cervix (Sections 3.5.1, 3.5.2, 3.5.3, 3.5.6). The presence of disease was also significantly more common in the 25-44 year age group, which probably reflects the skew in age distribution of the population as most of the women (67%) were within this age range. The HPV16 status appeared to be the most significant of these three cofactors, though the weighting was more significant in the equivocal group. The equivocal results may be due either to cross-reactivity with other HPV types or reflect the poor sensitivity of the FISH method where low copy numbers of virus might be present if the cytology result is negative (see Section 4.3) or a combination of both. These findings could be explained by smoking exerting an immunosuppressive effect on the premenopausal cervix (Barton et al, 1989) and allowing HPV16 or other related types to cause epithelial damage resulting in HPVI alone or some degree of dysplasia.

The venereal nature of cervical disease is highlighted by a history of gonorrhoea having a significant effect on the development of CIN (Section 3.5.3) or a major grade lesion (Section 3.5.5) compared to the colposcopically normal cervix. Other features, however, appear confusing, with a history of genital warts giving a protective effect to the normal cervix from developing HPVI alone (Section 3.5.2) but a significant effect

for CIN compared to those with HPVI alone (Section 3.5.4). A history of chlamydia would also appear to have a protective effect on the cervix with HPVI alone compared to those with CIN (Section 3.5.4). The significance of cofactors when comparing different groupings is, therefore, confusing and the most likely explanation is due to the small numbers in certain of the categories used for the logistic regression analysis.

4.3 COMPARISON OF METHODOLOGIES

Southern blotting hybridisation has been taken to be the 'gold standard' for HPV identification. It has been estimated that 0.1-1.0 viral genome per cell for an average of 10^6 cells would be required as a minimum for this method to give a positive result (Lancaster & Norrild, 1989). The Southern blot filters were only probed for HPV16 DNA.

Overall, the correlation of the FISH and Southern blotting methodologies, as assessed by the Kappa value, is poor and is especially disappointing in the normal cytology group. This is related to the false negative rate of the FISH result (29 in all, Table 54) when compared to the Southern result. This may reflect the low viral copy number within the sample, or small amounts of total DNA extracted, which in turn reflects a small amount of viral DNA available for hybridisation. The dilution experiment shows that $5x10^5$ cells, each with 5-10 copies of the virus, are required to give an unequivocal result using FISH and giving 7 days exposure of the autoradiograph. Taking the estimate that approximately 5pg of DNA is obtained from 1 diploid cell (Cornelissen et al, 1988), then the cervical scrapes would have contained between $2x10^4$ to $10x10^6$ cells per sample by extrapolation from the range of amounts of DNA obtained per cervical scrape (Table 50). [The mean would be $1.8x10^6$ cells with a median of $1.2x10^6$.] Each sample was then divided into 2 equal aliquots. The low cell

yield and resulting small amount of HPV16 may account for the lack of any signal on the autoradiograph after the high stringency wash and result in a high false negative rate with the FISH method.

False positive results have also been considered a problem with the FISH method because cellular debris causes some of the radioactively labelled HPV16 to remain on the filter after the high stringency washes. As a result, background noise is increased on the autoradiographs and makes their interpretation difficult. The discrepancy in the kappa results suggests that HPV16 is more commonly found in detectable copy numbers at the HPVI alone stage and may consequently be most infectious at this time. Indeed, Schneider et al (1987) reported that the highest amount of viral DNA is found in koilocytes.

McCance et al (1986b) developed the method used in this thesis and found a good correlation between the results of FISH using cervical scrapes from women being followed up for abnormal cytology and Southern blotting using biopsy material from the same cervices. The cell counts from the cervical scrapes ranged from $0.5x10^6$ to $10x10^6$ (average $3.85x10^6$). In samples from 24 patients, they found 10 positive for HPV16 by FISH and 11 positive by Southern blot. The results agreed in 9 cases. The sample size is small, no mention is made of equivocal results and the cases were those in which the copy number may have been expected to be high because of the presence of disease.

In contrast to McCance's study, Hallam et al (1989) compared 100 cervical scrapes and biopsies using dot blot hybridisation. They managed to extract 5-10μg of DNA from the scrapes for this study. The samples were from women with colposcopic abnormalities. Though the dot blot technique

is different from the FISH method and probably more sensitive than the latter (Cornelissen et al, 1988), there was a discrepancy in the results. Forty-two (42%) were positive for HPV16 DNA using the biopsy material, whereas only 33(%) were positive with the scrape. However, only 24 (57%) of the 42, who had a positive result with the biopsy, had a positive result with the scrape. In Cornelissen et al's study (1988), FISH was compared with the dot blot method in 51 women who had abnormal cytology. Thirty-nine (76%) were positive for HPV16 by dot blot compared to only 11 (22%) by FISH, implying that the FISH method is not as sensitive.

Melchers et al (1989a), developed the FISH technique further by denaturing the cellular material before application to a filter. The same workers presented data (Melchers et al, 1989b) comparing Southern blotting and their modified FISH technique using cervical scrapes in women who had had abnormal cytology, though there was no mention of the subsequent histological diagnosis. With the exception of one false negative result in the modified FISH group, the results agreed completely in the other 79 cases. Unfortunately, the comparison of the two groups was not repeated in the normal control group because the correlation had been so good in the abnormal group.

In summary, studies comparing methodologies for HPV identification have usually used material from abnormal cervices. The results have been variable as discussed above. Comparison of the FISH and Southern blotting method using normal cervical scrapes has not previously been addressed. The results in Section 3.6 suggest that whilst FISH may be of use where the viral copy number is expected to be high or where the amount of extracted DNA is greater than 10μg, its sensitivity in normal cervical scrapes must be brought into question.

4.4 PROGRESSION TO DISEASE

Only 2.7% of the women with a colposcopically normal cervix at the outset (9 out of 334) developed CIN of any grade over a minimum period of two years from the time of screening (Table 74). Only 4 (1.2%) of those cases who developed disease were HPV16 positive by FISH at the time of the original screening. In the 5 women who developed CIN 1, 3 were originally negative for HPV16, though 2 of these women had a subsequent positive result by FISH and the other one had an equivocal result (Table 75.1). Four women (1.2%) developed CIN 2 and they were evenly distributed between the orignal HPV16 positive and negative groups, with both patients in the control group (HPV16 negative) having a subsequent positive result (Table 75.2). It is also obvious from Tables 69, 70 & 75, that HPV16 status as determined by FISH changes from visit to visit. The serial results and transitional modelling data (Section 3.9), suggest that if the cervix is positive for HPV16 by FISH then it does not necessarily remain positive for the virus. This may be due to the transient nature of HPV infection or lack of sensitivity of the FISH method, as previously explained. Obviously, there are inherent problems with FISH but even in those colposcopically normal women who had Southern blot analysis on their cervical scrapes (Table 65), only 1 woman developed CIN 1 and she was negative for HPV16 by this hybridisation method.

Few lesions developed within the 3 years and none of these were as severe as CIN 3. This fact lends weight to the argument that repeat routine cervical cytology less than 3 yearly will, in general, be too frequent. If screening is performed at this interval then there should be no resulting serious morbidity, though this does not take into account the initial false negative cytology rate.

The logistic regression analysis in the normal group gives the age of first intercourse as the only significant factor in those developing CIN. This finding is similar to many other studies already discussed in the introduction, and suggests that the immature Transformation Zone suffers some insult at this time which predisposes it to the development of CIN at a later age. However, the numbers in the analysis are small and so caution is essential in laying too much emphasis on this interpretation.

The 56 women who started with HPVI alone at the beginning of the study were reviewed. Only 6 (10.5%) developed CIN and only 2 (3.5%) of these cases were CIN 2 (Table 77). It is difficult to know how much store to place on these findings as there can be wide inter-reporting variation with HPVI and CIN 1. These figures are in marked contrast to those of Evans & Monaghan (1986), Syrjanen et al (1986) and Campion et al (1986). In all of these studies, at least a 20% progression rate to CIN 3 from mild cervical atypia over 2 years was found. It could be argued that a) biopsy of the initial lesion altered its natural history and/or b) the natural history of these lesions, in the absence of abnormal cytology, may be different from the studies quoted. The logistic regression analysis on the women developing CIN in the HPVI group has been included for completeness and highlights parity as a significant factor. Few studies (Parazzini et al, 1989) have linked parity with cervical abnormalities but as the figures in Section 3.10.2 are small, the overall significance of these findings must be interpreted cautiously.

The two groups (Normal and HPVI) have been amalgamated and the results in Section 3.10.3 show evidence of overfitting because of the small numbers in the CIN group. The positive trend association with parity can be seen more easily by reviewing the row percentages in Table 79, where

there is an obvious linear trend down the table. This does not appear with Gravidity (Table 78). Caution must again be used in further interpretation of this data in view of the small numbers in each group.

In summary, the overall follow-up results are disappointing from a theoretical point of view that positivity for HPV16 DNA places the cervix at an increased risk of developing CIN, but are very reassuring for the women involved in the study and for the clinician who has to counsel these patients. These findings would agree with the common features of cancers that follow viral infections, namely (i) that only a small proportion of those who carry the virus (usually without symptoms) eventually develop a malignant tumour, and (ii) crude estimates of latency periods between primary infection and cancer appearance indicates that the process requires years or even decades of continuous host cell/virus interactions (zur Hausen, 1986). The follow-up period, therefore, needs to be extended almost indefinitely to discover if time is the overriding factor for any further lesions to develop.

4.5 CONCLUDING REMARKS

The belief that, mild atypia has a potential for progression to CIN 3 over a 2 year period especially if associated with HPV16 (Campion et al, 1986), has been the starting point for this study. If HPV16 could be identified in the normal cervix, does this place the cervix at an increased risk of developing CIN over the same time period of 2 years? The progression results (Section 3.10) do not support the theory that the presence of HPV16 on the normal cervix places it at an increased risk of developing CIN. In this natural history study, few patients developed an abnormality and as a result no obvious factors have been shown to affect short term progression of disease,

Therefore, the aims of the study design (Section 2.1) have been fulfilled to a limited degree. The risk of developing CIN 3 in colposcopically and cytologically normal women, who have been found to be positive for HPV16 by FISH, does not appear to be increased over a 2 year period. A strong association between HPV16 positivity and smoking has been demonstrated, but there was no evidence to suggest an association between HPV16 status and OCP usage. Smoking and HPV16 positivity are significant for the presence of any form of cervical disease, especially in the low grade lesions. No further conclusion can be made for CIN 3 lesions, due to the small population size with this type of lesion.

The comparison of Southern blotting and FISH has been shown to be poor especially where the viral copy number may be expected to be low, i.e., the normal cervix. The further use of FISH in large epidemiological studies must, therefore, be brought into question. Whilst it is a relatively quick method, the discrepancy in results makes it unsuitable in studies on the normal cervix. The FISH method of HPV16 identification is, therefore, an inadequate and insensitive method for early natural history studies.

The patients in this study need to be kept under review on an annual basis. The lack of positive results may reflect the relatively short time scale of the project and extended follow-up may produce more significant results. The women, who I consider of particular interest are those with evidence of HPVI alone on the cervix but with no cytological abnormality. It will be interesting to see if this is a transient situation or a precursor of more severe disease. With the recent DNA technological advances in the form of PCR, the reliability of the FISH result as regards varying HPV16 status will be reviewed using the PCR method to assess whether the

apparent transience of HPV16 positivity is real or just a reflection of the poor sensitivity of the FISH methodology in the normal cervix.

Some of the results which have been presented suggest some association between HPV16 and cervical disease. It is reasonable to assume that the hypothesis of HPV16 plus cervix equals abnormality is likely to be confounded by other cofactors, some of which may have not yet been elucidated.

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APPENDIX

Number [ ]

Initial Questionnaire Date: [:] [:] [:]

Time: [:]

Name: _____

Address: _____

_____

_____

Date of Birth: [:] [:] [:]

G.P.'s Name and Address:

_____

_____

_____

Marital Status: SINGLE/MARRIED/SEPARATED/DIVORCED/WIDOWED/COHABITS

Number of cigarettes smoked per day: [:]

Contraception currently used: PILL/MINIPILL/COIL/CAP/SHEATH/OTHER

How long have you been using this method? (approx.) [:] [:]

Years Months

Have you been on the oral contraceptive pill in the past? YES/NO

Which one(s) if you can remember?

_____

For how long approximately?

_____

QUESTIONNAIRE FOR PATIENTS IN THE STUDY

DATE ☐☐☐☐☐☐ STUDY NUMBER ☐☐

Place of birth: _____

_____

Single/Married/Cohabiting/Separated/Divorced/Widowed

Number of times married: ☐☐

RACE: White/Black/Caribbean or Central American/Oriental/
Middle Eastern/Indian or Pakistani/Other

Specify _____

SEXUAL HISTORY

Age at first intercourse: ☐☐

Total number of sexual partners: ☐☐

Current partner? YES/NO

What is his occupation?

_____

How many different sexual contacts have you had in the last three months? ☐☐

Any history of a sexually transmitted disease in the past? YES/NO

_____

Have you ever had an abortion? YES/NO

When did you last have sexual intercourse?

_____

SMOKING HISTORY

Have you ever regularly smoked cigarettes, cigars or a pipe?
(Life long non-smoker=0; current smoker=1; ex-smoker=2)

A smoker is someone who has smoked on most days for a year or more.
An ex-smoker is someone who has not been a smoker for at least 6 months.

SMOKERS AND EX-SMOKERS ONLY

How old where you when you started to smoke regularly? ☐☐

Ex-smokers, how old where you when you stopped smoking? ☐☐

How much do/did you normally smoke (when you last gave up)? ☐☐

Commercial cigarettes, number per day: ☐☐

Brand name: _____

Hand rolled cigarettes, number per day: ☐☐

Small cigars, number per day: ☐☐

Large cigars, number per week: ☐☐

Pipe tobacco oz. per week: ☐☐

MENSTRUAL HISTORY

Menarche: ☐☐

Cycle length: ☐☐

Abnormal bleeding:
IMB/PCB

Pregnancy details: _____

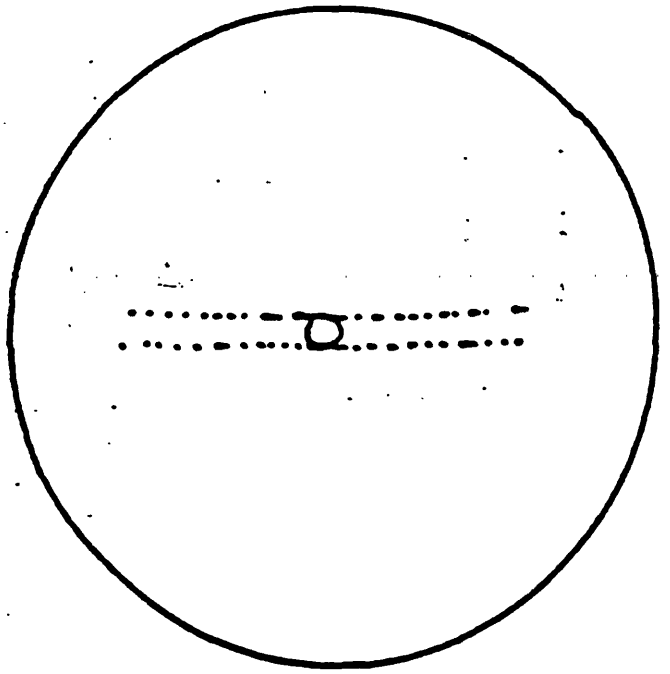
_____

MOSAICISM | PIGMENTATION.

VAGINA: NORMAL | MICROWARTS | ACETOWHITE | PUNCTATION.
MOSAICISM | PIGMENTATION.

CERVIX: COLPOSCOPY : SATISFACTORY | UNSATISFACTORY
UPPER LIMIT : SEEN | NOT SEEN.

BIOPSY FORMAL CALCIUM



BIOPSY LIQUID N_2

MARGIN : INDISTINCT | REGULAR STRAIGHT | ROLLED PEELING.

COLOUR : SNOWWHITE | GREY WHITE | DULL GREY WHITE

ABNORMAL VESSELS : UNIFORM FINE | NONE | PUNCTATION OR MOSAIC.

IODINE APPLICATION : POSITIVE | PARTIAL | REVERSE PUNCTATION OR NEGATIVE

IMPRESSION NORMAL | LNI | CIN I | CIN II | CIN III | MICRO | INVASIVE

MANAGEMENT.