AN INVESTIGATION INTO THE ASSOCIATION BETWEEN CYTOCHROME P450 AND GLUTATHIONE S-TRANSFERASE DETOXIFICATION ENZYME POLYMORPHISMS AND HUMAN ORAL SQUAMOUS CELL CARCINOMA

by

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ABSTRACT

Oral squamous cell carcinoma is the sixth commonest cancer in the world. Most patients who develop oral cancer are elderly males who are heavy users of tobacco and alcohol although the incidence is increasing in younger individuals and in those who neither smoke nor drink.

Approximately 80% of human cancers result from exposure to xenobiotics. Over the millennia Man has evolved complex families of detoxification enzymes to metabolise and eliminate these harmful compounds. Many of the genes that code for these enzymes are polymorphic, sometimes encoding enzymes with abnormal activity profiles. Numerous diseases have been shown to be more frequent in individuals with abnormal detoxification enzyme activity

This study investigated the association between polymorphisms in cytochrome P450 and glutathione S-transferase genes and disease susceptibility in 106 patients with histologically proven squamous cell carcinoma of the oral cavity.

The CYP2D6 PM phenotype was associated with a significantly increased risk of oral cancer ($p = 0.0012$). The CYP2D6 PM and HET phenotypes appear to be markers for a putative tumour suppressor gene at or close to 22q12. The EM phenotype is a risk factor in individuals who are heavy drinkers and smokers, possibly due to phase 1 activation of 4-(methylnitrosamino)-1-(3 pyridyl)-1-butanone.

DEDICATION

This volume is dedicated to my wife Josephine and my daughters Katherine and Sophie from whose company its completion kept me far too often.

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Finally, I thank all of the patients who consented to be entered into this study, many of whom are sadly no longer alive having succumbed to this most dreadful of diseases.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Page No

Page No

Page No

LIST OF TABLES

Page No

Page No

LIST OF ABBREVIATIONS

Abbreviation	Meaning
CI	Confidence interval
CYP	Cytochrome P450
CYP1A1	Cytochrome P450 1A1
CYP2D6	Cytochrome P450 2D6
DF	Degrees of freedom
dNTP	Di-nucleotide tri-phosphate
GST	Glutathione S-transferase
GSTM1	Glutathione S-transferase mu 1
GSTM3	Glutathione S-transferase mu **3**
GSTP1	Glutathione S-transferase pi 1
GSTT1	Glutathione S-transferase theta **1**
HR	Hazard ratio
LOH	Loss of heterozygosity
NAT	N-acetyl transferase
NF2	Neurofibromatosis type 2
OR	Odds ratio
PCR	Polymerase chain reaction
SD	Standard deviation
χ^2_n	Chi-square (n = degrees of freedom)

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Historical Perspective

Evidence for the occurrence of oral cancer in ancient civilisations is currently limited to the examination of a very few skeletal remains and papyri. The oldest cranial remains that show the stigmata of malignant disease during life date back some 5000 years (Wells, 1963). A skull dating from about 3,500 - 3,000 B.C. excavated from the Tepee Hissar site in Iran in 1931 by staff of the University of Pennsylvania shows evidence of massive destruction of the maxilla, zygoma and palate on the left extending into the floor **of** the orbit. Krogman (1940), suggested that the destruction is the result of extensive inflammation secondary to sinus infection. However, Wells (1963), proposed that the appearances are strongly suggestive of carcinoma. Certainly, the appearances **of** the skull as detailed in Figure 1 of Krogman's paper (Krogman,1940), are consistent with widespread bone destruction from an uncontrolled primary carcinoma of the maxillary antrum.

An ancient Egyptian skull dating from the 3rd-5th dynasty (2686 - 2494 B.C), (Mellersh,1995) and presently housed in the Duckworth Laboratory, University **of** Cambridge has approximately 26 osteolytic lesions involving either the inner or the outer tables of many of the cranial bones. The same skull also shows evidence of extensive destruction of the posterior hard palate, posterior antral wall, alveolus and pterygoid plates on the left side. There is thus strong circumstantial evidence that the skull lesions represent secondary deposits from a primary nasopharyngeal carcinoma (Wells,1963; Nunn, 1996).

Very few, if any, written accounts of oral carcinoma in the ancient world have survived. Although there are extensive references to the diagnosis and treatment of traumatic head and neck injuries in the ancient Egyptian medical papyri, in particular the

Edwin Smith surgical papyrus, none make any specific mention of tumours of this area (Christ,1979). Some authors (Martin, 1940), have interpreted references in the Ebers **Papyrus** (c 1500 B.C.) to "eating ulcer" of the gums and "illness of the tongue" to be descriptions of oral cancer, but others are more cautious (Christ, 1979; Nunn, 1996). While the paucity of data concerning oral cancer in antiquity may simply reflect the destruction and loss of informative remains and texts over the intervening millennia, it is likely that there was a lower incidence of cancer in ancient populations in comparison to modem ones due to a combination of limited life expectancy and reduced exposure to carcinogens (Christ, 1979; Nunn, 1996).

Hippocrates' (460 - 370 **B.C.)** is almost certainly referring to lingual carcinomas in his *Prorrheticon* in which he describes chronic ulcers on the edge of the tongue (Martin, 1940). In the same volume he exhorts his readers not to treat those who have internal cancer "since if treated they die quickly; but if not treated, they last a long time", and confines his advice on the treatment of tongue cancer to the elimination of any associated sharp teeth (Christ, 1979). The first account of surgical treatment for oral cancer is ascribed to Avicenna (**A.D**. 980 -1037) who reputedly excised carcinomas of the lip but allowed them to heal by secondary intention rather than attempting wound closure or reconstruction (Christ, 1979). He was doubtless describing a lingual carcinoma in his *Canon of Medicine* when he refers to "apostema dura (hard sore) of the tongue (Martin, 1940). Marchetti, Professor of Surgery in Padua, who some authors refer to as the grandfather of head and neck surgery, is credited with the first surgical excision of a lingual carcinoma in 1664 using cautery (Martin, 1940; Absolon *et al.,* 1962). However, it was not until Hams introduced the V-shaped incision and primary closure technique in 1835 that simultaneous resection and reconstruction for oral cancer became popular

(Absolon ***et*** *al.,* 1962). New surgical procedures designed to manage larger primary **tumours** and even nodal metastases rapidly developed. In 1836 Roux described the first combined lip split and midline mandibulotomy and in 1875 Baron Von Langenbeck was the first to excise enlarged regional lymph nodes in the submandibulartriangle in addition to a primary floor of mouth carcinoma (Absolon ***et*** *al.,* 1962). A seminal work describing the lymphatic drainage of buccal carcinoma and emphasising the necessity for radical removal of both the primary tumour and the regional lymph nodes was published by Polya and von Navratil in 1902 (Polya and von Navratil, 1902; McGregor, 1987). The importance of removing the regional lymph nodes in cases of oral cancer was also emphasised by Butlin (1845 - 19**12),** who advocated dissection of the contents of the anterior triangle of the neck at the same time as excision of the primary tumour (Butlin, 1909; Martin, 1940; Absolon ***et*** *al.,* 1962; Uttley and McGurk, 1996). Butlin's pioneering work culminated in the introduction of the block or radical neck dissection by Crile in 1906. In his classic paper (Crile, 1906), he described the results of 132 neck dissections and differentiated the radical block neck dissection from the block neck dissection. The former included the excision of the internal jugular vein and was performed for palpable nodal disease. The latter spared the internal jugular vein and was reserved for clinically negative necks. Current surgical techniques for the management of the regional lymph nodes have changed little since Crile's description.

The discovery of X-rays by Roentgen in 1895 was shortly followed by their application in the treatment of oral cancer. In 1903 Engman reported a case of lingual cancer treated by fractionated X-rays in which the local tumour was healed within a month and the patient was alive and well a year later (Martin, 1940). Brachytherapy for oral cancer was first introduced by Stevenson in 1914 who used needles containing either

radium or radon and implanted them into tongue carcinomas apparently with good results (Martin, 1940). The early teletherapy (external beam) sources were low energy (up to **300kv)** orthovoltage X-ray machines. The resulting X-rays were of low penetrance resulting in excessive skin damage. They were also preferentially absorbed by bone resulting in a high incidence of osteoradionecrosis (Henk and Langdon, 1985). Recent advances in radiotherapy techniques have been improved radiation sources including telecobalt machines, linear accelerators and iridium wires and improved treatment scheduling such as fractionation, hyperfractionation and afterloading. All of which have resulted in improved local control rates and reduced side effects such as skin damage and osteoradionecrosis.

The major surgical advances in oral cancer management over the last few decades have by and large been in the areas of reconstruction and rehabilitation. The first significant advance was the development of split skin grafts by Esser in 1917 (Esser, 1917), which resulted in improved healing of intraoral wounds following resection. However, despite their advantages, it soon became apparent that split skin grafts did not provide adequate bulk to reconstruct large defects and that they resulted in excessive contracture and deformity, especially when applied to the floor of the mouth and tongue (Christ, 1979). Numerous local and regional random and axial pattern flaps were rapidly devised in an attempt to overcome these limitations. The forehead flap, a local axial pattern flap based on the superficial temporal artery became the mainstay of intraoral reconstruction for many years (Edgerton, 1951; McGregor, 1963; Christ 1979). Although providing adequate and reliable soft tissue, the forehead flap was limited by its relatively short transfer distance and by the significant cosmetic donor site defect. Many of these problems were overcome by the deltopectoral flap popularised by Bakamjian in 1963 (Bakamjian, 1963). Large

amout of skin could be transferred over considerable distances and the donor site, although requiring skin grafting was on the chest and hidden by clothing. Flap reliability and length was found to be increased if the flap was delayed prior to transfer. Delay converted an essentially random flap into an axial one. The deltopectoral flap remained **the** workhorse flap for reconstructing head and neck defects for many years. However, while capable of providing good quality skin cover it was unable to provide the bulk of tissue required to reconstruct major defects in the floor of the mouth or of the tongue. This problem was overcome by the pectoralis major myocutaneous flap popularised by Ariyan (Ariyan,1979). However, its major limitation, like all pedicled flaps is that transfer distances and hence the defects that can be reconstructed are limited by the maximum length of pedicle available. It was not until the development and perfection of microvascular free tissue transfer techniques that it became possible to reliably reconstruct defects at sites remote from the donor site. In 1974, surgeons in Japan and **USA** simultaneously and independently reported on the successful reconstruction of head and neck defects using free groin flaps (Harashina ***et al.,*** 1974; Panje ***et al.,*** 1974). Perhaps the most important publication in the English literature in this area came in 1982 when Professor Muhlbauer reported his experiences while visiting burns units in the Peoples Republic of China in 1980 (Muhlbauer ***et al.,*** 1982). Surgeons there were harvesting fasciocutaneous flaps from the flexor surface of the forearm and anastamosing the radial artery and cephalic veins to suitable recipient vessels in order to reconstruct bum defects. The radial forearm flap or "Chinese flap" as it is often known was developed in 1978 at the Shenyang Military Hospital by Dr Yang Goufan, Dr Chen Baoqui and Dr Gao Yuzhi (Soutar ***et al.,*** 1983). The potential for this flap to reconstruct head and neck defects, particularly intraoral defects was instantly appreciated. The development and

popularisation of this flap is arguably the most important advance in reconstructive techniques to have emerged in recent times. The radial forearm flap has become the mainstay of intraoral reconstruction. Over the last decade numerous other free flaps have been developed enabling the surgeon to reconstruct both hard and soft tissue defects either singly or simultaneously. This explosion in reliable reconstructive techniques has resulted in vastly improved local control rates and improved quality of life for patients with oral cancer.

Oral cancer has claimed the lives of several eminent and world famous people including two presidents of the United States and Sigmund Freud. General Ulysses **S** Grant (1822-1885) was diagnosed as having a right tonsillar carcinoma with cervical node metastases in October 1844 from which he died 9 months later having received essentially no treatment (Renehan and Lowry, 1995). S Grover Cleveland (1837-1908), developed a lesion originally diagnosed as a squamous cell carcinoma of the left hard palate in May 1893 (Morreels Jr, 1967). Following a hemimaxillectomy performed on board a friend's yacht in July 1893 and a few minor interventions to fit and replace Obturators over the subsequent years Cleveland enjoyed a long disease free interval and died in June 1908 (Morreels Jr, 1967; Renehan and Lowry, 1995). Indeed, the long survival called into question the accuracy of the original pathology report. After numerous possibilities were proffered and refuted a consensus was reached in 1980 that the original lesion had been a verrucous carcinoma (Renehan and Lowry, 1995). Freud first sought help for ulceration of the right hard palate in 1923, although he had been aware of a problem since 1917 (Davenport, l993). This was initially treated by right partial maxillectomy in April 1923 but the tumour recurred due to incomplete excision. Subsequent right hemimaxillectomy and obturation 5 months later followed by numerous changes of Obturator gave Freud 16

years of reasonable health before he finally succumbed to his disease (Davenport, 1993). It is noteworthy that both President Grant and Sigmund Freud were heavy cigar smokers and chose to ignore their tumours before seeking treatment (Davenport, 1993; Renehan and Lowry, 1995)

1.2 Overview of Oral Cancer

Oral cancer is the sixth most common cancer in the world and is largely preventable (Parkin *et al.,* 1988; Raubenheimer *et al.,* 1989). It accounts for approximately 4% of all cancers and 2% of all cancer deaths world-wide (Boring *et al.,* 1993). It is the third commonest malignant neoplasm in developing countries and the eighth commonest in industrialised Western societies (Parkin *et al.,* 1988). In India it is the commonest malignant neoplasm, accounting for 20-30% of all cancers (Nair *et al.,* 1990). In excess of 1900 patients every year in the United Kingdom develop oral cancer and every year 900 patients die from this disease (Johnson and Warnakulasuriya, 1991). In the United States the annual new registration and death rates are 42,000 and 12,000 respectively (Boring *et al.,* 1993). With a death:registrationratio of 0.45 oral cancer is a disease of **high** lethality, comparable to that for carcinoma of the cervix (0.48) and greater than that for malignant melanoma (0.38) (Johnson and Warnakulasuriya,1993).

More than 80% of oral cancers are squamous cell carcinomas arising from the oral mucosa with the tongue being the commonest site (Johnson *et al.,* 1993; Smith,1995). While over 70% of all oral cancers arise in the so called "sump" area formed by the floor of the mouth, ventral and lateral surfaces of the tongue and mandibular alveolus, this area comprises only 30% of the surface area of the mouth. **A** possible explanation for this is the increased exposure of these non-keratinised and permeable tissues to putative xenobiotics and carcinogens pooling and collecting in the "sump" (Jovanovic *et al.,* 1993; Cawson *et al.,* 1996). **A** recent study by Thomson (1997) has also demonstrated that cells in the basal layer of human floor of mouth and ventral tongue epithelium have higher labelling indices than those in other oral mucosal sites as assessed by double labelling techniques using tritiated thymidine and bromodeoxyuridine followed by autoradiography and

immunocytochemistry respectively. This suggests that floor of mouth and ventral tongue epithelium has a higher innate proliferative capacity, which may render it more susceptible to malignant transformation.

The anatomical sites covered by the term "oral" include the lips and all intra-oral sites corresponding to the International Classification of Diseases ninth revision (ICD9) (WHO,1977)codes 140 (lip), 141 (tongue), 143 (gum), 144 (floor of mouth) and 145 (other non-specific sites), but exclude sites 142 (major salivary glands), 146 (oropharynx), 147 (nasopharynx), 148 (hypopharynx) and 149 (ill defined oral/oropharynx)(Johnson *et* ***al.,*** 1993). This definition of "oral" will be used throughout unless stated otherwise.

Oral cancer predominantly affects those in their sixties and seventies and is more common in males than females. In the series reported by Langdon *et al.* (1977), the mean age of presentation was 63.5 years for males and 60.6 years for females with a male: female ratio of 1.3:1. However, there is compelling evidence for an increasing incidence of oral cancer in younger subjects (Binnie *et al.,* 1972; Worrall,1995; Johnson *et al.,* 1996).

World wide, the major risk factors for developing oral cancer are heavy tobacco and alcohol consumption but a significant minority of patients with oral cancer are non-smokers and teetotal (Lemon ***et*** *al.,* 1964; Henk and Langdon,1985; Ehlinger *et al.,* 1993). Factors such as nutritional deficiencies, exposure to xenobiotic compounds, local trauma, viral, bacterial and fungal infections, immunological deficiencies and various potentially malignant lesions and conditions are also thought to be important (Johnson and Warnakulasuriya,1993). The marked variation in geographical and racial incidence together with the multiplicity **of** known and putative risk factors for oral cancer are highly suggestive that the disease is the result of a complex interplay between genetic susceptibility and exposure to environmental carcinogenic agents.

The last 40 years has seen tremendous advances in the surgical and non-surgical management of oral cancer. In particular, the advent and widespread adoption of microvascular reconstructive techniques allowing the opportunity for enhanced functional rehabilitation following major ablative surgery and the development of mega voltage radiotherapy machines resulting in enhanced tumour cell killing with reduced normal host cell damage has revolutionised patient management. However, despite improvements in the ability to control disease locally, the cure rate for oral cancer has improved little during this period. The overall ***5*** year survival rate remains depressingly low at around 35% even though oral cancer is a curable disease if patients are treated sufficiently early (Stell *et al.,* 1982; Ogden *et al.,* 1996). In a study of 194 patients over a 16 year period, Henk and Langdon (1985), reported an uncorrected overall ***5*** year survival rate of **32.8%.** In the same series of patients the uncorrected 5 year survival rate was 42.1% for women but only 22.9% for men (Henk and Langdon,l985). The enhanced survival in women may be related to their tendency to present earlier for treatment and with smaller tumours than men (Worrall and Corrigan, 1995). These depressing statistics suggest that alternatives to surgery and radiotherapy will need to be found if long term patient survival from oral cancer is ever going to be improved.

In many cases, invasive oral cancer is preceded by a potentially malignant lesion or condition. Potentially malignant lesions are those in which the oral mucosa locally has undergone sufficient cytological change as to render it more likely than normal tissue to become malignant. The majority, if not all potentially malignant conditions are systemic disorders that result in atrophy of the oral mucosa resulting in increased penetrance of carcinogens (Johnson *et al.,* 1993). At the present time it is not possible to differentiate the lesions that will ultimately progress to invasive carcinoma and as such warrant early

aggressive treatment from those that will regress and can safely be left alone. Likewise, it is not possible to accurately predict an individual patient's prognosis. Some patients with small localised tumours at the primary site at presentation develop early nodal and systemic metastases with a resultant poor outcome while others may have a large tumour at presentation which remains localised to the primary site and has a good outcome. The inability to predict the behaviour of these lesions results in some patients being under treated with a consequent risk of a sub-optimal outcome, while others may be over treated and suffer unnecessary morbidity. Greater understanding of the individual patient's genetic susceptibility to oral cancer should enable a more scientific management strategy to be developed resulting in improved quality of life and survival to say nothing of the potential for screening, early detection and possibly even prevention.

1.3 Epidemiology

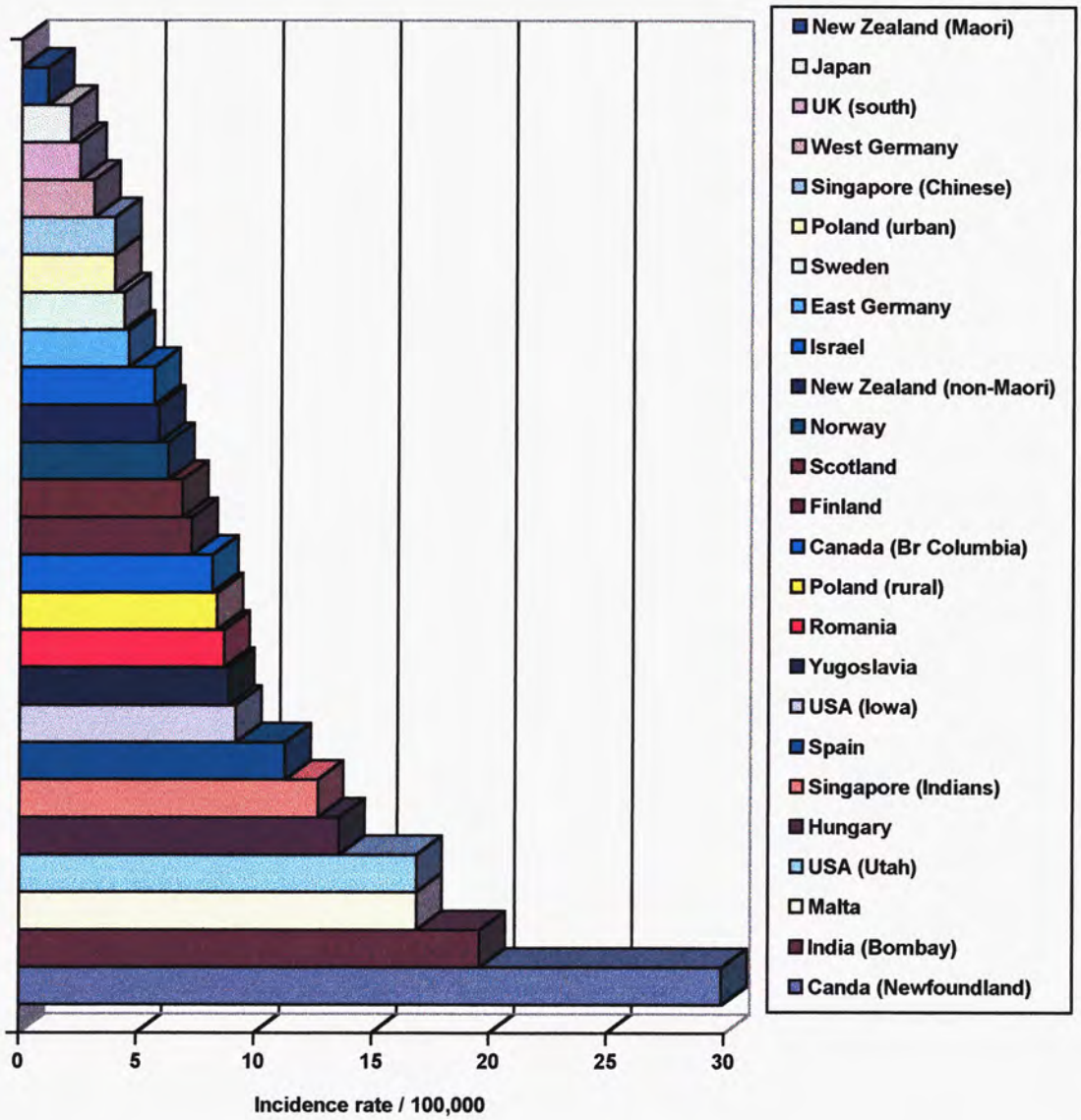
Incidence

There is a marked geographic variation in the incidence of oral cancer both between and within countries (Blot *et al.,* 1994) (Figure 1).

In Europe the male incidence varies between 16.9:100,000 in Malta to 6.9:100,000 in the northern UK and 2.5:100,000 in the southern UK. The mean UK annual incidence rate for both sexes is approximately 4.5:100,000. These variations can be almost entirely explained by the increased incidence of lip cancer in Malta compared to the southern UK consequent upon an increased exposure to actinic radiation. Similar variations are seen in America between Newfoundland (29.9:100,000) and British Colombia (8.2:100,000) again resulting from increased exposure to actinic radiation. Evidence for the protective role of racial cutaneous pigmentation exists in that the non-Maori population of New-Zealand has an incidence **of** oral cancer 5 times greater than that of the Maori population. In Asia the incidence varies between Bombay (19.6:100,000) and Japan (2.1:100,000). In this situation however, the variation is thought to be due to the widespread habit of Betel nut chewing practised by certain sections of the Indian population (Waterhouse et.al.,1976; Henk and Langdon,1985).

Although confined to a small and well defined anatomical area, the sites that together comprise the term "oral" as defined previously, show very different patterns of tumour incidence, behaviour and prognosis. It **is** thus misleading to equate incidence and survival figures pertaining to oral cancer as a whole (or worse still, "head and neck cancer") with those for the individual sites within the oral cavity (Table 1).

Figure 1



Annual age-adjusted incidence rates for oral cancer in males per 100,000 population. (Adapted from Waterhouse *et.al,* 1977).

Table 1

Site	Population Males	Females	Total
Oral cavity (ICD 140, 141, 143-5)	4.62	2.43	3.51
Salivary gland (ICD 142)	0.74	0.62	0.68
Pharynx (ICD 146-148)	2.59	1.05	1.81
Larynx (ICD 161)	7.12	1.40	4.22
All head and neck sites	17.76	9.42	13.53

Annual incidence of head and neck cancer per 100,000 population in the West Midlands Health Region 1988-92.
(Data from West Midland Cancer Registry).

Table 2

Site	Registry Bas-Rhin (France)	Bombay (India)	Newfoundland (Canada)	Trent (UK)
Buccal cavity & pharynx (ICD 140-9)	49.1	34.3	21.3	5.5
Lip (ICD 140)	0.7	0.3	15.1	0.8
Tongue (ICD 141)	7.4	9.4	1.5	1.1
Salivary gland (ICD 142)	0.8	0.5	0.5	0.6
Mouth (ICD 143-5)	13.5	6.5	1.7	1.3
Oropharynx (ICD 146)	10.7	3.5	0.8	0.4
Nasopharynx (ICD 147)	1.1	0.8	0.8	0.4
Hypopharynx (ICD 148)	11.9	9.9	0.8	0.6

World age-standardised mean annual incidence rates for malignant neoplasms per 100,000 males c1980.
(Data from Muir *et al*, 1987; Langdon and Henk, 1995).

The site specific oral cancer data also show wide geographical variation (Table 2). For example, the Bas-Rhin region in France has the highest recorded incidence of oral cancer in the world. This is thought to be due to the combined effects of alcoholic liver disease and the large quantity of home-brewed spirits containing toxic distillation by-products produced and consumed in this region (Binnie *et al.,* 1972; Henk and Langdon,l985). However, Bombay, which has the second highest incidence of oral cancer in the world, has a greater incidence of lingual carcinoma than does Bas-Rhin.

Regardless of geographical or anatomical site, oral cancer affects men more frequently than it affects women. The overall mean age at first presentation is in the range of 60-65 years, with men presenting approximately 5 years earlier than women (62.6 and 67.2 years respectively) (Henk and Langdon,l985; Shah *et al.,* 1990; Worrall and Corrigan,1995).

Since the turn of the century there has been a steady decline in the incidence of oral cancer. However, several authors have reported an increasing incidence in some oral cancers in both men and women (Hindle and Nally, 1991; Zakrzewska, 1994; Worrall, 1995; Hindle *et al.,* 1996). Analysis of all new oral cancer registrations in England & Wales over the last 20 years confirms this increasing incidence and also shows a trend towards first presentation in a younger age group (Table 3). It should be noted that these are raw data and are not standardised either for age, sex or population. Furthermore, while the percentage changes in incidence may be large the actual number of cases involved is small. Conclusions drawn from this data could thus be misleading. Nevertheless, it is instructive to note the changing pattern of disease over this time period.

There were 1731 new oral cancer registrations in 1989 compared to 1570 in 1971, an increase of 10.3%. Comparing the periods 1971-75 with 1985-89 there was a slight fall

in new registrations from 8313 to 8114 (2.4%). The male: female ratio has fallen from 2.1:1 in 1971 to 1.8:1 in 1989. Regardless of whether the data were compared between the single years 1971-1989 or the two 5 year periods 1971-75 & 1985-89 the overall trends for each oral cancer site were the same.

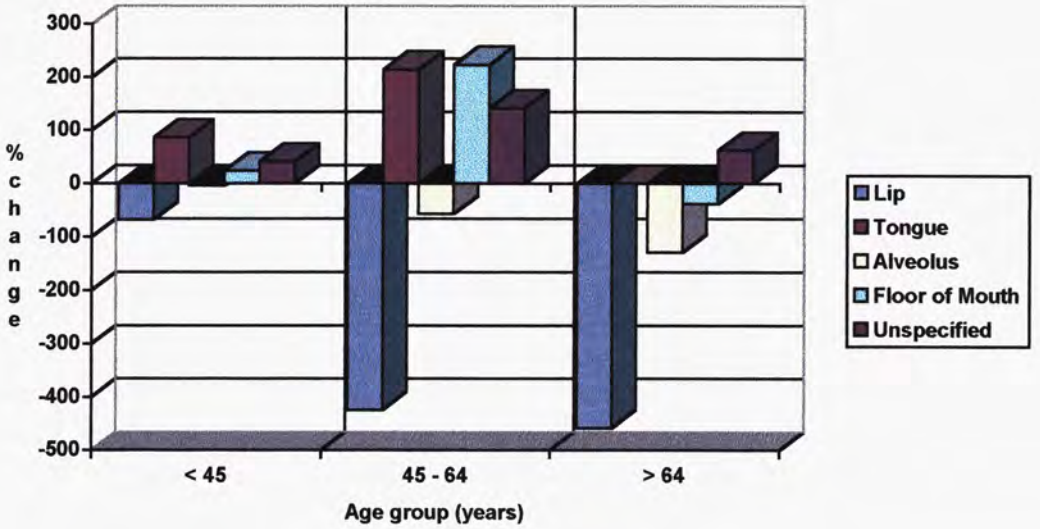
Table 3

Site	Age group (years)	Males		Females	
		1971-75	1985-89	1971-75	1985-89
Lip	< 45	106	38	16	6
(ICD 140)	45-64	705	281	66	47
	>64	1114	656	232	218
Tongue	< 45	107	193	56	96
(ICD 141)	45-64	573	787	370	319
	> 64	886	886	721	751
Alveolus	< 45	27	23	13	17
(ICD 143)	45-64	163	106	93	54
	> 64	290	161	237	202
Floor of mouth	< 45	28	52	13	14
(ICD 144)	45-64	340	563	103	153
	> 64	468	430	163	204
Unspecified	< 45	41	82	42	50
(ICD 145)	45-64	282	423	188	215
	> 64	503	565	367	522

New oral cancer registrations by site, sex and age group: 1971-1975 & 1985-1989. (Office of population Censuses and Surveys: Cancer Statistics Series MB1, No's 1, 2, 3, 4, 5, 18, 19, 20, 21, & 22).

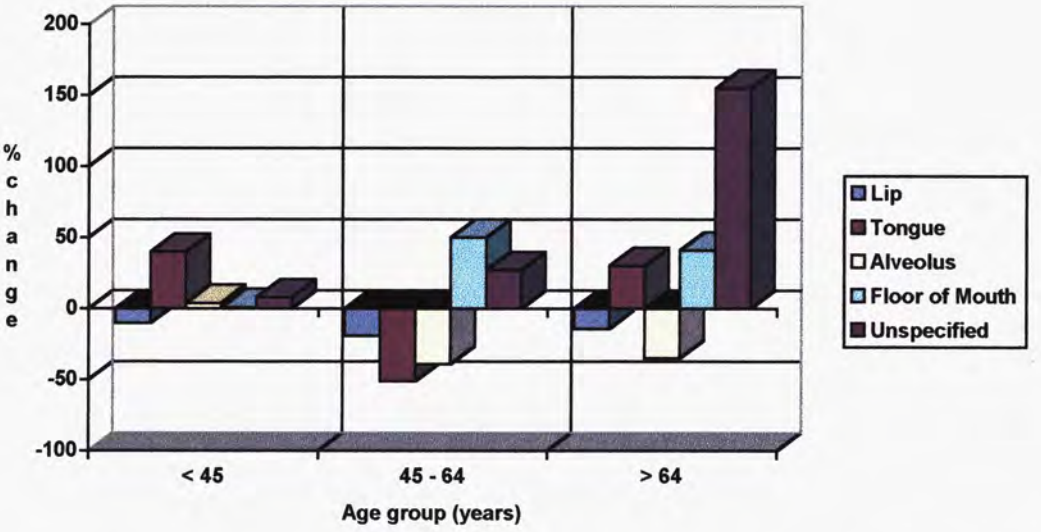
The incidence of carcinoma of the lip has declined in all age groups and in both sexes since 1971. **A** similar decline in alveolar carcinoma is seen in all male age groups but has increased in women under 45 years by over 30%. Carcinoma of the tongue and floor of mouth has increased markedly in males and females. This increase has been in a younger population than traditionally felt to be at risk. Floor of mouth cancer has seen the largest overall increase in both women (33%) and men (25%). Lingual and floor of mouth carcinoma has increased in men under 45 years of age (80.4%) and (85.7%) respectively (Figures 2 and 3).

Figure 2



Changes in the number of new oral cancer registrations in males by site and age group between 1971-1975 and 1985-1989.
(Office of population Censuses and Surveys: Cancer Statistics Series MB1, No's 1, 2, 3, 4, 5, 18, 19, 20, 21, & 22).

Figure 3



Changes in the number of new oral cancer registrations in females by site and age group between 1971-1975 and 1985-1989.
(Office of population Censuses and Surveys: Cancer Statistics Series MB1, No's 1, 2, 3, 4, 5, 18, 19, 20, 21, & 22).

Hindle and Nally (1991), have drawn attention to the increasing incidence of oral cancer particularly in women. They suggested that this may be due to a rise in alcohol consumption in men and to a rise in both tobacco and alcohol consumption in women.

More recently, Hindle ***et al.,*** (1996) have reported on the epidemiological trends in oral cancer in the UK from 1901 to 1990. This large study demonstrated that there have been significant increases in oral cancer incidence and mortality in younger males and a smaller increase in younger females over the last 30 years confirming the results of the previously cited smaller studies. Male mortality from oral cancer in the 35-64 year old age group rose from a century low of 1.67:100,000 in 1966-1970 to 2.91:100,000 in 1986-1990. Incidence rates in this group have increased from 3.61:100,000 in 1962-1966 to 5.52:100,00 in 1982-1986. During the same time periods incidence and mortality rates for males aged over 65 have fallen. A similar trend was noted in young females with mortality rising from 0.73:100,000 in 1971-1975 to 0.93:100,000 in 1986-1990 and incidence rising from 1.85:100,000 in 1962-1966 to 2.19:100,000 in 1982-1986 in the 35-64 year old age group. Again, the incidence and mortality rates for females aged over 65 years fell during these time periods. The increases in mortality and incidence are associated with the cohorts of patients born after 1911-1912. The authors suggest that the pronounced rise in alcohol consumption in the UK over the last 40 years may be responsible for the increases (Hindle ***et al.,*** 1996).

However, while tobacco usage is the most important cause of oral cancer, and there is an important synergism (multiplicative) with excessive alcohol consumption (La Vecchia ***et al.,*** 1997), a significant minority of patients with oral cancer do not have these risk factors. Ehlinger ***et al.,*** (1993), noted that 32.6% (78% of women) of their patients with oral cancer had a negative history for alcohol and tobacco consumption. There is no

doubt that many patients seen in oral cancer clinics are life time teetotallers and non-smokers.

Aetiology

It has been estimated that up to 80% of all human malignancies result from exposure to and mutational damage from environmental carcinogens (Doll and Peto, 1981). There is no reason to suppose that this is not equally applicable to the aetiology of oral cancer. It is likely that certain individuals are at increased risk of developing oral cancer not only because of increased exposure to mutagenic environmental agents consequent on their lifestyle, but also because of a reduced ability to deal adequately with these agents due to inherited genetic defects, notably in the loci coding for phase 1 and phase 2 detoxification enzymes.

The risk factors involved in the aetiology of cancer of the lip are to a large extent different to those involved in intra oral carcinoma. The vast majority of labial carcinomas involve the lower lip. The vermilion border and labial mucosa are exposed to carcinogens acting on both the oral cavity and the extraoral facial skin. Actinic radiation is the pre-eminent risk factor for labial carcinoma. The geographic variations in oral cancer incidence rates described previously can be explained almost entirely by variations in the amount of average daily sunshine to which the local population is exposed, and the degree of racial pigmentation of that population. Dark skinned races have considerably lower rates of lip cancer compared to fair skinned races living at the same latitude indicating the protective role of melanin (Anderson, 1971; Lindquist and Teppo, 1978).

Pipe smoking, in particular clay pipes has long been associated with lip cancer (Levin ***et al.,*** 1950). The combined effects of heat from the non-insulated pipe stem and the carcinogens in the tobacco are both felt to be causally involved. The high incidence of labial cancer in certain regions of Czechoslovakia is thought to be due to the combined carcinogenic effects of sunlight, spiced food and the smoking of short stemmed pipes **(Henk** and Langdon,1985). The fall in labial cancer incidence rates in UK males may be due **to** a reduction in pipe smoking (especially clay pipes) and a reduction in outside working (Hindle and Nally, 1991**;** Johnson and Warnakulasuriya,1993). It is likely that reduced exposure to actinic damage is also responsible for the fall in labial cancer in women, albeit from a much lower incidence base than in men.

Tobacco

Globally, tobacco consumption in all its various forms (smoking, chewing & snuff dipping etc.) is the commonest aetiological risk factor for the subsequent development of oral cancer (Elwood *et al.,* 1984; IARC, 1985; Blott *et al.,* 1988). In developing countries the use of tobacco and /or the areca nut produces chronic, potentially malignant lesions (leucoplakia, erythroplalua & submucous fibrosis) from which the majority of oral cancers arise (Daftary *et al.,* 1992). Conversely, in the developed Western countries, potentially malignant lesions or conditions are identifiable in only a minority of cases and the majority of oral cancers arise *de novo* from clinically normal oral mucosa (Table **4).** These cancers are more aggressive and have a poorer prognosis than cancers arising within pre-existing potentially malignant lesions, most notably from within areas **of** tobacco induced leucoplakia. (Henk and Langdon, 1985; Johnson *et al.,* 1993).

Table 4

Aetiology	Number	Percentage
None	108	55.7
Leucoplakia	39	20.1
Cigarette smoking	22	11.3
Mechanical irritation	14	7.2
Excessive alcohol	3	1.5
Syphilis	3	1.5
Betel nut chewing	1	0.5
Chronic hyperplastic candidosis	2	1.0
Pipe smoking	1	0.5
Other	1	0.5

Aetiological factors thought to be significant in a series of 194 patients with oral cancer (London, UK).
(Henk and Langdon, 1985).

In the Western world cigarette smoking is responsible for the vast majority of all tobacco related oral cancers. In Southern Asia, particularly the Indian sub-continent, both tobacco smoking and chewing habits are major aetiological factors. Bidi smoking and pan chewing are the commonest forms of tobacco usage in India. The Bidi is a cheap home made smoking stick comprising coarse tobacco wrapped in a temburni leaf. Pan is a small packet or quid containing areca nut, lime and usually tobacco wrapped in a betel leaf (Gupta,1984). It has been estimated that some 200 million people chew betel quid making this the single most important known aetiological risk factor for the development of oral cancer world wide (Johnson and Warnakulasuriya,1993).

The risk of developing oral cancer is directly related to the intensity of tobacco usage (Graham *et al.,* 1977; La Vecchia *et al.,* 1997) with heavy smokers (over 20 cigarettes or 5 cigars per day) having a six fold increased risk of developing the disease compared to non-smokers (Cawson *et al.,* 1996). Quitting smoking, especially for 10 years or more reduces the odds ratio for developing oral cancer almost to unity (Franceschi *et al.,* 1990). For those who both chew betel quid and smoke cigarettes the risk of developing oral cancer is 12 times greater than exists for non-chewers and non-smokers (WHO,1984).

Further evidence for the carcinogenic nature of tobacco derives from the fact that when tobacco is absent from the betel quid the incidence of oral leucoplakia is low (Johnson *et al.,* 1993). Addition of tobacco to the betel quid increases the relative risk of developing oral cancer by a factor of three (Warnakulasuriya and Johnson, 1991). The probable cause of this differential effect is that when chewed, the carcinogens in the tobacco such as polycyclic aromatic hydrocarbons (PAH), N-nitrosamines and epoxides are held directly in contact with the oral mucosa.

Oral cancer is rare in non-smokers. In a study of 47,866 Californian Seventh Day Adventists (a relatively non-smoking religious sect) between 1958 and 1963 only 1 male and **2** females died of oral or oropharyngeal cancer (Lemon *et al.,* 1964). Oral and oropharyngeal cancer deaths accounted for 0.4% and 0.5% of all male and female deaths respectively in this group. The solitary male was among a small sub-group of sect members who had smoked for up to 20 years. During the same time period 1,855 males and 595 females from the same geographical area in California who were not Seventh Day Adventists died from oral cancer. Representing 3.5% and 1.3% of all deaths in males and females respectively in this group.

Alcohol

The consumption of alcoholic beverages in excess is another major aetiological factor in the genesis of oral cancer. This is likely to be due to a local carcinogenic effect on the oral mucosa as excess alcohol intake is not associated with an increased incidence of carcinoma of the intrinsic larynx (Elwood *et al.,* 1984). In the mouth, alcohol may act as a solvent enhancing the transfer of carcinogens across the normally intact mucosal barrier (La Vecchia *et al.,* 1997). There are often confounding variables between smoking and alcohol because many heavy smokers are also heavy drinkers (Cawson *et al.,* 1996). In a recent study of 116 patients with oral squamous cell carcinoma 80% of men and 50% of women were smokers with 45% of each sex smoking over 20 cigarettes per day. In the same study 74% of men and 53% of women drank alcohol with 33% of men consuming over 40 units per week. Seven of the ten patients in the study who drank over 40 units per week also smoked over 20 cigarettes per day (Worrall and Corrigan, 1995). However, several studies have demonstrated that the consumption **of** alcohol is a risk factor for oral

cancer independent of tobacco smoking (Blott *et al.,* 1988; Elwood *et al.,* 1984). As with tobacco consumption there is a direct relationship between the amount of alcohol consumed and the development of oral cancer. There is evidence suggesting that it may not be the ethanol itself in the alcoholic beverage that is responsible for the carcinogenic effect, rather it may be due to contaminants or to by-products of ethanol metabolism such as acetaldehyde (MacSween, 1982; Ng *et al,* 1993). In addition to its primary aetiological role in oral cancer, alcohol acts synergistically with tobacco in an additive or multiplicative fashion (Elwood *et al.,* 1984). The relative risk for the development of oral cancer in both heavy drinkers and heavy smokers has been shown to be as high as 24 (McCoy and Wynder, 1979). In a study of 291 Italian patients with oral and oropharyngeal cancers Franceschi *et al* (1990), demonstrated that heavy drinkers and heavy smokers had an 80 fold greater odds ratio for cancer than light drinkers and smokers.

Potentially malignant lesions

Leucoplakia and erythroplakia are frequently referred to as premalignant or precancerous lesions. The implication being, that once present, there is an inevitable progression from a white or red patch through various stages of epithelial dysplasia and cellular atypia to invasive carcinoma. However, not all leucoplakias and erythroplakias will progress to invasive carcinoma. Some will remain static while others may regress completely (van der Waal *et al.,* 1997). It is thus more accurate to use the term potentially malignant lesions when referring to leucoplakia and erythroplakia (Johnson *et al.,* 1993).

Leucoplakia, defined as a white patch that cannot be characterised as, or associated with, any other disease or any physical or chemical agent except the use of tobacco, is the

commonest potentially malignant oral lesion (Axell *et al.,* 1984). The smoking and/or the chewing of tobacco are widely accepted to be the major aetiological factors for the development of oral leucoplakia (Pindborg,1980). Although the majority of leucoplakias world wide are induced by tobacco and local trauma, the factors involved in the causation of many of these lesions remains unknown. And while most leucoplakias simply reflect benign hyperkeratosis of the oral mucosa (Silverman Jr *et al.,* 1984), globally some 70% of all oral cancers arise from such lesions (Johnson *et al.,* 1993).

The true rate of malignant transformation is difficult to quantify with various studies quoting a range of between 0. 13 – 6% (Tradati ***et*** *al.,* 1997). Although the World Health Organisation consensus range is between 3 and 6% (WHO,1978;Johnson *et al.,* 1993), the actual rate may be significantly greater than this and may be a high as 15% (Tradati *et al.,* 1997) The nodular or speckled variety of leucoplakia caries a greater risk of malignant transformation than the commoner, homogenous type although homogenous leucoplakia in the floor of the mouth known as sublingual keratosis, is more prone to malignant change. In a study of 257 patients with oral leucoplakia followed up for an average period of 7.2 years, 45 (17.5%) subsequently developed oral squamous cell carcinomas. Although 73% of patients smoked at the time of diagnosis there was a higher rate of malignant transformation of leucoplakias in non-smokers (1**8%)** than smokers (15%) (Silverman Jr *et al.,* 1984). Banoczy and Csiba (1976), reporting on 500 leucoplakias found an overall malignant transformation rate of 9.6%. The transformation rate increased to 13.2%(9/68) in leucoplakias showing histological features of dysplasia. Of the 9 cancers, 8 arose in patients whose leucoplakia was managed conservatively and not surgically excised. These authors also noted increased malignant transformation rates for lingual leucoplakias.

Oral leucoplakia is positively associated with bidi smoking, betel quid chewing and the use of smokeless tobacco such as snuff dipping (Johnson *et al.,* 1993). Gupta (1984), surveyed over 12,000 tobacco users in Kerala, India to assess their tobacco habits and identify any associated oral lesions. Nearly 10,500 subjects either chewed betel quids or smoked bidis and the remainder both chewed and smoked. **A** highly significant dose-response relationship was demonstrated which was stronger for smoking than chewing. Leucoplakia was found in 20.7:1000 of all subjects, ranging from 11.3:1000 in those who smoked or chewed between 1 and 5 bidis or betel quids respectively per day, to 33.7:1000 for those who smoked or chewed 16 or more bidis or betel quids respectively per day. There is evidence that the leucoplalua associated with bidi smoking has **a** greater potential for malignant transformation than that associated with betel quid chewing (Mehta *et al.,* 1981). The stronger correlation between bidi smoking and leucoplalua, and the poorer prognosis for bidi associated leucoplakias may be due to the composition of the bidi smoke which has a significantly higher concentration of carcinogenic agents compared to traditional Western type cigarette smoke (Hoffman *et al.,* 1974).

As with the risk of developing oral cancer, the presence or absence of tobacco from the betel quid is important for the subsequent development of leucoplakia. The presence of tobacco in the betel quid increases the relative risk of developing leucoplakia 3 fold (Warnakulasuriya and Johnson,1991).

When considering the role of tobacco smoking in leucoplakia and oral cancer it is important to appreciate the differences between commercially produced cigarettes and the items smoked in developing countries. Despite the extensive evidence implicating cigarette smoking in the genesis of oral cancer, Western style tobacco smoke is a relatively weak carcinogen and it probably has a relatively limited influence on the incidence of oral

cancer globally (Henk and Langdon,l985). In contrast, tobacco smoking habits in the developing countries where the bidi or similar home made smoking sticks are employed are strongly associated with oral leucoplakia and cancer. The increased risks are due not **only** to the enhanced carcinogenic potential of the bidi smoke in comparison to Western cigarettes, but also to the vast numbers of people world wide who engage in these smoking habits.

Moreover, in Western countries, while tobacco smoking and chewing are potent causes of leucoplakia non-users of tobacco with idiopathic leucoplakia are at increased risk of developing oral cancer. This risk increases if there is histological evidence of epithelial dysplasia and/or the leucoplakia is on the tongue. Clearly, these high risk leucoplakias warrant aggressive treatment by surgical excision.

Since its original description in 1911 as a lesion occurring on the glans penis in elderly syphilitic men when it was termed erythroplasia, erythroplakia has become widely recognised as the other clinically significant potentially malignant oral lesion (Raubenheimer and De Villiers, 1989; Johnson *et al.,* 1993). It is defined as any lesion of the oral mucosa presenting as a bright red velvety plaque which cannot be characterised clinically or pathologically as any other lesion (Henk and Langdon,l985). Like leucoplakia the diagnosis is clinical and by exclusion. Areas of leucoplakia and erythroplakia may coexist, and the speckled areas in speckled leucoplakia are often separate areas of erythroplakia (Cawson *et ul.,* 1996). Erythroplakia is much rarer than leucoplakia but it caries a significantly greater risk of malignant transformation. The malignant transformation rate of erythroplakia is approximately 17 times that of leucoplakia and the majority if not all are areas of either severe epithelial dysplasia,

carcinoma *in situ* or invasive carcinoma (Shafer and Waldron, 1975). Of extreme clinical importance is the fact that erythroplakia is clinically indistinguishable from many innocent inflammatory conditions and that very small innocuous areas of erythroplakia may escape detection yet be the site of an invasive carcinoma (Shafer ***et al.,*** 1974; Raubenheimer and De Villiers, 1989).

Diet and nutrition

Dietary factors have long been implicated in the aetiology of colorectal carcinoma. There is a strong correlation between the intake of meat, particularly red meat and the incidence of colorectal carcinoma (Hill, 1997). The cooking process results in the formation of N-heterocyclic compounds from sugars, amino acids and creatinine contained in red meat *via* the Maillard reaction (O'Brien, 1997). Heterocyclic amines derived from precursor N-heterocyclic compounds are activated by the cytochrome P450 (CYP) CYP1A2 enzyme and subsequently undergo Phase 2 esterification by N-acetyl transferases (NAT) and Glutathione S-transferases (GST) resulting in the production of genotoxic species that have been demonstrated to be mutagenic in animals (Gooderham, 1997). Interestingly, the food carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-b] Pyridine (PhIP), which is the predominant heterocyclic amine present in the human diet, is capable of detoxification by animals but not by humans who almost exclusively activate PhIP *via* CYP1A2 (Gooderham, 1997).

Nutritional factors, in particular dietary deficiencies have been associated with an increased risk of developing oral cancer. The Patterson-Kelly syndrome was first described by Donald Rose Patterson and Adam Brown Kelly in 1919, and subsequently by

Henry Stanley Plummer and Porter Paisley Vinson in 1921, hence its alternative eponym of the Plummer-Vinson syndrome (Harding Rains and Ritchie,1984; Schusterman ***et al.***, 1991). This syndrome consisting of iron deficiency anaemia, sideropaenic dysphagia and post-cricoid carcinoma is a well recognised disease entity, particularly in Scandinavian women (Larsson ***et al.***, 1975). Various dietary micro-nutrients such as vitamins and trace elements have recently been shown to be protective against oral cancer. Frequent consumption of fresh fruit and vegetables is associated with a reduced risk (0.5-0.7) of developing oral and oropharyngeal cancer (La Vecchia ***et al.***, 1993). More specifically, the antioxidant nutrients ß-Carotene and vitamin E have been shown to produce regression of oral leucoplakia, while diets low in carotenoid intake result in an increased cancer risk (Garewal and Schantz, 1995). Evidence is emerging that prolonged and heavy consumption of foods rich in nitrites and nitrosamines such as preserved meats and fish significantly increases lifetime risk for the development of oral cancer (Johnson ***et al.***, 1996). Approximately 15% of oral and oropharyngeal cancers can be attributed to dietary deficiencies or imbalances (La Vecchia ***et al.***, 1997).

Published in August1980, the Black Report highlighted the association between social (occupational) class and health in the UK (Working Group on Inequalities in Health, 1980). The report showed that low socio-economic status is positively associated with increased morbidity and mortality. For example, in England and Wales in 1971 the death rate for adult men in social class 5 was almost two and a half times that of adult men in social class 1 (9.88/1000 and 3.98 /1000 respectively). Furthermore, an almost linear relationship was demonstrated between lower social class and rising standardised mortality ratio in adult males dying from malignant neoplasms (Working Group on Inequalities in Health, 1980, page 40).

Lower socio-economic status is also positively linked with a higher incidence of oral cancer (Table **5***)*. The excess oral cancer incidence seen in socially and financially disadvantaged patient groups is also likely to explain the time-honoured association between poor oral hygiene with a neglected dentition and an increased incidence of oral cancer (Graham ***et*** *al.,* 1960). Part, but not all of the increased oral cancer risk seen with diets low in fresh fruit and vegetables is likely to be due to the association between poor diet and low socio-economic status.

Table 5

Social Class	Number (N = 106)	Percentage
1	8	7.5
2	14	13.2
3	19	18.0
4	44	41.5
5	21	19.8

The social class of 106 patients with oral cancer treated at the North Staffordshire Hospital between 1986 and 1996.
(Worrall, unpublished data).

The traditional argument that it is the chronic trauma from broken teeth which leads to the development of an oral cancer is probably spurious. Rather, it is likely that numerous factors such as increased tobacco and alcohol consumption, poor nutritional status and crowded living conditions with increased exposure to environmental carcinogens and infectious agents all combine to produce the excess of oral cancers seen in this group.

Infections

Conflicting evidence exists for the role of various infectious agents in the aetiology of oral cancer. For many years syphilis has been felt to be an important cause of oral cancer, particularly lingual and labial carcinoma (Henk and Langdon,1985). During the 1920's patients with lingual carcinoma were up to ***5*** times more likely to have syphilis than other hospitalised patients (Fry,1929). It is possible that in the past the carcinogenic effects were due as much to the arsenic used in therapy as the disease itself (Smith,1973; Shafer ***et*** *al.,* 1974). It has been reported that the incidence of syphilis in the UK population is falling rapidly and that this reduction may account for a significant proportion of the fall in labial carcinoma currently occurring in the UK (Binnie *et al.,* 1983; Henk and Langdon,l985). While screening for current or previous syphilis infection was a routine component of the clinical assessment of patients presenting with oral cancer until fairly recently, many units have abandoned this procedure because of the perceived fall in disease prevalence. In one series of 197 patients with oral cancer only **3** (1.5%) had positive syphilis serology (Henk and Langdon *et al.,* 1977). However, one recent study found that ***5*** of 63 patients (8%) with carcinoma of the tongue tested positive for syphilis antibodies, indicating that the disease may be more prevalent than was thought to be the case and that routine screening for syphilis is probably warranted in this group of patients (Dickenson *et al.,* 1995).

Considering the high frequency of oral viral infections within the population it **is** not surprising that an aetiological role for viruses in oral carcinoma has been sought. Especially as there is compelling evidence linking specific viruses with squamous cell carcinoma of tissues outwith the oral cavity such as the uterine cervix (zur Hausen,1980). Of the many viruses that are potential candidates for oral carcinogenesis there is little or no

evidence at the present time for either the retroviruses, adenoviruses or the Epstein-Barr virus being involved either directly or indirectly (Cox *et al.,* 1991). Although Herpes simplex viruses (HSV) and the Human papillomaviruses (HPV) and antibodies to them are found in patients with and without oral cancer, and electron microscopy has failed to demonstrate viral particles within tumour cells (Chen and Hardwick, 1977), there is mounting data implicating these viruses in the aetiology of some oral cancers (Johnson and Warnakulasuriya, 1993).

HPV 16 can induce malignant transformation in human squamous epithelial cells (Munger ***et*** *al.,* 1989) and HPV infection is known to block p53 function following its degradation by the HPV-16 associated E6 protein (Johnson *et al.,* 1993). HPV-E7 protein binds to the retinoblastoma gene product and sequesters it thus enhancing its transforming activity (Johnson ***et*** *al.,* 1996). HPV may also integrate into or close to proto-oncogenes such as c-myc and ***ras*** and by upregulating them stimulate mitosis (Johnson ***et*** *al.,* 1996). Mutations at the loci coding for both retinoblastoma and p53 protein have been reported in a wide range of head and neck cancers, in particular tonsil carcinomas indicating a possible oncogenic role for HPV (Field, 1992). In the largest reported molecular epidemiological study of HPV infection in human oral squamous cell carcinoma, Balaram *et al.,* (1999, amplified tumour DNA using the polymerase chain reaction (PCR) and HPV specific primers and found viral DNA sequences in 74% of the lesions. Forty one percent of the HPV positive cases demonstrated infection by multiple HPV strains. The majority of their patients were also betel nut chewers and they propose that carcinogens derived from the betel quid act synergistically with HPV to cause neoplastic transformation.

There is thus increasing although at times conflicting evidence for a role for HPV infection in oral carcinogenesis. Although HPV 16 and 18 are the most frequent types

involved other less well investigated strains may also be important (La Vecchia *et al.,* **1997).**

Patients with oral cancer have raised titres of antibodies against HSV when compared to non-smoking controls but similar titres to smoking controls suggesting that cigarette smoking may predispose to mucosal HSV infection (Shillitoe *et al.,* **1982).** Cigarette smoking is known to suppress natural killer **(NK)** cell activity which is necessary **for** normal host defences against HSV infection (Ferson *et al.,* **1979;** Lopez *et al.,* **1985).** Strong evidence for an association between HSV infection and oral cancer comes from the finding of RNA complementary to HSV-DNA in oral squamous cell carcinoma tissue but not in normal mucosa taken from the same patients (Eglin *et al.,* **1983).** Moreover, immunisation against HSV prevents the co-carcinogenic activity of HSV and Dimethyl Benzanthracene in the hamster cheek pouch model (Park *et al.,* **1990).**

While it is tempting to speculate that HSV and HPV may be responsible for some oral cancers the evidence for their involvement in oral carcinogenesis remains essentially circumstantial. Given the ubiquitous nature of HSV and HPV infection within the population and the relatively low incidence of oral cancer, if they do have an oncogenic role it must be small. There is still the distinct possibility that tumour cells provide a convenient environment for viral infection and that the viruses are little more than innocent passengers (Scully *et al.,* **1985).**

Candida albicans, a normal oral commensal is not infrequently isolated from oral carcinomas which has lead many to speculate that it may have a role in oral carcinogenesis (Langdon and Henk, **1995).** Candida1 hyphae are intracellular parasites (Cawson and Rajasingham, **1972)** which have been shown to induce epithelial hyperplasia *in vitro* (Cawson'1973). O'Grady and Reade **(1992)** produced tongue and palate carcinomas in

experimental rats infected with *C. Albicans* following prior application of 4-nitroquinoline-1-oxide (4NQO). The oral mucosa in rats treated with 4NQO but not infected with *C. Albicans* did not develop carcinomas. The authors concluded that *C. Albicans* might participate in causing neoplastic transformation in humans.

Possible carcinogenic mechanisms include the elaboration of genotoxic metabolites (Krogh *et al.,* 1987) and /or the transfection of invaded epithelial cells by ras fungal oncogenes (Johnson and Warnakulasuriya, 1993). There is evidence that *C. Albicans* may be involved in the genesis of some potentially malignant lesions. The speckled leucoplakias are frequently infected with candida, and as previously discussed the incidence of malignant change is significantly greater in the speckled, non-homogenous leucoplakias than it is in the homogenous ones. Treatment with antimycotics often results in partial or complete resolution of these lesions with leucoplakias previously classified as severely dysplastic reverting to mild dysplasia.

Interestingly, although vaginal infection with *C. Albicans* is common, particularly in pregnancy where over 20% of women harbour the fungus (Llewellyn-Jones, 1986) there is little published literature on the association between carcinoma of the uterine cervix and C. *Albicans* infection. An Internet Medline search (1966-1987) of the terms "carcinoma of the cervix" and *"CandidaAlbicans"* produced 34,098 references of which only 2 discussed the association between infection and carcinoma or its' treatment. It would appear that the incidence of infection with *C. Albicans* is no higher in patients with cervical carcinoma than the normal female population unless treated by radiotherapy when carriage rates increase (Richter *et al.,* 1977; Talwar ***et*** *al.,* 1992).

As with HSV and HPV infections, it is likely that in the majority of cases the presence of candida in oral carcinomas simply reflects secondary infection by

opportunistic organisms. While antibodies to *C. Albicans* are present at high levels in patients with oral carcinoma, the titres are not significantly different to those detected in normal patients or those with non-malignant oral diseases (Winters *et al.*, 1984). The lack of evidence for an association between *C. Albicans* and carcinoma of the uterine cervix, a site with a **high** fungal carriage rate further suggests that it is unlikely to be of significance in oral carcinoma. Most authors are in agreement that if *C. Albicans* is involved in oral carcinogenesis it accounts for relatively few cases (Cawson ***et*** *al.*, 1996).

Xenobiotics and detoxification enzymes

All living organisms are continuously exposed to exogenous and endogenous toxic chemicals. While it is tempting to blame modem industrialised manufacturing processes and motor vehicle pollution for producing these compounds, many xenobiotics occur naturally in plants and have been present in the environment since life began (Hayes and Pulford, 1995). Flavinoids, glucosinolates and saponins present in various plants are rodent carcinogens and mould aflatoxin is one of the most potent carcinogenic agents known. Humans may consume 1.5 g of these naturally occurring plant pesticides each day. Furthermore, many toxic compounds arise as a result of endogenous metabolism. Cell membrane lipid peroxidation occurs following attack by reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide and the hydroxyl ion derived from various metabolic reactions including aerobic respiration and inflammation (Hayes and Pulford, 1995).

To protect themselves from somatic and genetic damage consequent on exposure to both exogenous xenobiotics and endogenous electrophiles, higher organisms have evolved

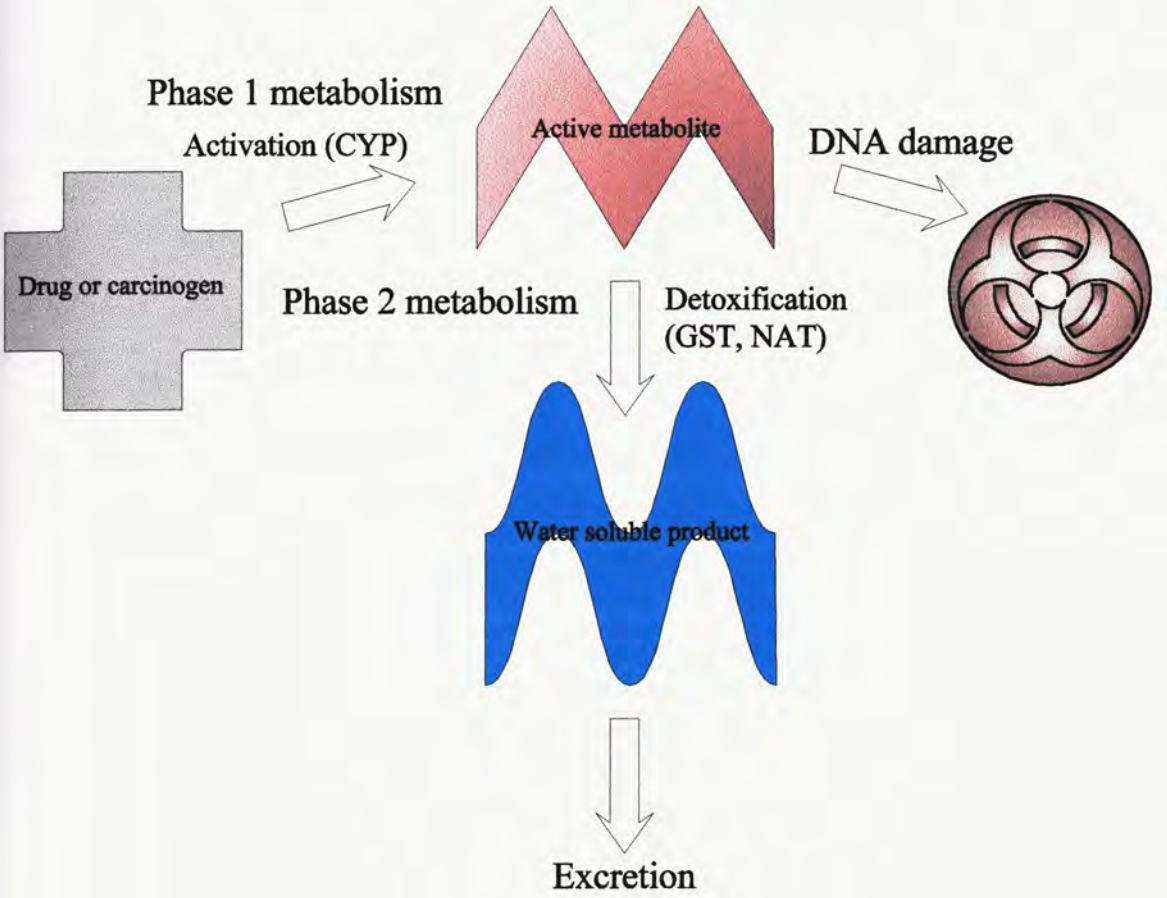
complex and complementary enzyme systems capable of detoxifying and eliminating these harmful compounds. Over the millennia there has been an evolutionary pressure, or molecular drive for organisms to continuously adapt their set of detoxification enzymes in order to metabolise ever changing plant phytotoxins (Ingelman-Sundberg and Johansson, 1995). In the process, several enzyme systems, including the *CYP*, GST and NAT have evolved to protect cells from chemical damage (Smith *et al.*, 1995). While these enzymes are able to metabolise xenobiotics and thus eliminate potentially harmful ingested exogenous compounds, many also play a major role in cellular homeostasis by detoxifying and eliminating endogenously produced chemicals. Detoxification enzyme activity is frequently increased following exposure to a wide variety of substrates indicating a pleiotropic adaptive response to chemical stress. In particular, GST is induced by environmental pollutants, phytochemicals, and raised intracellular H_2O_2 (Hayes and Pulford, 1995).

Oral cancer is thought to be the end result of a multi-phase process in which carcinogen induced genetic changes produce unrestrained cellular growth (Bongers *et al.*, 1995). The first step in this process involves the covalent binding of exogenous xenobiotic compounds to cellular DNA (Harris, 1991). The polycyclic aromatic hydrocarbon 9,10-dimethyl-1,2-benzanthracene (DMBA) reliably induces malignant transformation when applied to hamster cheek pouches (MacDonald, 1978; MacDonald and Pospisil, 1981) and industrial exposure to wood dust and organic chemicals are risk factors for oro-pharyngeal and laryngeal cancer (Johnson and Warnakulasuriya, 1993). However, it is the carcinogens present in tobacco products that are the major xenobiotics involved in oral carcinogenesis.

Human drug metabolism is classically considered as occurring in **2** distinct phases (Figure 4). In phase 1 metabolism, both endogenous and exogenous electrophillic

compounds are converted to reactive intermediary species, most commonly by *CYP* mono-oxygenases *via* the insertion of an atom of molecular oxygen (Smith *et al.*, 1995). The activated metabolites of phase 1 metabolism have an increased affinity for the phase **2** enzymes such as GST and NAT which ultimately results in enhanced excretion of the original compound from the body. However, certain compounds, notably many carcinogens are activated by *CYP* phase 1 metabolism and are converted to mutagenic electrophiles (e.g. epoxides) (Smith *et al.*, 1995). Following phase 1 metabolism, the activated intermediaries are rendered more hydrophilic by conjugation with glutathione (GSH), glucuronic acid or other polar groups *via* the phase **2** enzymes GST, UDP-glucuronyl transferase and NAT and ultimately excreted (Smith *et al.*, 1995). Variations in the activity of either the phase 1 or phase **2** enzymes could thus be expected to influence disease susceptibility by causing elevated levels of reactive and mutagenic metabolites either due to increased synthesis or reduced elimination or a combination of both. Thus, the carcinogens present in tobacco smoke for example, may lead to malignant transformation **of** oral epithelial cells if those cells are either unable to eliminate the carcinogens, or they convert them to more reactive and mutagenic compounds.

Figure 4



Pathways of drug metabolism.
(Adapted from Smith *et al,* 1995).

However, it is self evident that not all tobacco smokers develop oral cancer and not all oral cancer victims are smokers. It is therefore hypothesised that there must be an individual genetic susceptibility to, or protection from chemical carcinogen-induced DNA damage. The first clue to a possible explanation for this problem came in 1977 when, legend has it, a Professor of Pharmacology collapsed with severe hypotension while testing the new antihypertensive medication Debrisoquine ('Declinax' Roche) (Mahgoub *et al.*, 1977). He was subsequently found to be a so called poor metaboliser (*PM*) for the

cytochrome P450 2D6 (CYP2D6) phase 1 detoxification enzyme normally responsible for metabolising Debrisoquine. Since then it has been unequivocally established that many detoxification enzyme proteins are the products of genes that are polymorphic and are thus subject to variable expression and activity (Smith *et al.,* 1995).

CYP

The cytochrome P450s are a superfamily of hemoproteins which display a characteristic 450 nm absorption spectrum in the reduced CO-bound state (Crespi ***et al.,*** 1991; Ingleman-Sundberg,1995). They are divided into 12 families on the basis of their sequence homologies, those *CYPs* with greater than 40% similarity are largely grouped into the same family while those with over 55% homology are placed in the same subfamily. The *CYPs* are responsible for the phase 1 metabolism of a wide range of drugs, plant toxins, xenobiotics and endogenous compounds including steroids, fatty acids and cholesterol. Despite the fact that over 350 different *CYPs* have been cloned, it appears that only about ***6*** are important for human drug metabolism or homeostasis (Ingleman-Sundberg,1995).

The *CYP1A* gene family located at 15q22-ter has 2 functional genes, *CYP1A1* and *CYP1A2*. CYP1**A**1, which is predominantly an extrahepatic enzyme metabolises polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and benzo(a)pyrene while CYP1A2, which is liver specific metabolises nitrosamines and arylamines (Smith *et al.,* 1995). Currently, 3 *CYP1A1* polymorphisms have been described; a T-C transition in the 3' non-coding region, an **A-G** point mutation in exon **7** resulting in an isoleucine to valine substitution and an **AT-GC** transition in the 3' non-coding region (Smith *et al.,* 1995).

CYP1A1 expression is induced by tobacco smoke derived **PAH's** in the lungs of over **80%** of smokers and in Japanese but not Caucasian populations *CYP1A1* polymorphisms are associated with an increased risk of lung cancer (Smith *et al.,* 1995; Nebert *et al.,* 1996).

The CYP2D6 (debrisoquine hydroxylase) enzyme is involved in the metabolism of over 40 drugs and xenobiotics including antidepressants, neuroleptics, serotonin-specific compounds, ß-blockers, anti-arrhythmics and opiates (Marzo and Balant, 1996; Nebert *et al.,* 1996). It also metabolises and activates the carcinogenic tobacco smoke-derived nitrosamine, 4-(methylnitrosamino)-1-**(3** pyridyl)-1-butanone **(NNK)** producing agents capable of both methylating and pyridyloxobutylating DNA (Crespi *et al.,* 1991). **NNK** is the only procarcinogen reported that is activated by CYP2D6 (Crespi *et al.*, 1991).

Variation in CYP2D6 enzyme activity is based on an autosomal recessively inherited polymorphism at its gene locus on human chromosome 22q13.1 (Gough *et al.,* 1993; Ingleman-Sundberg and Johansson, 1995).

There are over 53 allelic variants of *CYP2D6* but only 3 are common (Marez *et al.*, 1997):

- *CYP2D6A* results from a base pair deletion in exon 5 producing a frame shift mutation
- *CYP2D6B* is due to a G-to-A transition at the intron 3 / exon **4** boundary which shifts the position of the **3'** splice site resulting in defective splicing prior to **mRNA** synthesis
- *CYP2D6D* which is due to an entire gene deletion.

Of these *CYP2D6B* is the commonest polymorphism accounting for over 70% of all PM genotypes (Gough *et al.*, 1990; Heim and Meyer, 1990; Smith *et al.*, 1995; Lear *et al.*, 1996; Nebert *et al.*, 1996).

Polymorphism appears to have resulted from homologous unequal cross-over of a 2.8 kb repeat region which flanks the active *CYP2D6* gene (Steen *et al.*, 1995). Inheritance of 2 functioning alleles produces the wild type or extensive metabolizer *(EM)* genotype (80-90% of the population) while a polymorphism resulting in no CYP2D6 enzyme activity results in the poor metabolizer *(PM)* genotype. A small, poorly categorised *EM* subgroup termed ultrarapid metabolizers (up to 5% of the population) (Steen *et al.*, 1995; Nebert *et al.*, 1996) possess enhanced CYP2D6 enzyme activity as a result of gene amplification (Steen *et al.*, 1995). *PM* frequency varies in different ethnic populations. Between 5% and 7% of Caucasians but only 1% of the Chinese population have the *CYP2D6* PM genotype (Johansson *et al.*, 1991).

GST

The glutathione S-transferases (GST) are a supergene family of phase 2 detoxification enzymes that play a central role in the metabolism of reactive Substrates, many of which are the products of previous CYP phase 1 reactions (Smith *et al.,* 1995; Strange and Fryer, 1997). The GST are intracellular cytosolic or membrane bound enzymes capable of catalysing a wide range of reactions with both endogenous and exogenous Substrates. Typically, they catalyse the conjugation **of** the sulphur atom **of** glutathione to the electrophilic centres of compounds such as arene oxides and organic halides (Bongers *et al.,* 1995; Strange and Fryer, 1997). Numerous carcinogenic compounds contain electrophillic centres including aflatoxin B_1, benzo[a]pyrene, 7,12 and dimethylbenz[a]anthracene (Hayes and Pulford, 1995).

The GST act co-operatively with numerous anti-oxidant agents including the C and E vitamins and the carotenoids playing a pivotal role in protecting cells from damage mediated by oxidative stress (Bongers *et al.,* 1995). GST mediated GSH-electrophile conjugates are less reactive, less hydrophobic, less likely to partition into membrane lipids and have a shorter half life than their parent compounds. However, the major biological value for GSH conjugation probably lies in its ability to produce compounds that can be exported from the cell by specific ATP-dependent GSH-conjugate efflux pumps called GS-X pumps (Hayes and Pulford, 1995). Because the GST are also involved in the detoxification of many cancer chemotherapeutic agents including cisplatin and alkylating agents, enhanced GST activity may result in reduced tumour cell killing by these agents resulting in the emergence of drug-resistant tumours (Sarkar *et al.,* 1997).

The dimeric cytosolic GST are divided into **6** classes alpha, mu, pi, theta, sigma and zeta on the basis of their sequence homology with members of the same class showing

a minimum of **65%** aminoacid homology (Smith *et al.,* **1995;** Board *et al.,* **1997).** While most work has focused on the activities of the cytosolic GST and their role in detoxification reactions and free radical scavenging, it is known that the membrane bound enzymes play a major role in protecting cell membranes from peroxidation damage (Bongers *et al.,* **1995;** Smith *et al.,* **1995;** Strange and Fryer, **1997)** (Table **6).**

Table 6

GST class	Chromosome	Number of genes	Carcinogen substrates
α GSTA	6p 12	>2	Aromatic amines, polycyclic aromatic hydrocarbons, lipid peroxidation products, chemotherapy drugs
μ GSTM	1p 13	5	lipid peroxidation products
π GSTP	11	1	Polycyclic aromatic hydrocarbons
θ GSTT	22	>2	Solvents
ζ GSTTZ	14q 24	?	?
Microsomal GST Mic	12	1	lipid peroxidation products

Some properties of the glutathione S-transferases.
(Data from Bongers *et al.,* 1995; Smith *et al.,* 1995; Strange and Fryer, 1997; Board *et al.,* 1997).

The first GST demonstrated to be polymorphic was the ***GSTM1*** locus (Board, 1981). This locus consists of 3 possible alleles: ***GSTM1****0, ***GSTM1 *A*** and ***GSTM1****B*. The ***GSTM1 ****O* is deleted, hence ***GSTM1 ****O* homozygotes (***GSTM1****null*), express no GSTM1 protein. ***GSTM1 *A*** and ***GSTM1****B* differ by just one base in exon 7 (Strange and Fryer,1997). There is evidence that ***GSTM1 *A*** and ***GSTM1****B* confer differing susceptibilities to diseases such as bladder cancer, Crohn's disease and multiple cutaneous basal cell carcinomas. ***GSTM1*** *A/B* but not ***GSTM1 A*** or ***GSTM1*** B appear protective against these diseases. Considering that the ***in vitro*** catalytic activities of the enzyme products of these 2 alleles are identical some other phenomenon must also be involved (Inskip ***et*** *al.,* 1995).

Similarly, the GSTM3 locus comprises ***2*** alleles, ***GSTM3 *A*** and ***GSTM3 *B*** which differ due to the deletion of 3 base pairs in intron ***6*** of ***GSTM3****B*. It has been suggested that ***GSTM3****B* but not ***GSTM3 *A*** is inducible, due to the presence in the former of the 5'-AAGATA-3' recognition site for the YY1 transcription factor (Inskip *et al.,* 1995). The intron ***6*** deletion being the only difference between the 2 alleles must thus influence the

YY1 recognition motif (Inskip *et al.,* 1995 ; Strange and Fryer,1997). It also appears that there is linkage disequilibrium between ***GSTM3*B*** and ***GSTM1 *A,*** with subjects of haplotype ***GSTM1*A/GSTM3*B*** expected to express a disproportionate amount of GSTM3 (Inskip *et al.,* 1995). It is thus important to recognise that the effects of ***GSTM1*** polymorphisms will be affected by Co-inherited GSTM3 polymorphisms. Disease susceptibility hypotheses centred around single mutated alleles are likely to be too simplistic (Yengi *et al.,* 1996).

Enzymes **of** the GST-a class are theoretically well placed to be involved in carcinogenesis as they display high activity against cumene hydroperoxide and 4-hydroxynonenal (Strange and Fryer, 1997). However, while there appears to be strong phenotypical evidence for the existence of polymorphisms in these enzymes to date, restriction fragment polymorphisms have only been reported in GSTA2 (Chen and Board, 1987; Strange and Fryer,1997).

Polymorphism has recently been identified at the GST-π locus (Ali-Osman *et al.,* 1995). Subjects with the *GSTPZ **AA*** genotype have an A to G transition in exon 5 resulting in **an** Ile (ATC) to Val (GTC) base change on both alleles at position 104 (Zimniak et al.,1994; Matthias *et al.,* 1998a). Introduction of valine into the GSTP1 enzyme protein close to its binding site increases affinity and activity for electrophillic substrates (Zimniak et al., 1994).

GSTPl is the most widespread of the human GST enzymes and is present in particularly high concentrations in cells of the gastrointestinal tract, lung, erythrocytes, platelets and foetal liver (Bongers *et al.,* 1995). GST-pi is highly inducible being found in elevated concentrations in experimentally induced malignant and premalignant human lesions (Zhang *et al.,* 1994; Bongers *et al.,* 1995). The GSTP1 enzyme is also able to

metabolise cigarette smoke derived carcinogens like benzo[a]pyrene diol epoxide and acrolein (Hayes and Pulford, 1995; Strange and Fryer, 1997).

The enzyme protein products of the *GSTT1* locus are responsible, amongst other things, for the metabolism of monohalomethanes, ethylene oxide and epoxides, all putative carcinogens present in cigarette smoke (Ketterer *et al.,* 1993, Strange and Fryer, 1997).

Board's group in Australia has recently reported the identification of 2 previously undescribed GST's, GSTZ and GSTT2. GSTZ is a dimeric enzyme composed of 2 24.2 **kD** subunits present in both plants and animals (Board *et al.,* 1997). The gene coding for the newly discovered theta class GSTT2 is polymorphic and located on chromosome 22 close to the *CYP2D6* locus (Strange RC, personal communication). The association between both GSTZ and GSTT2 and carcinogenesis is currently unclear.

1.4 Detoxification Enzyme Polymorphisms and Disease

Numerous examples in the plant and animal kingdoms testify to an organism's increased susceptibility to disease consequent upon altered expression at loci coding for detoxification enzymes. The GT112 strain of maize is GST *null* and lacks the ability to detoxify S-chlorotriazines rendering it very sensitive to herbicides containing this compound and differences in the expression of GSTA in the rat and the mouse are thought to be responsible for the selective toxicity of aflatoxin B_1 in the former and the resistance in the latter (Hayes and Pulford, 1995).

In addition to the slow metabolism of Debrisoquine consequent on the *CYP2D6 PM* genotype discussed previously, numerous other detoxification enzyme polymorphisms have been shown to confer altered disease susceptibility in humans. Patients with the *CYP2D6* PM genotype possess a 2-3 fold increased risk of developing Parkinson's disease (Smith *et al.,* 1992) and both astrocytoma and meningioma have been reported to be more frequent in patients who are *CYP2D6* PM or *GSTT1 null* (Elexpuru-Camiruaga *et al.,* 1995). Squamous cell carcinoma **of** the uterine cervix is positively associated with cigarette smoking and the *CYP2D6* EM phenotype is significantly higher in women with high grade cervical intraepithelial neoplasia (CIN), particularly in those who smoke than it is in controls (Warwick *et al.,* 1994b). Smokers who are *CYP2D6* EM are at increased **risk** of developing lung cancer possibly as a result of activation of a tobacco smoke derived carcinogen, while those with the PM and *HET* genotypes appear to be protected (Caporaso *et al.,* 1990; Crespi *et al.,* 1991). There is also some evidence that *CYP2D6* polymorphisms may also be associated with cancer of the urinary bladder, another malignancy positively linked to cigarette smoking (Chineegwundoh and Kaisarv, 1996).

In a study of 108 patients with non-small cell lung tumours Ryberg ***et*** *al.,* (1994), demonstrated that 32% of patients had a somatically acquired mutation at one or both p53 loci and that 91% of heavy smokers with GC transversion mutations were *GSTM1 null.* Similarly, in a study of ovarian cancers, Sarhanis ***et*** *al.,* (1996) demonstrated that 87% of tumours that reacted positively for p53 immunostaining were also *GSTM1 null.* These data suggest that GSTM1 plays a central role in the detoxification of the products of oxidative stress consequent on the exposure of cells to certain xenobiotics and that it protects p53 against carcinogen-DNA adduct damage *via* carcinogens such as benzo(a)pyrene (Strange and Fryer, 1997).

The first evidence for an association between *GSTM1 null* and lung cancer was provided by Seidegard *et al.,* (1986), who demonstrated a 1.6 fold increase in the frequency of this genotype in patients with lung cancer. Subsequent studies have frequently produced conflicting data perhaps reflecting the weak influence any single genotype has on this disease process. Meta analyses suggest that the *GSTM1 null* genotype confers a small increased risk for lung cancer (Strange and Fryer, 1997). While theses findings are consistent with the known biological activity of GSTM1 against PAH epoxides present in cigarette smoke it has also been shown that susceptibility to lung cancer in *GSTM1 null* individuals may be modified by dietary factors, particularly the serum levels of vitamin C and E (Hayes and Pulford, 1995).

Both the *GSTM1 null* and *GSTT1 null* genotypes have been shown to be associated with an increased risk of ulcerative colitis, Crohn's disease and cancer of the colon (Deakin *et al.,* 1996; Strange and Fryer, 1997).

High grade cervical intraepithelial neoplasia (CIN) has been shown to be more common in women who smoke and who are either, *GSTM1 null, GSTT1 null* **or** *CYP2D6*

EM(extensive metaboliser) than in controls (Warwick *et* al., 1994b). However, although women who are *CYP2D6* EM and smoke are at higher risk than controls of developing high grade CIN they have been shown to be less likely to progress to invasive carcinoma. Perhaps because of enhanced detoxification of an as yet unidentified chemical required for disease progression (Warwick *et* al., 1994a).

Yengi *et* al, (1996), have shown that GSTM3 is expressed in the basal layer of the epidermis and that the GSTM3 ***AA*** genotype is associated with an increased risk for the development of multiple cutaneous basal cell carcinomas in patients who also have skin type 1 or who are *GSTM1 null* or *CYP1A1* ***ml ml.*** They hypothesise that GSTM3, like GSTM1 plays an important role in free radical scavenging following ultra-violet (UV) light induced oxidative stress in the epidermis.

Polymorphism at the GSTM3 locus has been shown to influence susceptibility to laryngeal carcinoma but not pharyngeal carcinoma (Matthias *et* al., 1998b). The same study demonstrated that the GSTM3 subunit was strongly expressed in the laryngeal cilia while being undetectable in the anatomically close pharyngeal mucosa.

There is also evidence in support of the hypothesis that oral cancer risk is enhanced by certain detoxification enzyme polymorphisms. *GSTM1 null* and *GSTT1 null* genotype frequencies have been found to be significantly more common in patients with either oral cavity, pharyngeal or laryngeal cancer (Trima *et* al., 1995) (Table 7). Hung *et* al. (1997) reported that male Taiwanese patients with the combined *GSTM1 null* and/or *GSTT1 null* genotype had an increased oral cancer risk compared to those who were non-null for both *GSTM1* and *GSTT1* (OR = 4.6, 95% CI = 0.9 – 23.7). However, in contrast to Trima *et* al. (1995) they were unable to demonstrate a significant association between oral cancer risk and the individual *GSTM1* or *GSTT1* genotypes (Hung *et* al.,1997).

Table 7

Group	Polymorphism GSTTI *null*	*GSTM1 null*
Patients	45%	68%
Controls	36%	48%

Frequency of *GSTM1 null* in 186 patients with head and neck cancer and 42 controls and *GSTT1 null* in 127 patients and 42 controls.
(Trizna *et al.*, 1995).

Induction of squamous cell carcinoma in hamster cheek pouches by the application of **DMBA** has been shown to result in the production of GST-π positive cells (Zhang ***et al.,*** 1994). Significantly lower (p=0.003) levels of the high activity *GSTP1* ***AA*** genotype have recently been reported in patients with pharyngeal but not laryngeal cancer compared to controls and the GSTP1 enzyme subunit has been shown to be expressed widely throughout the mucosa lining the pharynx and larynx (Matthias et al., 1998a). These data suggest that GSTP1 influences susceptibility to squamous cell carcinoma of the upper aerodigestive tract and that subjects with the *GSTP1* ***AA*** genotype are better able to detoxify environmental carcinogens and hence are at lower risk of developing pharyngeal cancer.

Immunocytochemical staining of both normal and progressively more dysplastic oral mucosa leading up to invasive squamous cell carcinoma has demonstrated that GSTP1 expression mirrors the degree of tissue abnormality present (Table **8).** Plasma GSTPl levels also reflected the extent of the primary disease with higher levels being found in higher stage disease (Table 9).

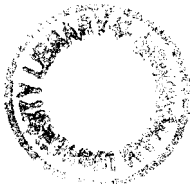


Table 8

HIstological diagnosis	Staining intensity +	++	+++	+++
Mild dysplasia	40%	60%	0	0
Moderate dysplasia	10%	40%	50%	0
Severe dysplasia & CIS	0	0	80%	20%
SCC	0	0	10%	90%
Epithelial hyperplasia	60%	40%	0	0
Normal oral epithelium	60%	60%	0	0

GSTP1 staining intensity and oral mucosal lesions.
+ = weakly positive, ++ = moderately positive, +++ = strongly positive, ++++ = very strongly positive.
(Adapted from Zhang *et al.,* 1994).

Table 9

Histological diagnosis	GSTP1 Mean (ng/ml)	Range (ng/ml)	% positive	N
SCC – Stage 1	33.2	17-63	50	12
SCC – Stage 2	33.4	10-60	64.3	14
SCC – Stage 3	45.1	13-87	70.6	17
SCC – Stage 4	47.2	21-98	77.8	18
Recurrence	43.0	20-78	76.9	13
Benign tumour	19.5	8-28	0	12
Normal controls	17.8	5-30	0	78

GSTP1 levels in patients with oral SCC, benign tumours and normal controls.
Positive levels were taken as >30.4 ng/ml (mean + 2SD of normal control levels)
(Adapted from Hirata *et al.,* 1992).

Sarkar ***et*** *al.* (1997) have demonstrated that GSTP1 expression is significantly higher in recurrent oral carcinomas than in primary oral tumours again suggesting a possible clinical role for GSTP1 in prognosis prediction. However, the same authors also demonstrated that GSTPl expression in normal oral mucosa is comparable to that seen in tumour tissue taken from the same patients suggesting that elevated GSTP1 expression may be a function of increased carcinogen exposure (in this case betel nut) rather than a marker for oral cancer per *se* (Sarkar ***et*** *al.,* 1997).

There is thus mounting evidence to suggest that allelic variation at the loci responsible for the production of many of the phase 1 and phase **2** enzymes described above significantly influences individual susceptibility to certain cancers, particularly those known to be tobacco related.

However, while molecular epidemiological techniques have been employed to construct phylogenetic trees that suggest that these detoxification enzymes evolved in order to metabolise environmental toxins (Johansson ***et*** *al.,* 1991; Ingleman-Sundberg, 1995) one must be wary of misinterpreting these findings. Surely these enzyme systems are not in existence solely to detoxify the by-products of human excesses! Moreover, as cancer overwhelmingly affects subjects already well into, if not past their fertile years, it is hard to reconcile how an evolutionary selection advantage is conferred on individuals who inherit enzymes that reduce their cancer risk. A more plausible explanation is that the detoxification enzymes exist because they are vital to normal cellular growth and homeostasis. The fact that they may also detoxify xenobiotics is an added bonus.

Although individual phase 1 or phase 2 enzyme polymorphisms may well influence disease susceptibility, it is more likely that inheritance of a combination **of** specific

polymorphisms at phase 1 and phase 2 enzyme loci is qualitatively a more important risk factor. Moreover, these multiple polymorphisms probably also need to act in concert with exposure to environmental or endogenous carcinogens such as tobacco smoke or alcohol. Such a cascade would be compatible with the current hypothesis that cancer is the end result of a multi-stage process involving loss of normal function, DNA damage and accumulation of adverse genetic and epigenetic events ultimately resulting in unrestrained growth and invasive carcinoma (Ryberg *et al.*, 1994; Sidransky, 1995).

In the final analysis the *raison detre* for the presence of the detoxification enzyme system may be unimportant. If the identification of certain allelic variations identifies individuals at high risk for the development of certain cancers and facilitates better prognosis estimation, treatment and survival we can use them to improve the management of our patients.

Given that abnormal detoxification profiles of xenobiotics and their metabolites are associated with increased malignant potential, by what mechanism do these reactive intermediaries exert their mutagenic effects? There is mounting evidence indicating an association between detoxification enzyme polymorphisms and mutations and amplifications of oncogenes and tumour suppressor genes.

1.5 Cancer Genetics

Cancer is a genetic disease, and carcinogenesis is now held to be a multi-stage process involving the progressive accumulation of genetic alterations in previously normal cells ultimately resulting in malignant transformation (Field, 1992; Sidransky, 1995; Califano ***et al.,*** 1996, Sidransky and Hollstein, 1996). Approximately 6-10 separate genetic events are required to transform a normal cell into a malignant one (Sidransky, 1995).

Cancer cells are distinguished from their normal counterparts by virtue of their immortality, transformation and in many cases their ability to metastasise (Lewin, 1994). These properties, which constitute the malignant phenotype, arise from aberrant cellular growth control and interaction processes (Partridge, 1995). Moreover, while the cellular composition of normal mucosa is polyclonal, being derived from numerous and diverse stem cells, carcinomas are monoclonal, indicating an origin from a single neoplastic cell that has undergone clonal expansion (Fearon and Vogelstein, 1990; Califano ***et al.,*** 1996).

There are 2 main classes of cancer genes; the oncogenes and the tumour suppressor genes although cancer results from the complex interplay of may environmental and genetic factors and is thus a polygenic disease (Field, 1992; Lewin, 1994; Partridge, 1995). Oncogenes are identified by genetic alterations that result in a gain of function, whereas tumour suppressor genes are identified by mutations that result in loss of function (Lewin, 1994).

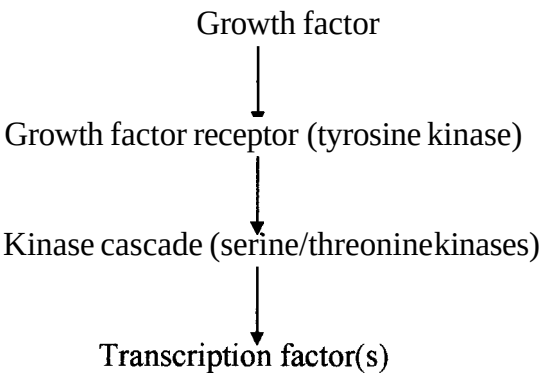
Oncogenes

The term oncogene is potentially misleading because it implies that it is always associated with malignancy, whereas oncogenes are DNA sequences found in normal cells, where they play a vital role in regulating growth and differentiation. It is only when they become altered either qualitatively or quantitatively that they develop oncogenic potential (Field, 1992). They are perhaps better referred to as proto-oncogenes (Lewin, 1994). Activation of proto-oncogenes can result from mutation following exposure to chemical carcinogens irradiation or viruses (Field, 1992; Partridge, 1995).

There are 2 broad categories of oncogenes; cellular oncogenes *(c-onc)* and viral oncogenes *(v-onc)*. **As** described above, *c-onc* 's or proto-oncogenes are specific DNA sequences that code for proteins whose function is to regulate cell growth and differentiation. The *v-onc* genes are sequestered in the form of RNA following a retroviral infection of a mammalian cell. Following reverse transcription of viral DNA, the viral genome is incorporated into the host DNA which may be close to a proto-oncogene. During viral replication part of the host DNA may become incorporated into the viral genome. If this host DNA contains a proto-oncogene sequence the retrovirus will now have the capacity to transform subsequently infected host cells as it now carries a viral oncogene.

The ability of viral oncogenes genes to transform normal cells together with the very close sequence homology between *v-onc* **'s** and their corresponding *c-onc* suggests that they act by mimicking normal cellular growth systems but without the usual tight control and restraint that is characteristic of normal cell differentiation. The proto-oncogene protein products are **known** to be involved with several aspects of cell growth control, in particular the regulation of DNA transcription following activation of a trans-

membrane cytosolic receptor (Lewin, 1994; Partridge, 1995). They include; growth factors, growth factor receptors, signal transduction cascade proteins and transcription factors acting in a cascaded sequence:

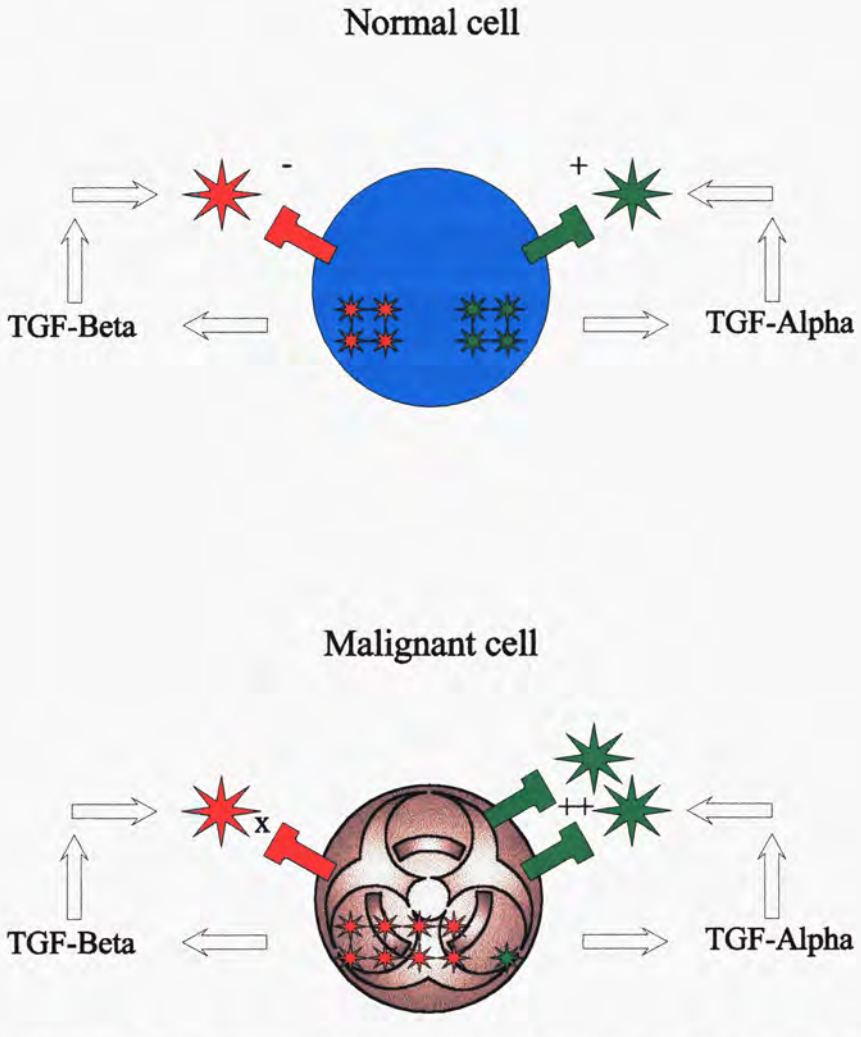


Normal and malignant epithelial cells secrete transforming growth factor alpha (TGF-a) and related ligands, a small polypeptide proto-oncogene product which stimulates cell growth by binding to a family of cell surface epidermal growth factor receptors (EGF). These cells also secrete TGF-ß which has a growth inhibitory effect on normal epithelial cells. However, TGF-ß is normally secreted in an inactive form and activation of TGF-ß, perhaps ***via*** protease digestion or acidification plays a crucial regulatory role in the control of cell proliferation (Partridge, 1995). Cell surface EGF receptor expression in oral squamous cell carcinomas is related to its state of differentiation and inversely proportional to the level of TGF-a secreted suggesting that binding of TGF-a to the EGF receptor is responsible for stimulating cell proliferation. Furthermore, growth and proliferation of many head and neck squamous cell carcinoma cell lines is not restrained by TGF-ß, possibly due to failure of TGF-ß activation, EGF receptor down regulation or subsequent failure **of** activation of the post-receptor kinase cascade (Figure 5) (Partridge, 1995).

It is self evident that there must be some difference between a proto-oncogene and its associated *v-onc* and "activated' *c-onc* otherwise life could not exist. It appears that differences in both structure and amount are responsible for the transforming potential of the *v-onc* genes and activated oncogenes.

There are **3** active genes in the oncogenes derived from the *c-ras* family: *N-ras, H-**ras*** and *K-Ras.* Each codes for a 21 kD protein (p21) on the inner cell membrane that binds guanosine triphosphate (GTP) in its active state. The *v-ras* and *c-ras* sequences differ by only a single amino acid, most commonly at positions 12 or 61. Glycine is normally found at position 12, if mutation results in its replacement by any amino acid other than proline an activated oncogene results. The mutated *rus* gene produces a permanently active p21 protein resulting in uncontrolled activation of the kinase cascade (Lewin, 1994; Partridge, 1995). *Rus* mutations have been demonstrated in approximately half of all colorectal carcinomas and large adenomas but in fewer than 10% of small adenomas (Fearon and Vogelstein, 1990; Field, l992). These data suggest that *rus* mutations are important in the early stages of colonic carcinogenesis (Field, 1992). Although *H-ras* mutations are uncommon in oral carcinomas in the western World they have been demonstrated in over one third of such lesions occurring in India (Field, 1992).

Figure 5



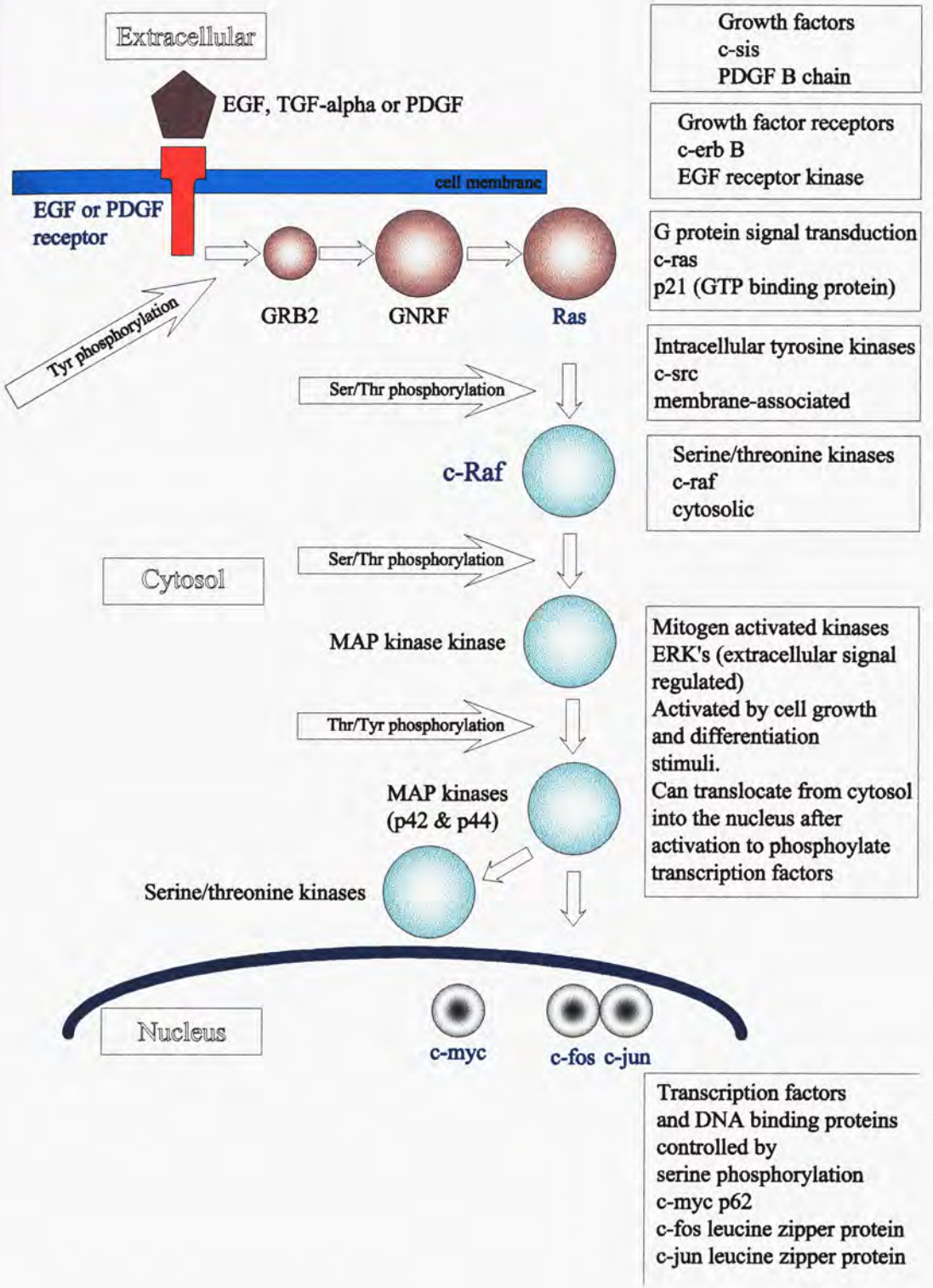
Schematic detailing proposed TGF-α/TGF-β positive and negative control mechanisms for the regulation of cell proliferation.
(Adapted from Partridge 1995).

As discussed previously, betel nut chewing is a major aetiological factor for oral squamous cell carcinoma in India and it is possible that carcinogens in the betel quid could be responsible for the *H-ras* mutations. Not all active oncogenes or v-onc **'s** have differing sequence homologies from their c-onc counterparts. The myc gene is a nuclear proto-oncogene coding for the 62 **kD** p62 protein involved in DNA and cell replication. If a retrovirus inserts close to the c-myc locus it can cause increased expression of p62 because virus controlled c-myc transcription is more efficient than normal transcription due to the enhanced promotion afforded by the long terminal repeat (LTR) sequences of the retrovirus. The c-myc locus may also be involved in a reciprocal translocation with the immunoglobulin heavy chain (IgH) locus on chromosome 14 which also results in increased expression and elevated levels of p62. If colonies of immature B lymphocytes are subjected to p62 hyper-expression because of a failure to turn off c-myc they are unable to differentiate into mature cells resulting in the formation of a lymphoma (Lewin,1994). Bcl-2 and c-myc, both play an important role in cell apoptosis and are detectable at an early stage in the change from normal to dysplastic mucosa. Bcl-2 appears to be able to protect cells from programmed cell death, while c-myc activation is frequently associated with malignant transformation (Scott ***et*** ***al.,*** 1996). Like *H-ras,* members of the c-myc gene family have not been shown to be important in head and neck cancer in the western World but have been shown to be amplified in over one third of Indian head and neck cancers (Field,1992).

Two other nuclear proto-oncogenes, which like c-myc code for DNA binding proteins, are c-fos and c-jun. The fos and jun oncogene proteins form a heterodimer which is a component of the AP-1 transcription factor (Partridge, 1995). We can now construct a

flow schematic detailing the interactions between proto-oncogenes which helps to explain their action following mutation of their normal cellular homologues (Figure **6).**

Figure 6



Signal transduction cascade involving oncogene products and oncoproteins.
(Adapted from Lewin 1994 and Partridge 1995).

Tumour suppressor genes

Unlike the oncogenes which introduce a gain of function leading to uncontrolled cellular growth and proliferation, abnormalities of the tumour suppressor genes are oncogenic because they result in loss of normal function (Partridge,l995). While oncogenes can be visualised as "dominant" genes inasmuch as only one proto-oncogene allele needs to be mutated for it to become an oncogene, tumour suppressor genes are "recessive" because both normal alleles need to be defective for tumorigenesis to occur. The loci coding for the production of the retinoblastoma (Rb) and p53 proteins are currently the best characterised tumour suppressor genes (Lewin, 1995).

Rb

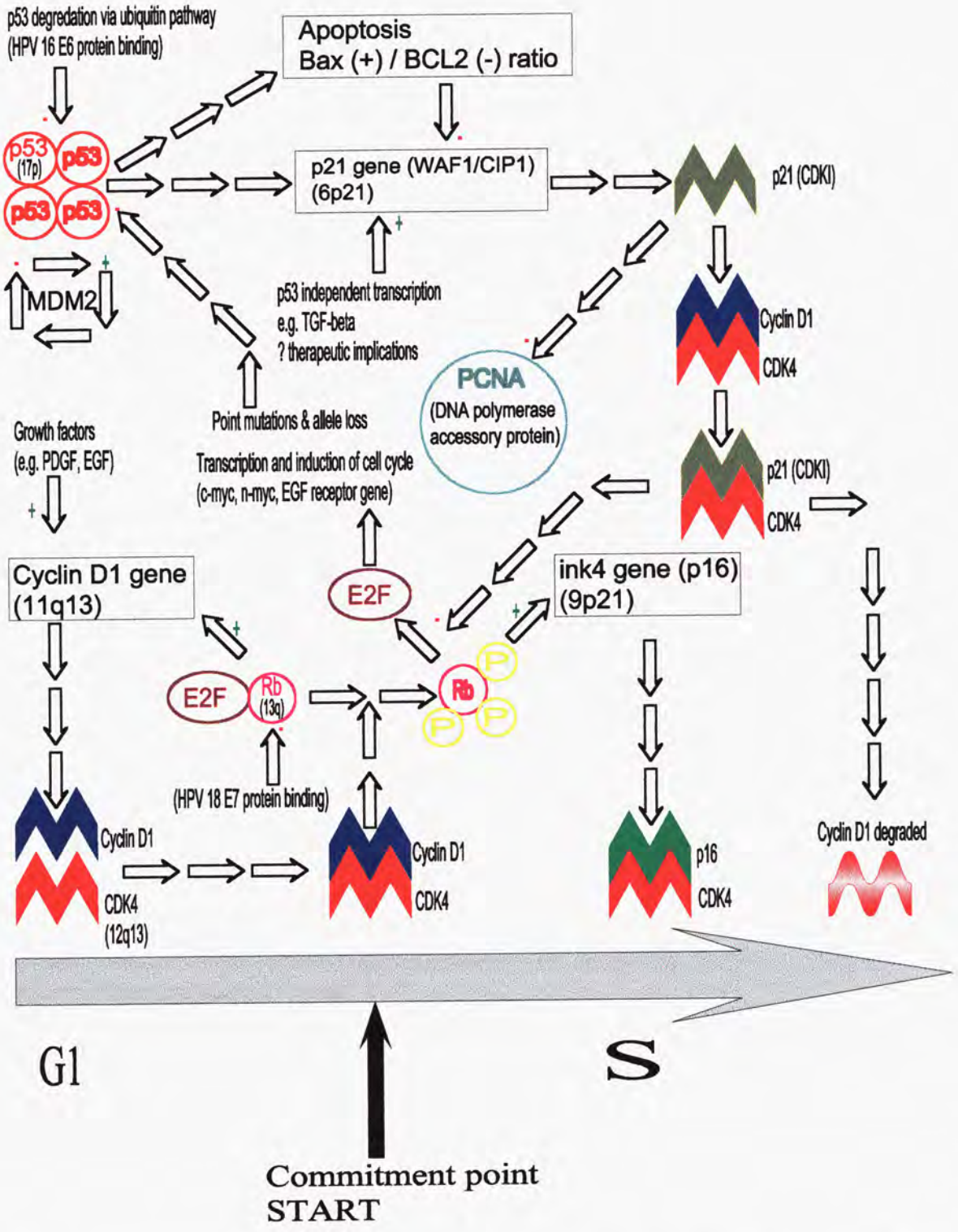
The Rb gene is located at 13q14. If both Rb alleles are inactive the affected individual will inevitably develop a retinoblastoma. Affected individuals may have the inherited form of retinoblastoma in which one Rb allele caries a germ line mutation and a subsequent somatic mutation in the retinal cells results in loss of both alleles. Alternatively, in the sporadic form of retinoblastoma both inherited Rb alleles are normal but somatic mutation of the retinal cells results in loss of both alleles.

Rb is a nuclear phosphoprotein that in its dephosphorylated form sequesters the E2F transcription factor and inhibits cell proliferation by blocking the cell cycle in the G_1/S phase. Phosphorylated Rb releases the cell cycle block allowing the cell to enter the S phase of mitosis. It is hypothesised that allelic loss at both Rb loci removes one of the cell cycle control blocks permitting unimpeded cell proliferation (Sidransky and Hollstein, 1996).

Transcription of the cyclin D1 (CCND1) gene, located on chromosome 11q13 which codes for the proto-oncogene cyclin D1, is induced and positively regulated by various growth factors (Betticher *et al.,* 1995; Lewin, 1995). The activated cyclin dependent kinase CDK4 complexes with cyclin D1 and inactivates the Rb protein by phosphorylation in the process releasing the previously Rb bound E2F transcription factor. Free E2F then activates several genes required for cell division. By competitively binding **to** CDK4, the p16 protein inhibits CDK4-cyclin D1 complex formation and induces accelerated turnover of unbound cyclin D1 during **S** phase (Betticher, 1996). Alternate binding and release of cyclin D1 to CDK4 thus controls the cell cycle and associated transcription events. Cyclin D1 thus appears to be a growth factor sensor and initiator of the cell cycle and drives it through the first restriction (START) point acting *via* the down regulation of the Rb protein (Figures 7a and 7b) (Betticher *et al.,* 1995; Betticher, 1996; Betticher *et al.,* 1996).

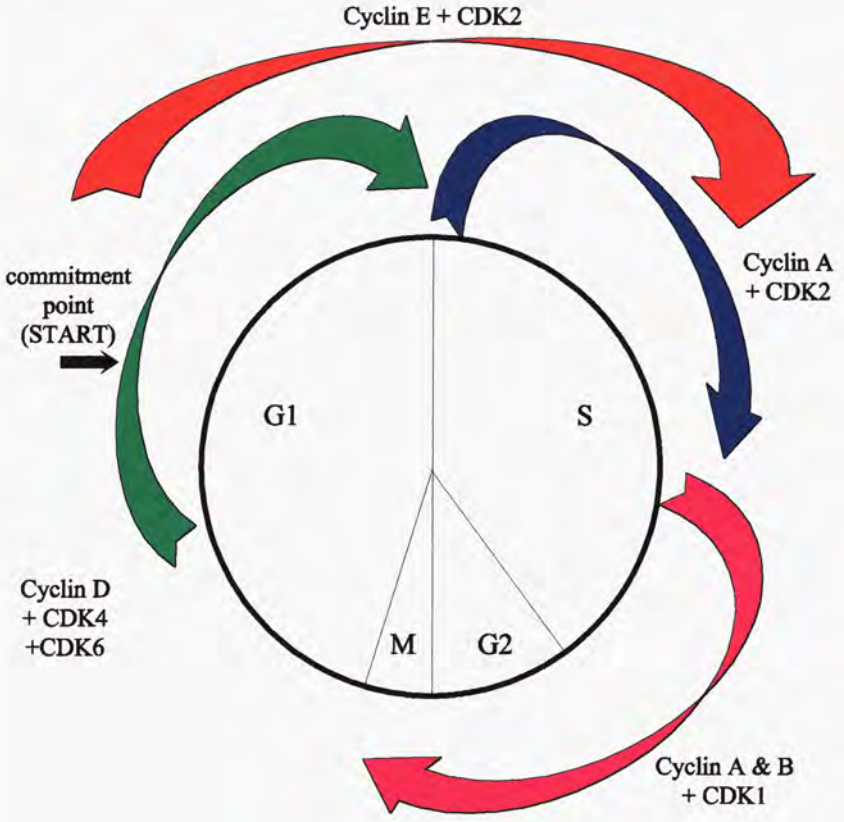
Cyclin D1 is potentially oncogenic in that its overexpression will result in induced cell proliferation (Betticher *et al.,* 1995). The *1*1q13 locus containing the CCND1 gene is one of the most commonly amplified loci in human carcinomas and cyclin D1 over expression in head and neck cancer is associated with local invasion early regional lymph node metastasis and poor prognosis (Michalides *et al.,* 1995; Bellacosa *et al.,* 1996; Betticher *et al.,* 1996). However, cyclin D1 is likely to be one of many factors responsible for normal cell cycle regulation as it has been shown that in cyclin D1 "knocked out" mice cell proliferation continues virtually unchanged (Betticher, 1996).

Figure 7a



Diagrammatic representation of G1/S cell cycle control by phosphorylation of Rb protein and CDK4 sequestration by p16.
(Adapted from Betticher, 1996).

Figure 7b



Diagrammatic representation of cyclin and cyclin dependent kinases (CDK) interactions and the cell cycle.
(Adapted from Betticher 1996).

Defective control of the formation of CDK4-Cyclin D 1complexes is another potential cause for abnormal cell proliferation. The ***ink4*** gene codes for the production of p16 the critical inhibitor of CDK4-Cyclin D1 complexes and is located on chromosome 9p21 (Serrano *et al.,* 1993). The 9p21-22 locus is the most commonly deleted region (66%) in head and neck cancer (Sidransky, 1995).

P53

This tumour suppressor gene is defective in approximately half of all malignant neoplasms (Sidransky and Hollstein, 1996).

The p53 gene is located on human chromosome 17 and codes for a 53 **kD** nuclear phosphoprotein which was first discovered in SV40-transformedcells (Lewin, 1994; Partridge, 1995). Mutation of the p53 gene is the most common genetic event implicated in human carcinogenesis (Iggo *et al.,* 1990; Ryberg *et al.,* 1994; Shintani *et al.,* 1995). Wild type p53 protein is present in minute concentrations in normal cells and is virtually undetectable. Mutation at the p53 locus results in the production of an abnormal protein, which unlike wild type p53 is unable to bind to the T-antigen of SV40. Furthermore, the mutated protein is stable and hence detectable by immunocytochemistry (Lane and Benchimol, 1990; Lewin, 1994; Partridge, 1995). In the Li-Fraumeni syndrome, a rare form of inherited cancer, both p53 alleles are mutated resulting in a non-functioning protein (Lewin, 1994). The T-antigen present in SV40 infected cells is required **for** viral replication and entry into S phase (Partridge, 1995). The fact that wild type p53 binds to SV40 T-antigen interfering with its ability to promote viral replication **is** indicative that the function of p53 may be to control the cell cycle by binding to a cellular homologue of

SV40 T-antigen (Lewin, 1994). Wild type p53 is known to bind to sequence sgecific double-stranded **DNA,** whereas all the mutated p53 proteins isolated to date have lost this **DNA** binding capacity (Lane, 1992; Wieczorek ***et al.,*** 1966). **DNA** binding is regulated by the allosteric structure of a basic region in the p53 protein located within the carboxy-terminal 30 amino acids (Yao ***et al.,*** 1966). The mutations involve a single amino acid substitution at an arginine target either in regions directly responsible for **DNA** binding (class 1) or areas that stabilise the **DNA** binding domain (class 2) (Wieczorek ***et al.,*** 1966). Mutant p53 is capable of binding with members of the cytoplasmic heat shock proteins (hsp 70) accounting for the high levels of extra-nuclear mutant p53, while wild type p53 is confined to the nucleus (Iggo ***et al.,*** 1990). **As** well as its **DNA** binding function, wild type p53 also functions as a potent transcription activator (Lane,1992). Mutant p53 is known to act as a dominant-negative, which, by forming inactive hetero-oligomers with wild type p53 overwhelms the normal protein (Lane,1992; Lewin,1994). This effect means that even heterozygotes with only one mutant p53 allele may have significantly reduced wild type p53 function (Iggo ***et al.,*** 1990). **A** similar oligomerization function probably accounts for the binding and subsequent inactivation of p53 by HPV 16 E6 protein (Field, 1992; Lewin,1994) which may be one of the methods by which HPV 16 induces malignant transformation in infected cells. Both point mutations and hetero-oligomer formation abolish normal p53 function.

Of fundamental importance to the understanding of the role of p53 in cellular homeostasis was the finding that **DNA** damage mediated by chemical carcinogens or ultra violet irradiation results in the accumulation of cellular p53 as a result of post-translational stabilisation (Maltzman and Czyzyk, 1984). Wild type p53 suppresses the replication **of**

genetically damaged cells by both arresting and promoting exit from the cell cycle (Sidransky and Hollstein, 1996).

There are 2 checkpoints in the cell cycle at which arrest can occur. The G1/S checkpoint controlled by the p53-dependent induction of p21, an inhibitor of the cyclin E-cdk2 complex, and the G2/M checkpoint which is regulated *via* p34 kinase (Yao *et al.*, 1966). Normally, following DNA damage such as strand breakage, p53 accumulates and arrests the cell cycle at the G1/S checkpoint thereby allowing time for DNA repair and/or apoptosis if repair fails (Sidransky and Hollstein,1996). Conversely, if p53 is mutated, or inactivated consequent on binding of viral proteins or xenobiotic attack etc, G1 cell cycle arrest is not possible. Cells with mutated p53 pass through the G1/S checkpoint and accumulate at the G2/M checkpoint (Ya0 *et al.,* 1966). DNA damage and mutations are allowed to occur resulting in rapid selection out of malignant clones (Lane,1992).

Not only does mutated p53 result in increased cell cycling and proliferation but it also confers resistance to tumour cell killing by irradiation or cytotoxic drugs because of the reduced vulnerability to apoptosis. This situation is reflected in the observation that tumours demonstrating a high incidence of p53 mutations such as lung and colon cancers respond poorly to anti-cancer chemotherapy and irradiation. Conversely, tumours displaying low rates of p53 mutations such as testicular cancer and Wilms' tumour are successfully treated by these modalities **(Yao** *et al.,* 1966).

Between 75% and 85% of lung cancers demonstrate damage at one or both p53 alleles (Partridge, 1995), many of which are G-T transversions, a process known to be inducible by exposure to benzo(a)pyrene present in cigarette smoke (Iggo *et al.,* 1990). However, p53 mutations were not found in carcinoid tumours, which have a different aetiology and behaviour (Iggo *et al.,* 1990).

Scott *et al.* (1996), found that p53 mutation occurred in 46% of colorectal carcinomas, but was never detected in normal large bowel mucosa (Scott *et* ***al.,*** 1996). They also reported that p53 overexpression was a late event in the carcinogenesis process of colorectal tumours, only being found in highly dysplastic adenomas and invasive carcinomas.

Overexpression of p53 has also been demonstrated in oral squamous cell carcinomas (Gusterson *et al.,* 1991; Partridge, 1995). Shintani *et al.,* .(1999, demonstrated p53 overexpression in 40.2% of oral squamous cell carcinomas, whereas there was no detectable p53 in 10 biopsies of normal oral mucosa ($p<0.05$). Interestingly, p53 was also overexpressed in 34.8% **of** biopsies taken from the macroscopically normal epithelium adjacent to invasive squamous cell carcinomas and in 18.7% of oral leucoplakias (Shintani ***et*** *al.,* 1995). Similarly, nuclear p53 overexpression has been reported in tobacco snuff-induced non-malignant oral lesions but not in normal mucosa taken from patients who did not use snuff (Wedenberg *et al.,* 1996). Increased p53 expression was also strongly associated with increased Ki-67 nuclear staining. The Ki-67 antibody reacts strongly with **2** nuclear proteins that are present throughout the cell cycle except G_0 and the intensity of Ki-67 staining has been shown to be positively correlated with cell proliferation (Wedenberg *et al.,* 1996).

Not only have p53 mutations been demonstrated in a large number of head and neck cancers, but also in a high proportion of microscopically normal mucosal biopsies taken from these patients (Ogden, 1996). This finding has important therapeutic implications because it indicates that histologically clear tumour excision margins may still contain cells that are destined to become malignant. Brennan *et al.* (1995b) demonstrated that in ***25*** cases of head and neck squamous cell carcinoma with histologically clear

resection margins 52% were positive for a p53 mutation in at least one margin. Thirty eight percent of the cases with p53 positive resection margins developed a local recurrence compared to 0% of the p53 negative cases. Moreover, 21% of histologically clear cervical lymph nodes were shown to contain the same p53 mutations as the primary tumour (Brennan *et al.,* 1995b). These data, together with the observation that polysomies of chromosomes 7 and 17 are present in both head and neck squamous cell carcinomas and in macroscopically normal oral mucosa adjacent to the primary tumour but significantly less frequently ($p<0.001$) in normal controls (Choi and Chung,1996), lends support to the "Field Cancerization" theory (Slaughter ***et*** *al.,* 1953).

The relative contributions of hereditary and environmental events ultimately resulting in these "normal" mucosal p53 mutations are presently unclear although recent work by Brennan *et al.* (1995a) suggests that different p53 mutations are involved. In a study involving 129 patients with head and neck squamous cell carcinoma 57% of patients who both smoked cigarettes and drank alcohol had a p53 mutation compared to only **17%** of patients who neither smoke nor drank. Furthermore, all of the p53 mutations in the non-drinking non-smoking group occurred at sites containing cytidine phosphate guanosine dinucleotides (CpG) compared to only 23% in the drinking and smoking group (Brennan *et al.,* 1995a). Defects at CpG sites are endogenous mutations resulting from deamination and methylation (Sidransky, 1995).

E-cadherin, a putative tumour suppressor gene located at 16q22 plays a central role in cell-cell adhesion. Down regulation of E-cadherin is associated with tumour invasion and metastasis and 16q22 translocations have been reported in head and neck squamous cell carcinomas (Field, 1992). E-cadherin expression has also been reported to be reduced in poorly differentiated carcinomas (Johnson *et al.,* 1996).

Allelic loss and tumorigenesis

In 1990 Fearon and Vogelstein proposed a model for colorectal tumorigenesis based on sequential and progressive genetic alterations involving oncogenes and tumour suppressor genes (Figure **8a).** They hypothesised that **4** or ***5*** mutations are required for the formation of a malignant tumour and that although genetic alterations often occur in a defined sequence, it is the accumulation of genetic changes that is important, rather than the exact order in which they occur (Fearon and Vogelstein, 1990)

Figure 8a

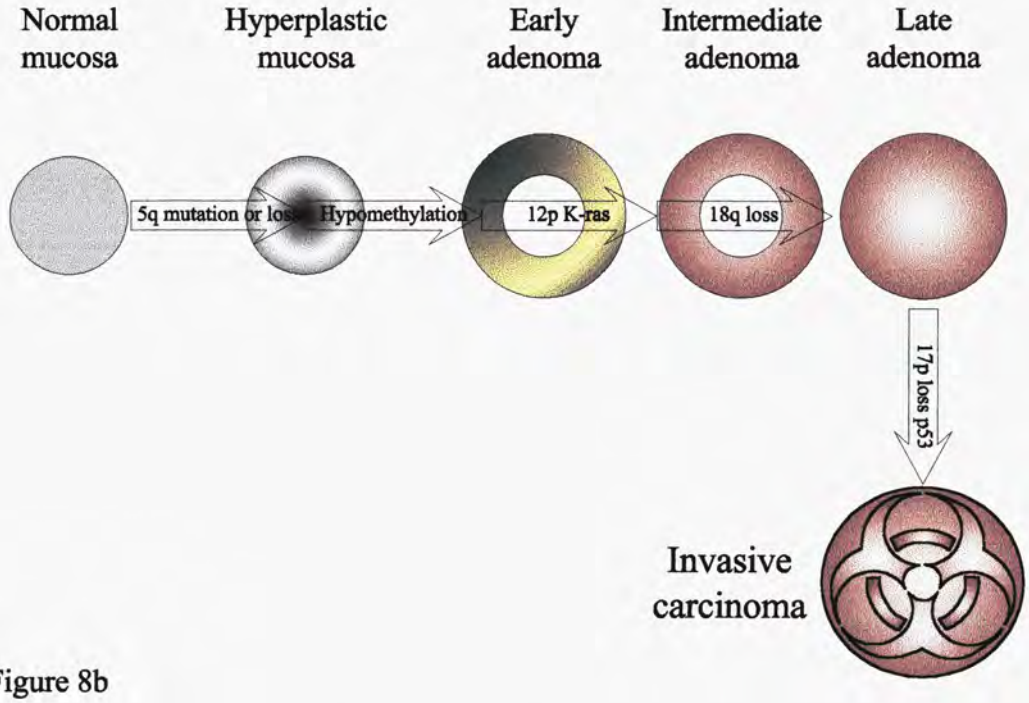


Figure 8b

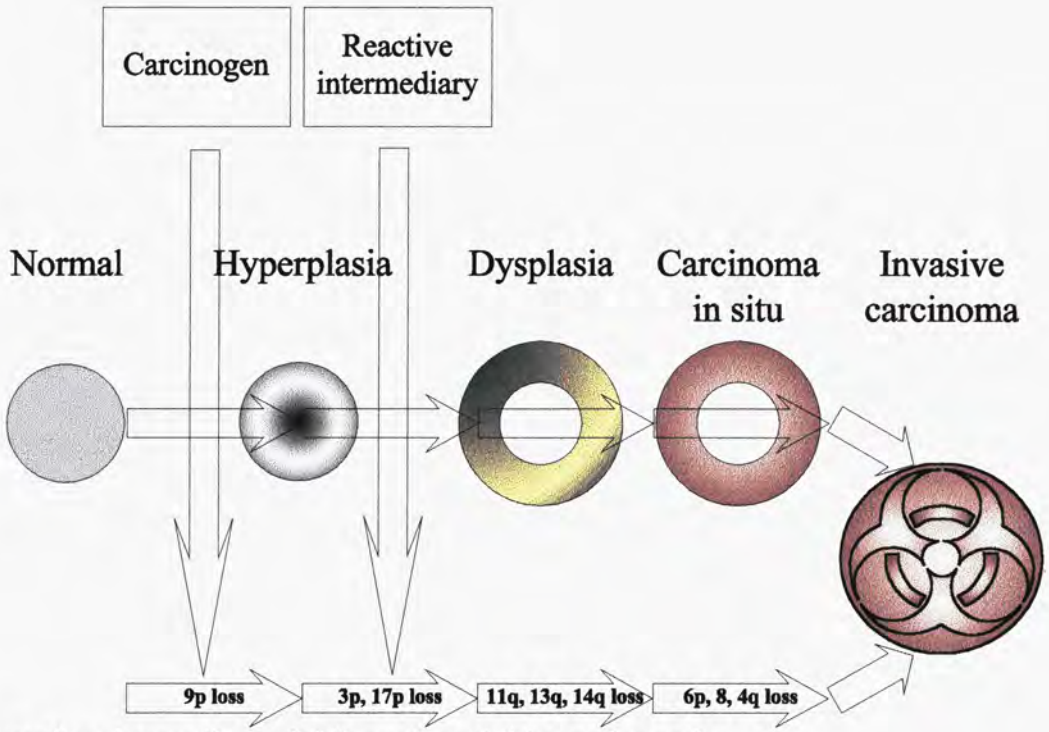


Figure 8a - A genetic model for colorectal tumorigenesis.
(Adapted from Vogelstein 1990).

Figure 8b - A hypothetical model for oral tumorigenesis.
(Adapted from Califano *et al* 1996).

Several chromosomal loci, known to code for proto-oncogenes and tumour suppressor genes, are commonly lost (loss of heterozygosity, LOH) in malignant head and neck tumours (Table 10).

Table 10

Gene locus	Gene product
9p21	p16
17p13	p53
13q	Rb
11q13	Cyclin D1

Chromosome loci showing frequent LOH and their associated proto-oncogenes and tumour suppressor genes.

Chromosomes 3p and 9p are subject to frequent LOH in head and neck cancers marking them as sites for putative tumour suppresser genes (El-Naggar et al., 1995; Field *et* al.,1995). LOH at 3p has been demonstrated in 53% of potentially malignant lesions (Emilion *et* al.,1996) and in 71% of oral squamous cell carcinomas (Partridge *et* al.,1996). In a study of 48 primary oral squamous cell carcinomas, Partridge *et al.* (1996) demonstrated a significant association between the number of regions on 3p demonstrating allelic loss and disease-free survival in early stage disease. They suggested that determination of LOH at 3p may be useful in individual prognostic prediction and treatment planning.

Following a recent study of LOH in 87 mucosal lesions of the head and neck ranging from benign hyperplasia to early invasive carcinoma, Califano *et al.* (1996), hypothesised a genetic progression model analogous to Fearon and Vogelstein's colorectal carcinoma model. The highest LOH frequency in benign hyperplastic tissue was at the 9p21 locus marking this as one of the earliest detectable events in head and neck

carcinogenesis. Furthermore, over 30% of histopathologically benign lesions demonstrated some genetic alterations. It thus seems likely that apparently normal mucosa and adjacent areas of epithelial dysplasia, carcinoma *in situ* or invasive carcinoma are all derived from a single clone of cells (Califano *et al.,* 1996). This observation provides a plausible explanation for the phenomena of locally recurrent and persistent disease and for the field cancerisation theory (Slaughter *et al.,* 1953). Later genetic events include mutations at the 3p, 17p, 13q and 11q13 loci (Sidransky, 1995, Califano **et** *al.,* 1996). One may thus envisage a composite molecular damage and genetic progression flow diagram superimposed on the traditional histopathological model of carcinogenesis (Figure 8b).

Support for the role of genetic polymorphisms and mutations in oral carcinogenesis is provided by recent work that demonstrated a significantly increased risk for developing head and neck cancer if a first degree relative had squamous cell carcinoma of the head and neck (Foulkes *et al.,* 1996). This extensive retrospective study investigated the incidence **of** squamous cell carcinoma of the head and neck in 1429 first degree relatives of 242 patients with head and neck cancer and 934 first degree relatives of the spouses of 156 patients with head and neck cancer who formed the control group. The relative risk for developing head and neck cancer if a first degree relative had also had the disease was 3.79. Interestingly, there was no significant excess risk demonstrable if a first degree relative had contracted cancer at sites outwith the head and neck. Twenty patients in the series **(8%),** had multiple primary tumours of the head and neck, the adjusted relative risk for their first degree relatives developing head and neck cancer was 7.89 compared to 3.53 in the 222 patients who had a single head and neck primary tumour ($p = 0.009$). Mention has already been made of the increasing number of younger patients developing oral cancer. It might be expected that these younger patients would be more likely to have a

positive family history of head and neck cancer but the study found no evidence to **support** this view (Foulkes ***et al.,*** 1996).

1.6 The Polymerase Chain Reaction

Following the pioneering work of Watson and Crick in 1953 (Lewin, 1994), it is known that the structure of DNA comprises a double helix of 2 parallel strands built from repeating units of the 4 deoxynucleotides deoxyadenylate **(A),** deoxythymidylate (T), deoxyguanylate (G) and deoxycytidylate (C) shaped somewhat like a ladder. It was also appreciated that the deoxynucleotides were arranged such that the deoxyribose-phosphate units formed the "sides" of the ladder, while the purine and pyrimidine bases are linked *viu* hydrogen bonds to form the "rungs". Because of the 3 dimensional structure of their binding sites, **A** will only bond with T and C will only bond with G. As a result of the complementarybase pairing arrangement the 2 single DNA strands are better described as being antiparallel (Figure 9a).

Following the discovery of DNA polymerase by Arthur Kornberg in 1955 and restriction endonucleases in the 1970's, Frederick Sanger developed a DNA sequencing technique which produced short fragments of previously added oligonucleotide primers that had been extended following the addition of nucleotide triphosphates and dideoxy nucleotide triphosphates catalysed by DNA polymerase. While revolutionary for its time, dideoxy sequencing (the Sanger technique), was laborious and time consuming as only one short fragment DNA copy could be produced from each single stranded DNA template. Unsatisfactory as it was, dideoxy sequencing was still faster than DNA cloning in plasmids which was the other standard technique used for human DNA analysis at the time (Mullis, 1990; Grainger and Madden, 1993). **A** new technique was required which could produce large amounts of DNA rapidly and reliably.

Figure 9a

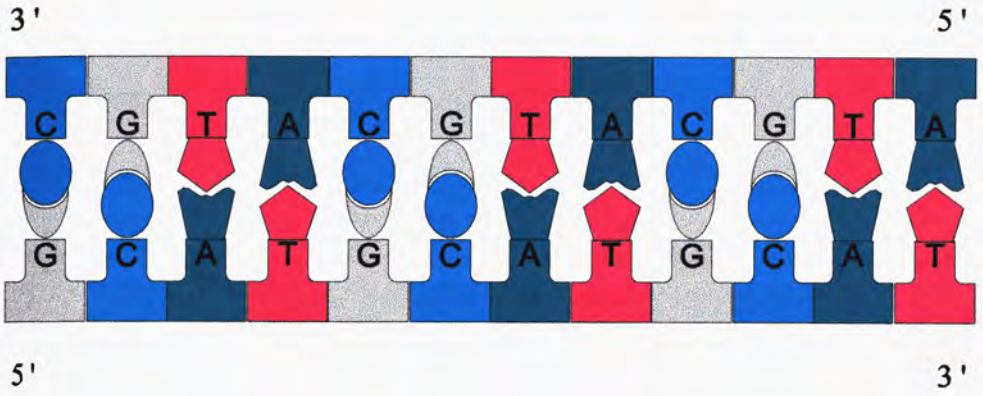


Figure 9b

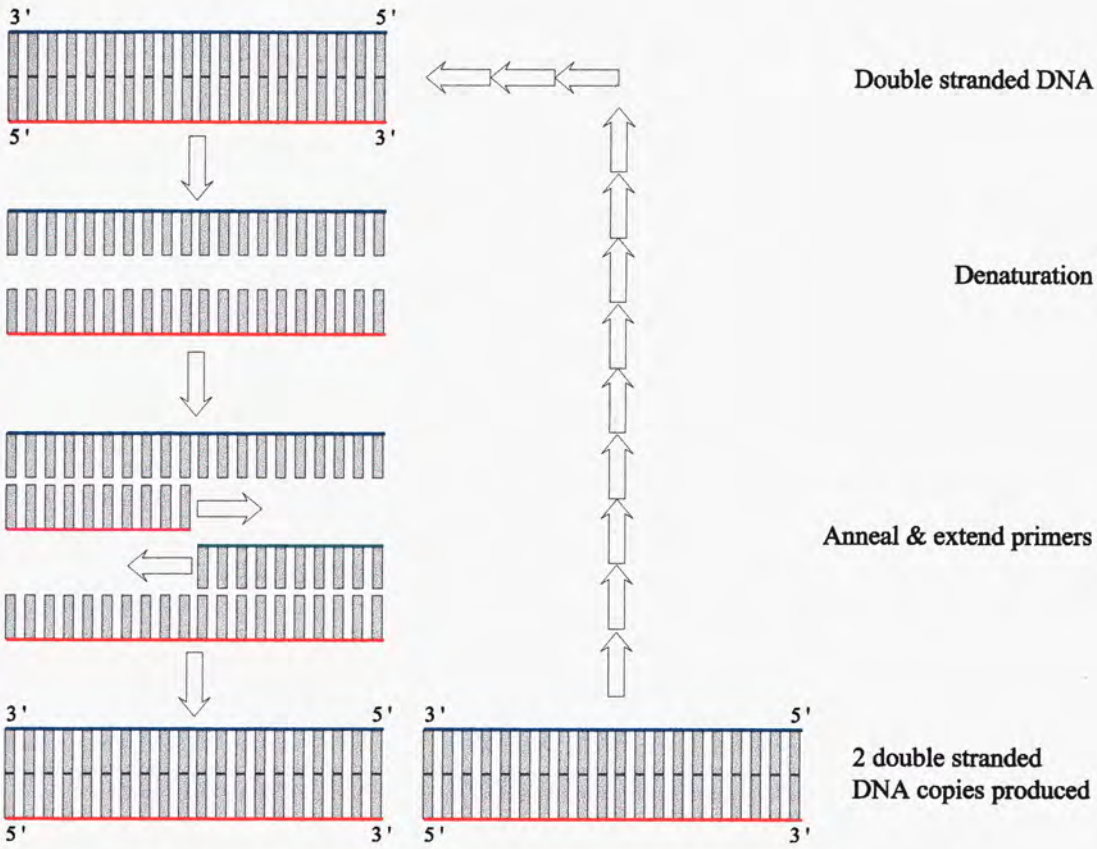


Figure 9a - Complementary base pairing in antiparallel DNA nucleotides. A = Adenine, T = Thymine, C = Cytosine, G = Guanine. (Adapted from Mullis 1990).

Figure 9b - Diagrammatic representation of a single PCR cycle. (Adapted from Grainger and Madden 1993).

In April 1983, Kary Mullis, a biochemist with the Cetus Corporation, had an idea that was to earn him a Nobel Prize in 1993 and revolutionise the study of molecular genetics (Mullis, 1990; Grainger and Madden, 1993). Mullis' stroke of genius was to add 2 primers comprising nucleotide sequences that allowed them to bind to either side of a targeted base pair. One primer would bracket the target base on one DNA strand while the other primer would bracket the target base on the opposite and complementary DNA strand (Mullis, 1990). As DNA polymerase will only catalyse the addition of a deoxynucleotide triphosphate (A, C, T or G) to the 3' end of an oligonucleotide primer bound to a complementary single strand DNA template, primer extension proceeds in opposite directions across the targeted base pair. This results in an exponential production of DNA (Erlich *et* ***al.,*** 1991) and has become known as the polymerase chain reaction (PCR). There are 3 discrete sequences that together constitute 1 PCR cycle (Sigg *et* ***al.,*** 1991; Grainger and Madden, 1993):

- Double stranded target DNA (dsDNA) is denatured by heating to 94-98°C producing 2 single stranded DNA (ssDNA) templates
- The reaction mixture is cooled to 40-60°C allowing oligonucleotide primers each approximately 20-30 bases long and which are present in excess to anneal to the target flanking regions on the ssDNA templates
- After raising the reaction temperature to about 72' C, DNA polymerase catalyses primer extension from the 3' end by the sequential addition of deoxynucleotide triphosphates (dntp's) complementary to the ssDNA template sequence. Extension continues along the length of each ssDNA template in a 3'-5' direction until 2 complete copies of the original dsDNA is completed.

The first cycle is now completed and the whole sequence can be repeated *ad infinitum,* although usually *20-30* cycles is sufficient to produce enough dsDNA for investigation (Figure 9b).

The performance of all 3 sequences in order completes 1 PCR cycle and the number of DNA copies that can be made equals 2^n where n = the number of PCR cycles completed (Wright and Wynford-Thomas, 1990). If 20 cycles are completed, theoretically over 10^6 DNA copies will be produced (Mullis, 1990; Erlich *et al.,* 1991) However, in practice about 25 cycles are required to produce 10^6 copies because the DNA yield is not 100% in every cycle (Sigg *et al.,* 1991).

Since publication of the first clinical application of PCR by Saiki *et al,* in 1985 who reported its use in the prenatal diagnosis of sickle cell anaemia following PCR amplification of a ß-globin Dde 1 restriction fragment, numerous improvements and amendments to the original technique have been reported.

The DNA polymerase originally used by Mullis was derived from *Escherichia culi (E. coli).* Although the enzyme worked well, it was inactivated by the high temperatures required to produce strand separation of the dsDNA prior to primer binding. This meant that fresh DNA polymerase had to be added after the denaturation phase of every PCR cycle which greatly increased overall reaction time and the manual input required (Erlich *et al.,* 1991). Furthermore, although the *E. coli* DNA polymerase was capable of amplifying a DNA fragment by some 200,000 times, because of poor specifícity, only about 1% of the final PCR product contained the sequence of interest. The poor yield required the addition of a further post-PCR step to isolate the targeted sequence. Part of the reason for the poor yield was that at the relatively low reaction temperatures required to ensure *E. coli* DNA polymerase activity, there was a high percentage of non-specific

pimer binding. At low annealing temperatures primers will bind to non-targeted template sequences which are subsequently amplified thus reducing the purity of the PCR product (Erlich *et al.,* 1991; Grainger and Madden, 1993).

Again it was the scientists at the Cetus Corporation who made a breakthrough. They isolated the DNA polymerase from the bacteria *Thermus aquaticus (Taq),* whose natural habitat is the hot springs and geysers of Yellowstone National Park. The optimal temperature for the *Taq* polymerase is 72°C, although its activity is virtually unaffected even at the 94°C required for dsDNA strand separation (Erlich *et al.,* 1991; Sigg, 1991). It was now unnecessary to manually add fresh polymerase to each PCR cycle and the whole reaction became fully automated. Furthermore, specificity was increased dramatically, both because of the inherent properties of the *Taq* polymerase and also because non-specific primer binding was virtually eliminated at the higher reaction temperatures achievable consequent on using *Taq.* The increased purity of the final PCR products meant that they could be easily detected using simple electrophoretic techniques and visualised as ethidium bromide stained bands under *UV* light, thus removing the need for the post PCR product isolation step (Erlich ***et*** *al.,* 1991; Grainger and Madden,1993).

Further recent improvements to the PCR technique and equipment include but are not restricted to (Erlich *et al.,* 1991; Grainger and Madden, 1993):

- Performing the PCR in thin walled capillary tubes which allows rapid temperature cycling and enables a single cycle to be completed in 20 s instead of about 5 minutes when using traditional plastic reaction vessels
- The development and use of 2 new extreme thermophillic DNA polymerases *Thermococcus litoralis* (VENT *polymeruse)* and *Pyrococcus furiosus* which are more specific than *Taq polymeruses*

- Hot starting, requiring the addition of an essential reaction reagent only after the reaction temperature has exceeded 70°C which minimises non-target amplification and primer-dimer formation.

There are many other enhancements too numerous to detail, and every laboratory, if not every experienced PCR scientist will have there own tips, tweaks and techniques to improve specificity and final PCR product yield.

1.7 Immunohistochemistry

Immunohistochemistry is a technique used to identify specific target cells or cellular components by means of an antigen-antibody reaction. The 3 dimensional structure of an antibodies' variable domain allows it to specifically bind to a complementary area or epitope on the antigen *viu* hydrogen bonds, electrostatic forces and van der Waals' forces.

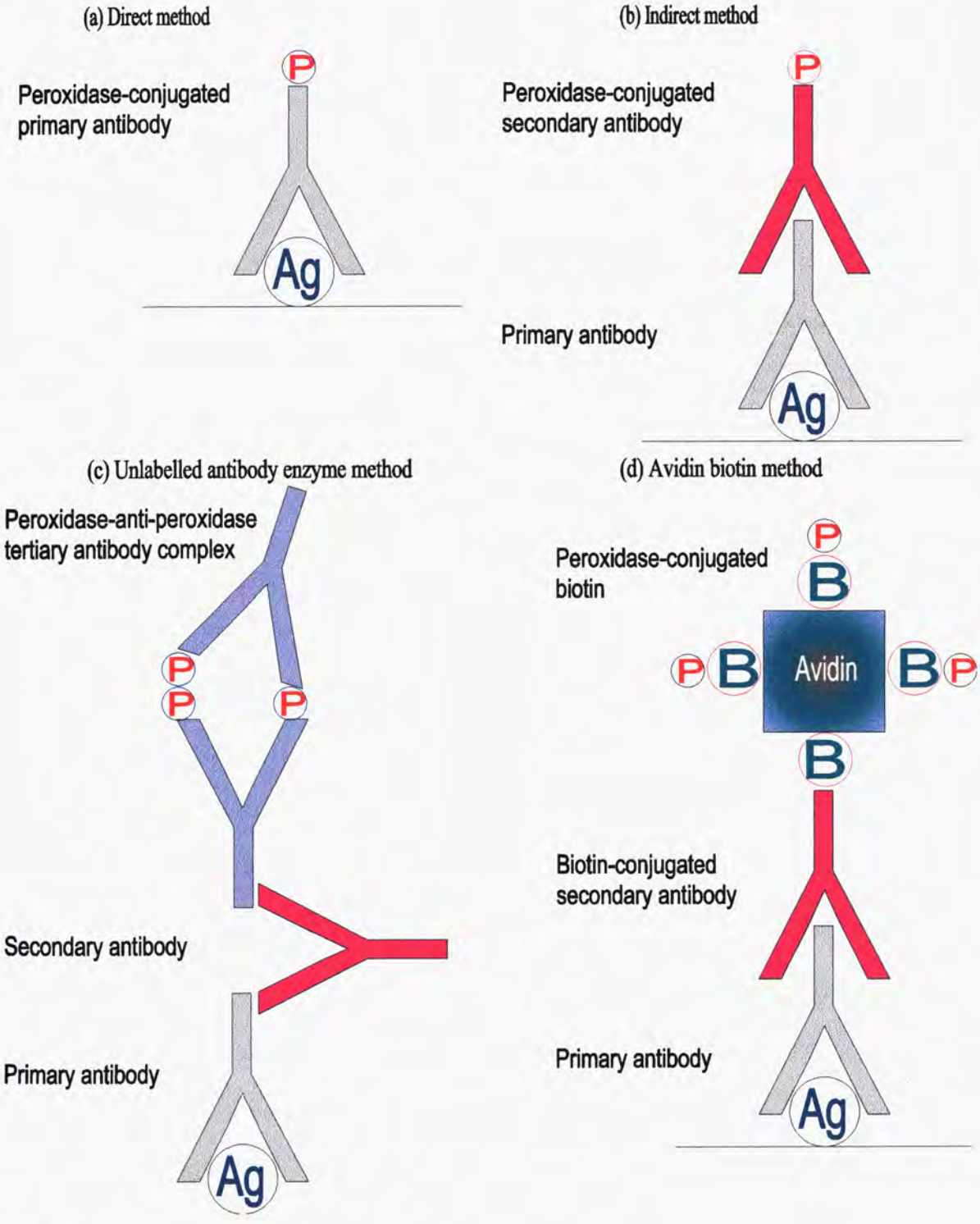
The antibodies used for immunohistochemical studies may be obtained either from animal sera following immunisation with the target antigen or from cell cultures of target antigen specific antibody secreting plasma cells. **As** animal immunisation results in the production of several antibodies, each of which is specific to a particular antigen epitope antibodies derived from this source are polyclonal and are likely to cross-react with non-target antigens in tissue sections (Robinson *et al.,* 1990). Specificity is increased and cross-reactivity decreased by the use of monoclonal antibodies derived from cell cultures. Kohler and Milstein (1975)' demonstrated that mouse myeloma cells could be fused with mouse spleen cells using the Sendai virus. When the spleen cells were harvested from a mouse that had previously been immunised with sheep red blood cells the resulting hybrid myeloma-spleen cells produced anti-sheep red blood cell antibody. By cloning the hybrid cells they were able to produce large quantities of antigen-specific monoclonal antibody. Commercial production of monoclonal antibodies involves careful screening to identify antibody producing hybrids without cross-reactivity and subsequently continuously culturing them in ascitic fluid (Robinson *et al.,* 1990).

If the antibody is labelled or tagged in some way then the site of an antigen-antibody reaction can be visualised in a tissue section following incubation with a specific chromogen. The label can either be conjugated onto the primary antibody that reacts directly with the target antigen (direct method) or else it can be conjugated to a secondary

antibody that displays specificity for the globulin fraction of a primary intermediary or bridging antibody (indirect method) (DeLellis *et al.,* 1979) (Figure 10).

Many substances including enzymes, colloidal metals, fluorescents and radioisotopes can be conjugated to antibodies and used as tracers or labels. Of the available labels, enzymes are the most frequently used and horseradish Peroxidase is the commonest enzyme (DeLellis *et al.,* 1979; Robinson *et al.,* 1990). Peroxidase is utilised in the **peroxidase-anti-Peroxidase** system. In this indirect method, **2** anti-Peroxidase antibodies and **3** Peroxidase molecules combine to form a labelled tertiary antibody used to locate a target antigen ***via*** a bridging secondary antibody that can combine to both the primary and tertiary antibodies (Figure 10c).

Figure 10



P = Peroxidase, B = Biotin, Ag = Antigen

Diagrammatic representation of four immunoperoxidase methods.
(Adapted from (Robinson *et al.*, 1990).

Use of the unlabelled antibody enzyme complex methods, in particular the peroxidase-anti-peroxidase technique enables the identification and classification of undifferentiated neoplasms with greater accuracy than either the indirect peroxidase method or radioimmunoassay (DeLellis *et al.,* 1979). Nonetheless, in certain situations immunoperoxidase techniques can give false negative results because the immunohistochemical reagents used may be too large to penetrate the formalin-fixed target cells in sufficient concentrationsto produce a visible reaction. The use of agents such as Trypsin to pre-treat sections can often "unmask" previously inaccessible intracellular antigens and reduce the false negative detection rate (Curran and Gregory,1978). Antigen unmasking can also be achieved in many tissues following exposure to microwave irradiation (Kirkpatrick and Marshall, 1992).

Increasing the number of tracer labels that can potentially bind to a single target antigen also increases immunohistochemical sensitivity and specificity. The Avidin-Biotin complex (ABC) (Hsu *et al.,* 1981) method utilises the exceptional affinity of the 67 kdal egg white protein avidin for the low molecular weight vitamin H (biotin). Each avidin molecule can bind 4 biotin molecules and up to 150 biotin molecules can be attached to a single molecule of antibody resulting in a high tracer:antibody ratio and increased reaction sensitivity (Robinson *et al.,* 1990). In the ABC reaction (Figure 10d) biotin-labelled peroxidase molecules containing free avidin binding sites are brought into contact with the target antigen *via* a secondary anti-avidin antibody and avidin (Hsu *et al.,* 1981; Robinson *et al.,* 1990). Unfortunately, avidin will also bind to any lectin-like proteins present in tissue sections resulting in an increased false positive rate because of non-specific binding. This disadvantage can be largely overcome by substituting avidin for Streptavidin, a 60 kdal protein isolated from the broth of ***Strptomyces*** *avidinii* (Robinson *et al.,* 1990).

The use of immunohistochemical techniques like the ABC reaction in conjunction with “unmasking” procedures such as enzyme digestion and microwaving permits the identification and localisation of low concentrations of target antigen facilitating both clinical diagnosis and research.

1.8 Prognostic Indicators

A fundamental requirement for successful cancer management is the ability to accurately predict the likely future course of disease for every individual patient. Only when a patients' prognosis has been ascertained can an individualised, logically based treatment plan be formulated. Numerous classification systems have been developed in an attempt to provide such prognostic data. Unfortunately, no single system currently available is sufficiently discriminating to enable it to reliably and reproducibly predict an individual patients prognosis. These deficiencies can, and do present patient management dilemmas. Recognition of the limitations of current classification systems was a major impetus for this study.

Clinical classification and staging

Tumours may be classified according to anatomical site and size, velocity of growth, histological grade or a combination of all these parameters (Langdon and Henk,1995). Both the TNM (Tumour, Node, Metastases) and the AJCC (American Joint Committee on Cancer) classifications are based on the anatomical size and site of the primary tumour and cervical lymph node metastases and the presence or absence of systemic metastases. Based on work first published in 1944 (Denoix,1944), the TNM classification (Union Internationale Contre le Cancer,1987) is now in it's fourth major revision (Table 11) and exactly corresponds to the AJCC third edition (American Joint Committee for Cancer Staging and End Results Reporting, 1992).

Table 11

T	Primary Tumour
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
TIS	Carcinoma in situ
T1	Tumour 2cm or less in greatest dimension
T2	Tumour more than 2cm but not more than 4 cm in greatest dimension
T3	Tumour more than 4cm in its greatest dimension
T4	Tumour invades adjacent structures e.g. through cortical bone, into deep (extrinsic) muscles of tongue, maxillary sinus, skin
N	Regional Lymph Nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2	Metastasis in a single ipsilateral lymph node, more than 3cm but not more than 6 cm in greatest dimension, or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension, or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, more than 3cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.
N3	Metastasis in a lymph node more than 6 cm in greatest dimension
M	Distant Metastasis
MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

UICC-TNM classification for lip and oral cavity cancer (ICD-0 140, 141, 143-145).
(Union Internationale Contre le Cancer, 1987).

Following assessment of the primary tumour, regional lymph nodes and distant sites, the patient is assigned the appropriate TNM score. In order for the TNM classification system to be of help in assessing prognosis it must group together combinations of T, N and M into homogenous groups which can be shown to have similar survival rates (Langdon and Henk,1995). Numerous studies have shown that in the majority of cases large tumours with evidence of metastatic spread have a poorer prognosis than tumours that remain localised to the primary site, and that the presence of cervical lymph node metastases is an adverse prognostic indicator (Hibbert ***et al.,*** 1983; Davis,1985; Zatterstrom ***et al.,*** 1991). The derived TNM stage groupings reflect these clinical and epidemiological findings such that provided the study group is **of** sufficient size stage 1 tumours have a better prognosis than stage 4 tumours (Rich and Radden,1984), (Tables 12 and 13).

Table 12

Stage	T classification	N classification	M classification
1	T1	N0	M0
2	T2	N0	M0
3	T3	N0	M0
	T1, T2, T3	N1	M0
4	T4	N0, N1	M0
	Any T	N2, N3	M0
	Any T	Any N	M1

UICC-TNM stage grouping for lip and oral cavity cancer (ICD-0 140, 141, 143-145).
(Union Internationale Contre le Cancer, 1987).

Table 13

Stage	Number of cases	Percentage	5 year survival rate
1	31	26	86%
2	22	19	43%
3	28	24	33%
4	37	31	16%

TNM stage grouping distribution and crude 5-year survival rate of 118 patients with oral squamous cell carcinoma.
(Rich and Radden, 1984).

The most important conclusions to be drawn from tables 12 and 13 is that the presence of regional lymph node metastases (N1 or above) significantly reduces 5 year survival rates.

Although the TNM and AJCC systems are widely accepted internationally as the two standard cancer classification systems (Phillips,1989), they suffer from several major deficiencies. Despite there being strong evidence that both histological and cytological features of a tumour influence its likely future behaviour, with well differentiated tumours having a better outcome than poorly differentiated tumours (Cawson *et al.*, 1996), neither TNM or AJCC systems take any account of the histopathological grading (Langdon and Henk, 1995). In an attempt to amalgamate histopathological data with clinical findings Rapidis *et al.*, (1976), devised a new grading system which added site **(S)** and Pathology categories to the existing TNM system resulting in the **STNMP** system. Several studies have found the **STNMP** system to be capable of more accurate prognostic prediction than the standard TNM system. Many patients previously classified by TNM into stages 2, 3 or 4 being are effectively "upstaged" by STNMP to stages 1, 2 or 3 (Evans *et al.*, 1982; Langdon and Henk, 1995), (Table14). However, in a study of 124 Australian patients with oral squamous cell carcinoma, Rich and Radden (1984), were unable to demonstrate any prognostic superiority for the **STNMP** system.

Table 14

	TNM		STNMP	
Stage	% of sample	% 5 year survival	% of sample	% 5 year survival
1	17.7	50.0	30.8	51.5
2	12.9	50.0	25.3	40.7
3	61.3	26.3	32.7	21.6
4	8.1	20.0	11.2	8.3

Distribution and crude 5-year survival figures for TNM and STNMP classifications. (Langdon and Henk, 1995).

A major European collaborative study involving 1021 German, Austrian and Swiss patients with carcinoma of the lips, oral cavity and oropharynx utilising both clinical and pathological determinants identified the following **7** factors that were prognostically relevant for individual patients (Platz ***et al.*, 1983):**

- Patient age
- Tumour site
- Tumour size
- Tumour infiltration
- Tumour differentiation
- Regional lymph node status
- Systemic metastasis status.

By assigning scores to the above items the authors have constructed a database that can be used to predict the likely prognosis of individual patients depending on the treatment modality employed. The final DOSAK database (Deutsch-Osterreichisch-Schweizerischer Arbeitskreis) uses ***6*** of the **7** determinants (tumour differentiation was

omitted following further multivariate analysis) and consists of 192 unique treatment dependent prognostic indices

The most significant criticism of the TNM, AJCC, **STNMP** and DOSAK systems is that they are heavily reliant on subjective clinical examination of the neck in order to assess the presence or absence of regional lymph node metastases. This deficiency is of paramount importance because of the significant adverse effect that regional lymph node metastases confer on survival rates and because clinical examination of cervical lymph nodes, even when supplemented with CT imaging has been shown to be error prone (Woolgar *et al.,* 1994). The 5 year survival rate of patients with oral cancer who have metastases in the regional lymph nodes is approximately 20% (Teichgraeber and Clairmont,1984). Yet two recent studies have demonstrated that clinical examination **of** the neck for cervical lymphadenopathy has a false negative rate of between 27% and 34%, and a false positive rate of between 31% and 40% (Shah *et al.,* 1990; Woolgar *et al.,* 1994). Preoperative CT imaging often fails to improve the accuracy of nodal staging because in many cases occult nodal metastases are microscopic and only detectable by thorough post-resection histological examination (Woolgar *et al.,* 1994). Any preoperative assessment t e c h q u e that can substantially improve on the clinical and radiological assessment of the cervical lymph nodes will potentially be a major prognostic and hence therapeutic advance.

Histopathological grading

The degree of cellular atypia present in a squamous cell carcinoma can be used to grade its degree of differentiation. In other words, by how much it resembles normal oral mucosa. Tumours may be classified as well differentiated, moderately differentiated,

poorly differentiated or anaplastic according to their histological appearance (Holm et al., 1982). The cells in well differentiated tumours are readily recognisable as epithelial cells many of which produce keratin indicating a high degree of normal functioning whereas poorly differentiated tumours display marked cellular pleomorphism and no keratin formation (Lighthelm *et al.,* 1989).

There is a strong association between the histological grade of a tumour and its subsequent behaviour, the 5-year survival being proportional to the degree of tumour differentiation. In their series of 194 patients with oral squamous cell carcinoma Langdon ***et*** *al.,* (1977) found the 5-year survival to vary between 11.8% for poorly differentiated tumours to 40.3% for well differentiated tumours. Unfortunately, any histopathological grading system relies on the biopsy being representative of the tumour as a whole, many of which display marked heterogeneity of structure, and is ultimately dependent on the subjective opinion of the pathologist examining the specimen (Zatterstrom ***et*** *al.,* 1991; Cawson *et al.,* 1996). The best prognostic information is obtained if only the cells at the invading margin are graded (Bryne, 1991). Furthermore, some authors have failed to find an association between tumour grade and subsequent behaviour, particularly the development of lymph node metastases (Nason ***et*** *al.,* 1990).

Attempts have been made to reduce the subjectivity of histopathological grading systems while improving prognostic prediction by introducing quantitative and objective scoring criteria. In a retrospective study of biopsies taken from 66 patients with oral squamous cell carcinoma Nason *et al.* (1990) demonstrated that following a semiquantitative assessment of 8 parameters relating to the tumour cell population and the tumour-host relationship (cytoplasmic differentiation, nuclear pleomorphism, mitotic count, mode of invasion, inflammatory cell reaction, desmoplasia, vascular/lymphatic

invasion and level of invasion) patients assigned a high malignancy score (>16) were significantly ($p < 0.001$) more likely to develop cervical lymph node metastases than those with a low malignancy score (<14).

Tumour thickness and tumour cell nuclear DNA content have also been shown to be related to prognosis. Patients with buccal squamous cell carcinomas less than 6mm thick have a significantly better survival rate ($p < 0.001$) than those with tumours thicker than 6mm regardless of TNM stage (Urist et al., 1987). Nondiploid tumours have a poorer prognosis than diploid tumours (Zatterstrom *et al.*, 1991).

Immunocytochemical detection of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis has been shown to be a good prognostic indicator of future tumour behaviour. ODC levels are elevated in potentially malignant and malignant oral lesions and ODC positivity is associated with cervical lymph node metastasis and poor outcome (Williams *et al.* 1995).

Tumour markers

The concept of a serological test for cancer is an appealing one because it would enable early detection of previously undiagnosed tumours. Markers such as oncofetal protein and human chorionic gonadotrophin have a role to play in detecting and monitoring tumours at inaccessible sites. However, the oral cavity is readily examined and easy to biopsy and so in theory at least there should be little need for such laboratory tests. Nonetheless, a reliable laboratory serological test would prove invaluable for detecting recurrences and perhaps for estimating tumour burden and hence prognosis (Johnson *et al.*, 1996). At the present time no such test exists.

Tumour necrosis factor alpha *(TNF-α)*, a pleiotropic cytokine has been shown to be intimately associated with several local and systemic effects seen in patients with cancer. Examples include cachexia and neoplastic bone destruction (Parks *et al.,* 1994). There is experimental evidence to suggest that the cachexia is secondary to the hypertriglyceridemia consequent on inactivation of endogenous lipoprotein lipase (LPL) resulting from increased serum levels of *TNF-α,* a known inhibitor of LPL (Nakajima *et al.,* 1995). *TNF-α* levels have been shown to be significantly higher ($p<0.001$) in patients with head and neck cancer than in controls (Gallo *et al.,* 1992) and animal studies have shown that *TNF-α* levels are elevated in experimentally produced oral carcinomas but are undetectable in normal control animals (Nakajima *et al.,* 1995). In one study serum *TNF-α* levels were found to be 100 fold higher in head and neck cancer patients than in controls Moreover, the *TNF-α* levels fell dramatically after treatment (Soylu *et al.,* 1994). Serum *TNF-α* levels may thus prove to be useful diagnostic and prognostic markers for oral squamous cell carcinoma.

TNF-α induces the synthesis of matrix metalloproteinases, which together with cathepsins and plasminogen activators form the main classes of proteases produced by tumour cells enabling them to lyse extracellular matrix components facilitating invasion and metastasis (Verspaget, 1998). Several of these proteases have been shown to be prognostic markers for solid tumours of the breast, stomach, colorectum, cervix kidney and lung (Duffy, 1996; Verspaget, 1998). Nielsen *et al.* (1998) have shown that high serum concentrations of plasminogen activator inhibitor-1 are associated with shorter survival in patients with colorectal cancer. This finding is surprising as logically it could be expected that the inhibitor would counteract the lytic activity of the protease and should thus be associated with a better outcome. However, it would appear that in most cancers

plasminogen activator inhibitor-1 regulates focal extracellular matrix breakdown, cellular adhesion and migration *via* clearance **of** activator-inhibitor complexes and adhesion molecule binding thus modulating the proteolytic process (Verspaget, 1998).

Pi class GST is the most ubiquitous of the human GST enzymes and is present in particularly high concentrations in cells of the gastrointestinal tract, lung, erythrocytes, platelets and foetal liver (Bongers *et al.,* 1995). High levels of GST have recently been reported in serum samples of patients with oral cancer. Hirata *et al.* (1992), found a highly significant difference in plasma GST levels ($p<0.001$) between patients with oral cancer and those with benign oral diseases or normal healthy control patients. Of great clinical interest is the fact that GST levels fell to and remained below the cut off level (30.4ng/ml) in patients with no evidence of recurrent disease. However, GST levels either failed to fall or fell and rose rapidly above the cut off levei in those with recurrent or persistent disease. Elevated plasma GST levels were found in the majority of patients with recurrent disease well in advance of clinically detectable tumour. Assay of plasma GST-π levels is thus a useful marker for patients with oral cancer for monitoring tumour burden and postoperative recurrence (Hirata *et al.,* 1992).

1.9 Management of Oral Cancer

Treatment

As with all other medical and surgical specialities, the advances in the diagnosis and management of oral cancer is advancing at such a rapid rate that it is impossible for any one clinician or speciality to be able to manage oral cancer patients in isolation. Modern management demands that patients are seen and managed in a multi-disciplinary "joint clinic" consisting of surgeons, oncologists and paramedical support staff (Rapidis *et **al.,*** 1980). Indeed, current UK Department of Health guidelines on the setting up and running of cancer centres demand that such facilities not only exist but are held at least weekly (Calman, 1995).

The fundamental question to be answered when formulating a treatment plan for a patient with oral cancer is whether he or she is potentially curable and thus whether treatment is aimed at being curative or palliative. It is axiomatic that each patient is an individual and thus needs to be offered a tailor-made treatment plan, a treatment plan that **is** deemed suitable for a relatively young, fit and healthy person may be totally inappropriate for an elderly, infirm and medically compromised patient. While these points are obvious, it is all too easy to loose sight of them if one adopts a "cure at all costs" approach to management. Thus, while modern surgical and reconstructive techniques give the surgeon the ability to resect and reconstruct virtually any oral cancer, many of them should not be so treated. The advice of the Arabian physician Avicenna **(A.D.** 980-1037) (Martin,1940), "The cure of a disease must never be worse than the disease itself' **is** particularly relevant here (Langdon and Henk, 1995).

Before embarking on a curative resection the surgeon must satisfy him **or** herself of the following points. That there is a good chance of achieving a cure, that all of the tumour

can be removed together with a surrounding margin of normal tissue, that function can be restored to as near normal as possible and that the final cosmetic result will be acceptable to the patient (Langdon and Henk, 1995; Cawson ***et al.,*** 1996). If these conditions can not be satisfied, all other things being equal the patient is better served with a palliative management strategy. However, no matter whether the management intention is cure or palliation it is vital that all known risk factors including alcohol, tobacco and local trauma are removed (La Vecchia ***et al.,*** 1997; Tradati ***et al.,*** 1997).

The size and extent of the primary tumour together with the status of the cervical lymph nodes, i.e. the stage of the tumour, to a large extent dictates the therapeutic strategy that must be employed in each case. For T1 and early T2 squamous cell carcinomas confined to the lining mucosa, surgery and radiotherapy offer an equal chance of cure. The actual therapeutic modality employed will thus often depend on local expertise and patient preferences (Cawson ***et al.,*** 1996). Larger tumours are seldom eradicated by radiotherapy alone and usually require primary surgery and postoperative radiotherapy. Thus most clinicians employ postoperative radiotherapy if the primary tumour microscopic resection margins are less than 5mm, if there has been extracapsular spread of disease in one or more cervical lymph nodes or if more than 2 lymph nodes are involved.

In some cases small T1 tumours may be excised and then either allowed to heal by secondary intention or be closed primarily. Small tumours of the tongue and lip fall into these categories. Hemimaxillectomy cavities and orbital exenteration defects can often be reliably reconstructed using a temporalis muscle axial pattern local flap (Bradley and Brockbank, 1981). Larger tumours require larger, more mutilating resections and leave larger defects requiring subsequent reconstruction. The distant pedicled flaps such as the pectoralis major myocutaneous flap (Ariyan, 1979) are extremely useful where large soft

tissue bulk is required such as following subtotal glossectomy and radical neck dissection. However, in many situations, particularly for floor of mouth and smaller tongue cancers, the radial forearm flap provides excellent quality thin, pliable skin which improves postoperative tongue and floor of mouth function (Soutar *et al.,* 1983).

Radiotherapy is ineffective at killing tumour cells within bone. If there is evidence of bone invasion the involved bone must be resected, followed by reconstruction in the majority of cases (Langdon and Henk,1995). When bone, particularly large sections of mandible need to be excised and reconstructed, the choice lies between using alloplastic materials such as titanium reconstruction plates or bone. Reconstruction plates have the advantage of simplicity and reduced operative time which can be an important consideration, particularly in enfeebled patients, but bone remains the "Gold Standard' for reconstructing mandibular defects, particularly in young people (Schusterman *et al.,* 1991). Bone can be transferred as a non-vascularised graft in either block, or cortico-cancellous "mush" form held in trays and covered with a pectoralis major flap (Phillips *et al.,* 1991; Langdon and Henk,1995), or as a microvascular free bone graft, with or without overlying soft tissues (Soutar *et al.,* 1983). Microvascular bone flaps have significant advantages over free bone grafts with respect to graft survival, particularly in previously irradiated tissues (Schusterman *et al.,* 1991). Numerous donor sites are available for harvesting and transfer, but the radius (Soutar *et al.,* 1983), and fibula (Lyberg and Olstad, 1991; Serra *et al.,* 1991) either as bone only, or as osteo-fasciocutaneous flaps are ideal sources. Use of these flaps enables the surgeon to perform a one stage mandibular and soft tissue reconstruction and facilitates the subsequent placement of osseointegrated implants allowing the patient to wear implant retained dentures and a return to near normal masticatory functioning.

Recently, exciting advances in the field of distraction osteogenesis and bone transport have made the dream of growing and moving segments of a patients' own mandible to reconstruct a post-surgical defect without the need to resort to grafts and flaps **a** reality (McCarthy *et* ***al.,*** 1992). Over the next few years, modifications and enhancements to equipment and techniques are set to make distraction osteogenesis the reconstruction revolution **of** the latter half of the decade, in the same way that the radial forearm flap has revolutionised reconstruction in the first half.

If there is clinical and/or imaging evidence **of** cervical node metastasis the majority **of** patients should receive a neck dissection, especially where surgery is to be employed to treat the primary site. There is still controversy however over how best to treat the NO neck in patients with oral cancer. The adverse prognostic significance **of** cervical node metastases has already been mentioned, as has the high false positive and false negative rate of clinical neck examination. The debate centres on whether one should perform an elective, prophylactic neck dissection in these cases, or adopt a "wait and see" policy, only treating the neck if and when nodal metastases declare themselves. Unfortunately, there can be no all encompassing solution to this dilemma. While nodal metastases reduce 5 year survival rates by 50%, no prospective controlled trial has shown a survival advantage **for** elective treatment, and neck dissection is not without morbidity (Langdon and Henk,1995). Not uncommonly, NO patients will receive an elective functional neck dissection in order to facilitate access to the blood vessels in the neck if a microvascular free flap is being used to reconstruct the primary site.

With the N+ neck the consensus is that some form of neck dissection needs to be performed (if it has been decided to treat the patient surgically rather than with radiotherapy). There is however, continuing debate on what type of neck dissection should

be carried out. The classical radical neck dissection (Crile,l906), to this day remains the benchmark operation for cervical node clearance in that all levels in both triangles are cleared of lymph nodes. Unfortunately, postoperative morbidity is not inconsequential with this operation which results in shoulder weakness consequent on transection of the spinal accessory nerve and an obvious cosmetic deformity. In a retrospective review of 1,234 patient with oral squamous cell carcinoma over a 21 year period Shah *et al,* (1990), demonstrated that in selected cases, the performance of an appropriate modified neck dissection would have resulted in only 4% of patients being left with occult nodal disease. However, until data from a prospective randomised trial is available most surgeons will still opt for a classical radical neck dissection in all N+ necks, and an appropriate modified neck dissection or expectant management in patients with NO necks (Langdon and Henk,1995). What is required is the ability to predict which patients who present with NO necks will develop nodal metastases and do badly. Unfortunately, to date there is no routine clinical or laboratory investigation that can provide this data. Hopefully, continuing refinement of the histopathological, cytological and molecular biological assays discussed previously will help to answer these questions.

In the UK, chemotherapy is principally used for attempted palliation in end stage large, inoperable tumours. Early studies demonstrated that few if any patients were cured by chemotherapy, especially when it was used as monotherapy (Jones and Gore,l995). For although tumour response rates are often high, this does not translate into improved long term survival and previous metanalyses have failed to show any benefit from adjuvant chemotherapy regimes (Munro,1995). However, a recent metanalysis of 51 published trials did show a survival advantage if chemotherapy was used as a single agent synchronously with radiotherapy (Munro,1995).

The UKHAN 1 trial, which started recruiting head and neck cancer patients in 1990 randomises patients into 1 of 4 treatment arms:

- Radiotherapy alone
- Radiotherapy + simultaneous Chemotherapy
- Radiotherapy + subsequent Chemotherapy
- Radiotherapy + simultaneous & subsequent Chemotherapy.

The aims of this trial are to assess the effectiveness of outpatient single agent chemotherapy, in this case Methotrexate, combined with radiotherapy in the treatment of head and neck cancer. Patient accrual is not expected to be complete until 1999. Since the start of UKHAN 1 studies like those reported by Munro (1995), have drawn attention to the possible survival advantage of synchronous Cisplatin and radiotherapy in the management of head and neck cancer and a further UKHAN trial is being considered to assess this new data. We must await the outcome of these important trials before passing final judgement on the role of chemotherapy in the management of oral cancer (UKHAN 1 Newsletter, Autumn 1996).

Two recent studies have shown that in the UK approximately half of all patients with oral cancer who are treated by Oral & Maxillofacial Surgeons receive multimodality treatment consisting of preoperative surgery and postoperative radiotherapy and approximately one third are treated by surgery alone (Webster and Worrall, 1995; Worrall and Corrigan, 1995) (Table 15).

Table 15

Treatment	Unit	
	Leeds (UK)	Stoke-on-Trent (UK)
Nil	1.8%	0
Surgery + radiotherapy	50%	41.9%
Surgery only	32.7%	33.3%
Radiotherapy only	13.7%	14.3%
Radiotherapy + chemotherapy	0.9%	9.5%
Chemotherapy only	0.9%	1.0%

Treatment modalities employed by 2 UK Oral & Maxillofacial Surgery units in the management of patients with oral carcinoma.
(Adapted from Webster and Worrall,1995; Worrall and Corrigan,1995).

The future

As discussed previously, oral cancer is a genetic disease and current treatment modalities have been singularly unsuccessful in extending 5 year survival rates. Surgery, and to a lesser extent radiotherapy and chemotherapy are fairly crude instruments with which to attack a disease that operates at the molecular level. It is hoped that in the future, development and refinement of emerging molecular biological and gene therapy techniques may produce a range of effective, non-mutilating and non-invasive therapeutic options. There is already evidence that such techniques may be feasible.

The herpes simplex virus thymidine kinase (HSV ***tk)*** gene will sensitise transduced cells to the drug ganciclovir (GCV **[9-(1,3-dihydroxy-2propoxyrnethyl)** guanine]). GCV is a purine analogue which is phosphorylated by the protein product of HSV ***tk*** to monophosphate GCV. Monophosphate GCV can be phosphorylated by normal human thymidine kinase into triphosphate GCV which is cytotoxic (Goebel ***et al.,*** 1996). In an ***in vitro*** experiment using head and neck squamous cell carcinoma cell lines (Hep-2, FaDu

and SCC-4) infected with an adenovirus containing the HSV ***tk*** gene (Ad.RSV ***tk)*** dramatic tumour cell killing ensued following the subsequent administration of GCV. Moreover, substantial cytotoxic effects were observed using therapeutic concentrations of GCV (20μmol/L) (Goebel ***et*** *al.,* 1996).

Recent work has shown that unrestrained cellular proliferation and resistance to apoptosis consequent on loss of G1/S cell cycle control can be prevented. Enhanced irradiation induced apoptosis in p53 deficient mice has been demonstrated following caffeine induced activation of the p34 kinase controlling the G2/M check point (Yao ***et*** *al.,* 1966). In an ***in vitro*** experiment the DNA binding affinity for class 1 mutated p53 protein was shown to be significantly enhanced following substitution of the mutated amino acid (Thr284) with arginine (Wieczorek ***et*** *al.,* 1966). Experimentally at least, it appears that it is possible to rescue cells from p53 mutation and in so doing restore cell cycle control enabling DNA repair or apoptosis to occur as appropriate and thereby reducing unrestrained growth. The future therapeutic possibilities of these experimental findings are very exciting.

Quality of life

A major consideration when planning and implementing a curative treatment protocol is that postoperative function should be as near normal as possible. However, if cure is the goal, the desire to restore normal functioning must never sway the surgeon into performing a less than ideal oncological resection for fear of leaving tumour behind. Nonetheless, when assessing the results of any treatment regimen, simple estimation of the length of survival is no longer acceptable. The quality of life experienced by patients is as important, if not more important than the quantity of life gained (Langdon and Henk,1995).

Providing a high quality of life for our patients is of pre-eminent importance when it has been decided that patients are beyond cure and are entered into a palliative care protocol. Palliation does not mean that patients are simply supported through their last days, it may involve tumour debulking to improve pain control and cosmesis both surgically and with radiotherapy or chemotherapy.

Quality of life, is a vague, almost nebulous concept that is difficult to define and means different things to different people (Calman, 1984). Nonetheless, there is general agreement that it is not a single entity, but multifactorial and that in order to measure it several different but interrelated facets of a patients life need to be assessed. It has been suggested by Aaronson *&.al.* (1988), that these include:

- Physical complaints - somatic sensations, disease symptoms, side effects
- Social functioning - anxiety, depression
- Psychological distress - quality and quantity of relations with others
- Functional status - activity level, vocational activity.

In addition to the above parameters, it is likely that quality of life is more than simply the sum of its component parts. Any instrument used to assess quality of life should be able to be completed by the patient as they are the best judge of how their disease and its treatment has impacted on their lives. Furthermore, it should be simple and quick to complete, valid and capable of reflecting changes over time (Jones ***et*** *al.,* 1991).

The European Organisation for Research into the Treatment of Cancer (EORTC) has developed a modular questionnaire consisting of both general and disease specific modules (Aaronson *et al.,* 1988). The EORTC QLQ-C30 consists of 30 general questions covering 5 function scales, 9 symptom scales and overall quality of life (Aaraonson ***et*** *al.,*

1993). Patient responses on the questionnaires are linearly transformed into a 0 - 100 scale such that a score of 100 for the functional scales and quality of life indicates maximum functioning (good), while a score of 100 for the symptom scales indicates maximum symptoms (bad). **A** recently developed head and neck specific questionnaire, the H&N35 (Bjordal *et al.,* 1994) which covers 13 aspects of patient well being is currently being evaluated internationally (see Figure 26).

A recent study used the EORTC QLQ-C30 questionnaire to compare the quality of life of 44 patients with oral cancer who were at least 1 year post treatment with 47 control patients who were attending for treatment of non-malignant oral conditions (Mellor and Worrall,l996). There was no significant difference in overall quality of life or any symptom scales between cases and controls, but cases had significantly poorer physical and role functioning than controls (Tables 16 & 17).

Table 16

	PF	RF	EF	CF	SF	QOL
Cases n = 44	75.4	67.8	71.7	78.1	77.4	69.7
Controls n = 47	85.5	81.9	72.3	83.7	79.3	67.7
p value	0.042	0.026	NS	NS	NS	NS

EORTC QLQ-C30 functional scales: cases v controls.
PF = physical functioning, RF = role functioning, EF = emotional functioning
CF = cognitive functioning, SF = social functioning, QOL = quality of life
(Mellor and Worrall, 1996)

Table 17

	FA	NV	PA	DY	SL	AP	CO	DI	FI
Cases n = 44	30.8	10.2	27.4	20.7	32.6	24.4	10.3	6.6	12.6
Controls n = 47	26.8	6.4	28.3	24.0	31.1	7.7	9.4	10.8	15.9
p value	NS	NS	NS	NS	NS	0.003	NS	NS	NS

EORTC QLQ-C30 symptom scales: cases v controls.
FA = fatigue, NV = nausea & vomiting, PA = pain, DY = dyspnoea, SL = insomnia
AP = appetite, CO = coughing, DI = diarrhoea, FI = financial problems
(Mellor and Worrall, 1996).

There was no significant difference in any of the scales with respect to stage of disease at presentation or treatment regimen instituted. However, patients with lower stage disease and those treated solely by surgery tended to have higher quality of life scores than those with higher stage disease and those treated with surgery plus radiotherapy.

1.10 Aims of the study

It will be apparent from this brief introduction that oral squamous cell carcinoma has a high morbidity and mortality despite recent advances in many treatment modalities. One of the major obstacles **to** improving outcome is the difficulty in accurately predicting tumour behaviour and prognosis in individual cases. Numerous studies have shown that many diseases, including certain cancers are associated with particular phase 1 and phase 2 detoxification enzyme polymorphisms.

This study had 2 main aims:

- To investigate whether oral squamous cell carcinoma susceptibility is associated with polymorphism at the gene loci coding for the cytochrome P450 CYP2D6 and CYP1A1 phase 1 detoxification enzymes and the glutathione S-transferase GSTM1, GSTM3, GSTT1 and GSTP1 phase 2 detoxification enzymes.
- To ascertain whether the presence or absence of particular CYP and GST polymorphisms in patients with oral cancer can be used to improve prognosis prediction and augment existing patient and tumour specific factors to refine individual management strategies.

There were two interrelated tasks necessary for the setting up and conduct of this study:

- Designing, developing and implementing a computerised oral cancer database to record and analyse individual patients' demographics, risk factors, tumour characteristics, treatment and outcome
- Determination of *GSTM1, GSTM3, GSTT1, GSTP1, CYP2D6* and *CYP1A1* detoxification enzyme genotypes in peripheral blood samples of patients with oral squamous cell carcinoma and the correlation of the genotype with the clinical and pathological features of each patient as recorded in the database.

Chapter 2

PATIENTS AND METHODS

2.1 The Database

The ability to assign patients with similar disease profiles into groups based on disease stage facilitates treatment planning and prediction of prognosis. However, TNM stage groupings take no account of other important data such as patient risk factors, treatment modality employed, complications, quality of life, recurrence and survival times etc. In order to ensure that patients are receiving optimum treatment, it is vital that this data is collected. Data analysis can then be used to audit results against national and international standards and targets.

Although the treatment for oral cancer has advanced rapidly in recent years with the advent of new ablative and reconstructive techniques, the need to audit the results of treatment has only recently been generally accepted. Unfortunately, even when there is a desire to audit one's own results, the information systems available may be woefully inadequate and the required data almost impossible to obtain.
Furthermore, if novel diagnostic or treatment methods are to be assessed for usefulness and efficacy, it is mandatory that patients' epidemiological, treatment and outcome data is available for analysis.

Central to testing the hypothesis of this study was the need to correlate our oral cancer patients' detoxification enzyme genotype data with their exposure to environmental carcinogens such as cigarette smoke and alcohol, and their clinical course and outcome. The lack of a suitable existing database to achieve these aims, together with the success of a previous project to design a computerised surgical logbook (Worrall, 1992), were the catalysts to designing and implementing a computer database to record and analyse data on patients with oral cancer.

The idea for the design of an oral cancer database first occurred to me during 1994. The first prototype database was written later that year (Worrall, 1994), and used in a pilot project to retrospectively analyse data on oral cancer patients over a ***5*** year period (Worrall and Corrigan, 1995). However, it was the realisation that the detoxification enzyme project required a comprehensive database capable of linking clinical events with research data that was the stimulus for further development and refinement. It also became apparent that as the project required a multi-centre approach, the database would also need to be capable of being installed in other units.

I also wished to be able to produce a simple, user-friendly system that would encourage clinicians involved in the management of patients with head and neck cancer to record data on their patients in a standardised manner. **A** recent national survey of consultants who treat head and neck cancer in the UK has demonstrated a pressing need for such a system. Edwards *et al* (1997) found that only 4% (37/919) of consultants actively engaged in the management of patients with head and neck cancer used a standardised method **of** data collection. Of even greater concern was the finding that 14% of the consultant stated that they did not routinely record the clinical stage of the tumour in the patients' hospital records!

Widespread uptake of an agreed national head and neck cancer minimum dataset and the adoption of a standardised method of data collection and audit such as the database described below would ensure that vital data concerning risk factors, treatment and outcome is collected for all patients with head and neck cancer. For a rare disease like oral cancer, with only about 2000 new cases per year in the UK this approach is crucial if we are to improve the diagnosis, treatment and outcome for these patients and ensure that an acceptable standard of treatment is available throughout the UK.

In order to fulfil its designated role as a clinical and research tool, there were several requirements that were fundamental to the design of the database:

- It must be simple to use to encourage data input by non-medical staff
- It must be able to record both clinical and research data
- It must be cost effective to purchase and implement in other units.

It was decided that the database should be written using Microsoft® Access 2 ™ running under Microsoft Windows ™ 3.1 or higher. It is relatively straightforward to design complex relational databases in Access 2 and to provide a friendly and intuitive user interface. A basic core database was written onto which specific modules for research purposes were added. These included modules for calculating body composition and recording detoxification enzyme genotype data. By using a mainstream database, which was likely to be supported by a major software manufacturer for some considerable time in the future, it was felt that the program would be able to be installed by the majority of interested clinicians at little extra cost.

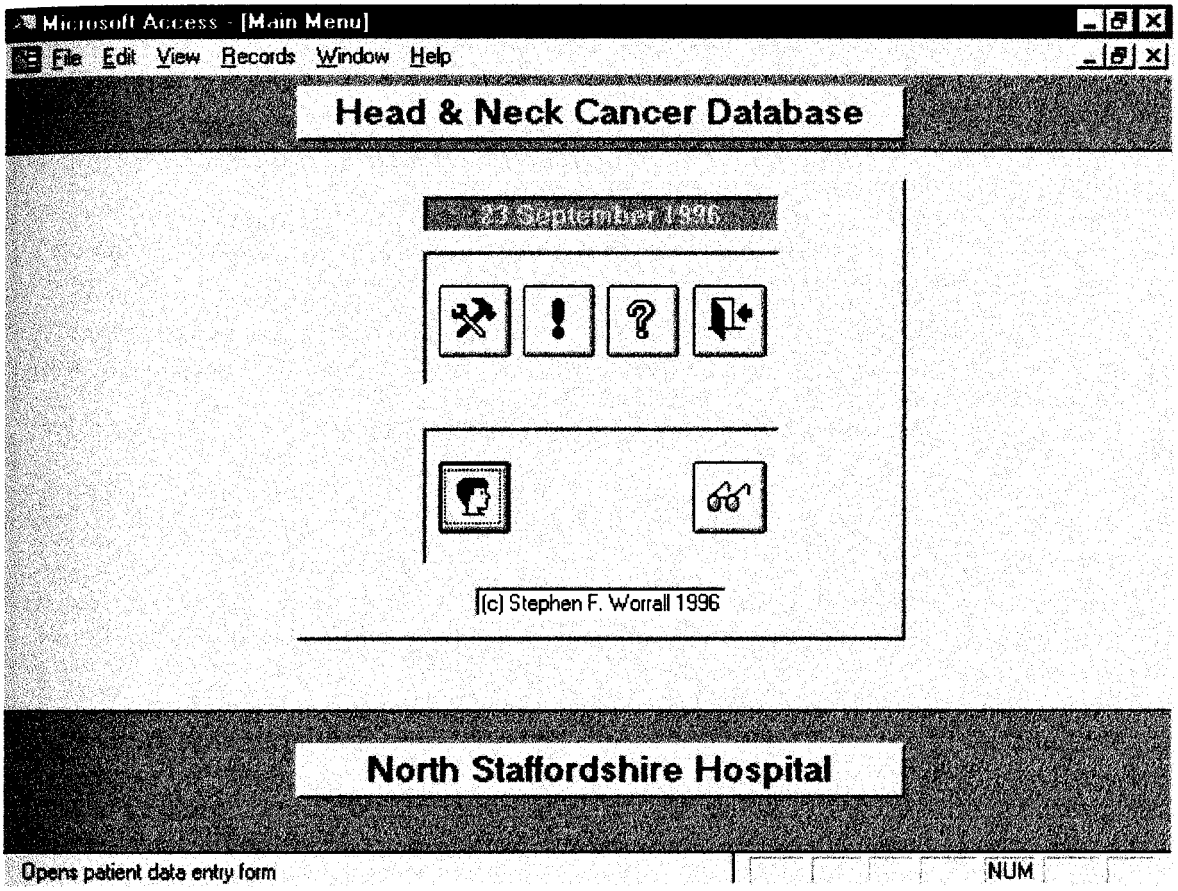
In order to improve usability and reduce typing errors, extensive use was made of choice boxes, selection lists, automatic field calculations and graphics. Several dozen pre-set queries, searches and reports were written, all of which are accessed by selecting choice buttons. Detoxification enzyme data were recorded alongside patients' clinical details affording easy and rapid correlation between genotype and disease and enabling direct output into spreadsheet and statistical analysis software.

Photographs of patients can also be stored directly in the database using new digital image technology and a Kodak DC20 digital camera (© Eastman Kodak Company).

Appreciating that the majority of computer users seldom read instruction manuals, a comprehensive on-line hypertext help file was written in standard Microsoft format. This was authored using Microsoft Word ® **2** together with the Windows' Little Helper document template written by Mike Hardaker (O M Hardaker, 1991). The file was then compiled using the Microsoft help compiler HC31.EXE from the Microsoft Windows Software Development Kit ®

Figures 11-27 are screen shots of all the main data entry screens in sequential order, together with the main Help file screen and examples of some database reporting functions. Certain details have been removed from these screen shots to preserve patient confidentiality. Figure 17 details the detoxification enzyme data entry screen listing the selected patients' detoxification enzyme genotype together with PCR details.

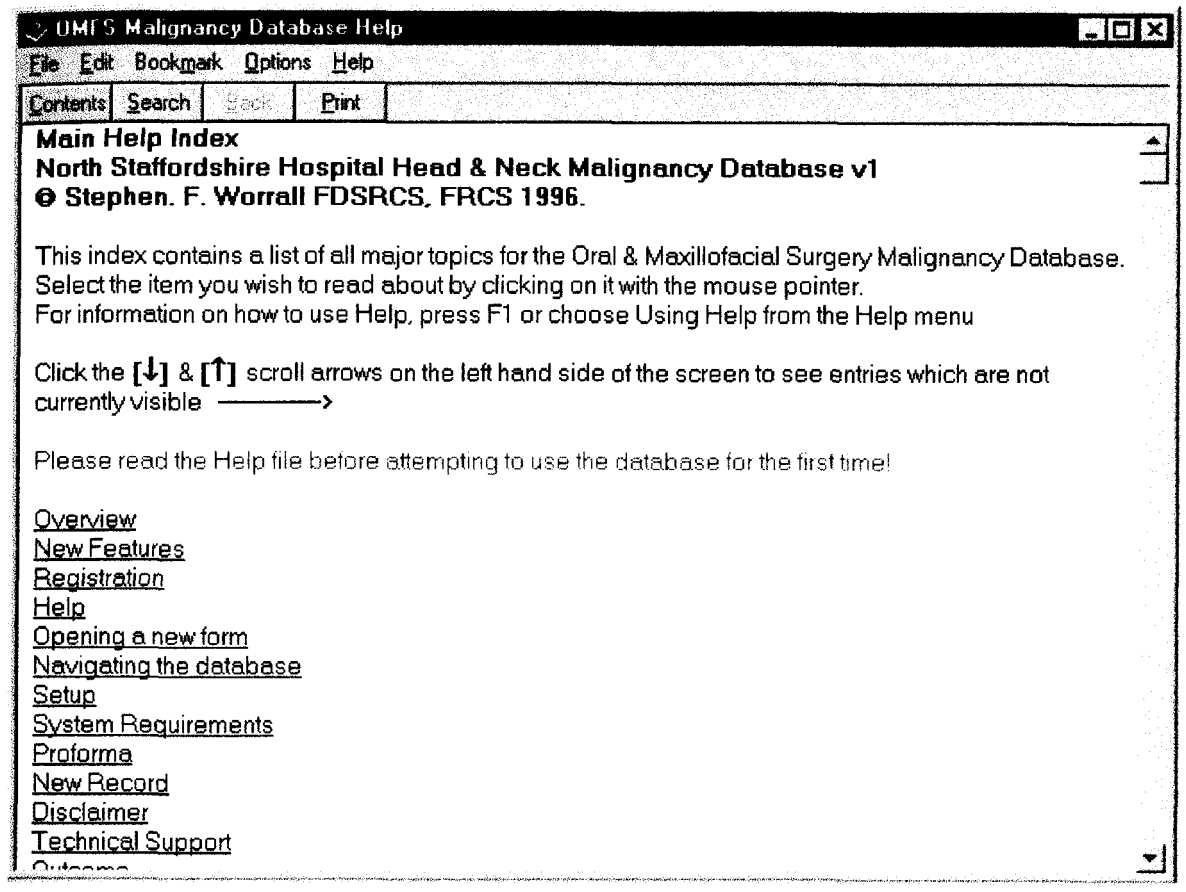
Figure 11



Database Main Menu.

The top row command buttons from left to right open: System Information, Technical Support Information, Help and Exit the database. The bottom row command buttons from left to right open: Patient Details form and Reports Menu.

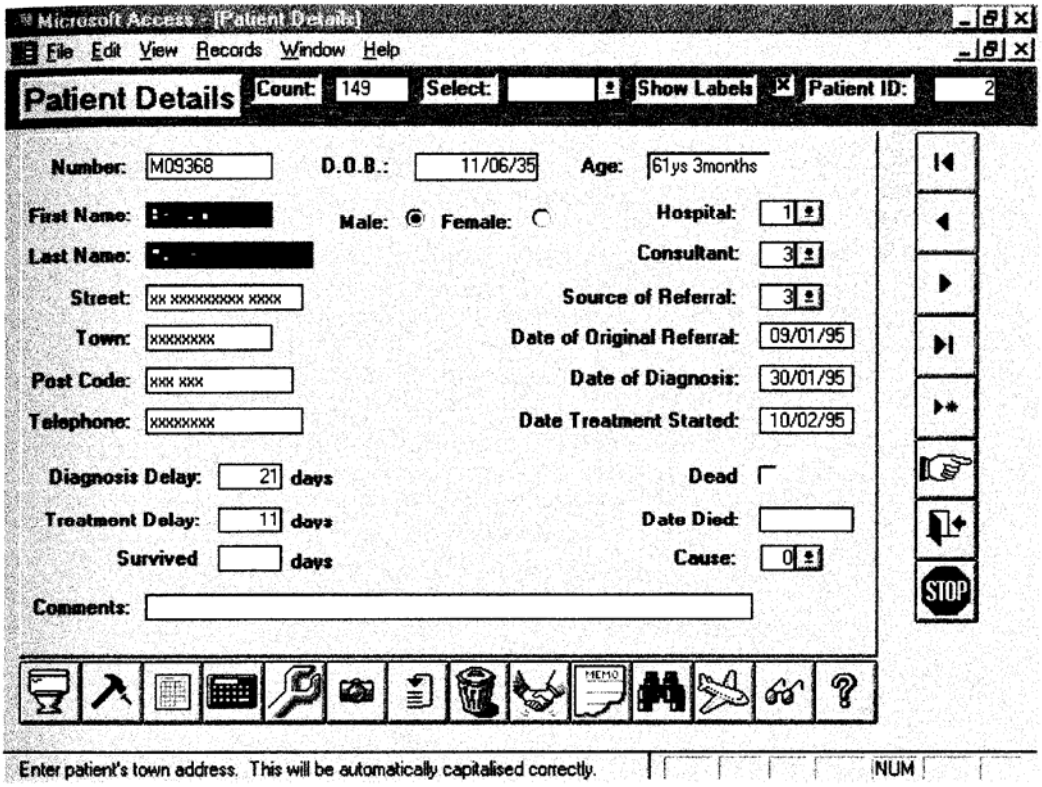
Figure 12



Database Help.

There is an extensive on-line hypertext help file conforming to the Microsoft ™ Help standard conventions and styles providing comprehensive details of all database functions and actions. By selecting the underlined words or phrases the user **is** instantly shown additional relevant information.

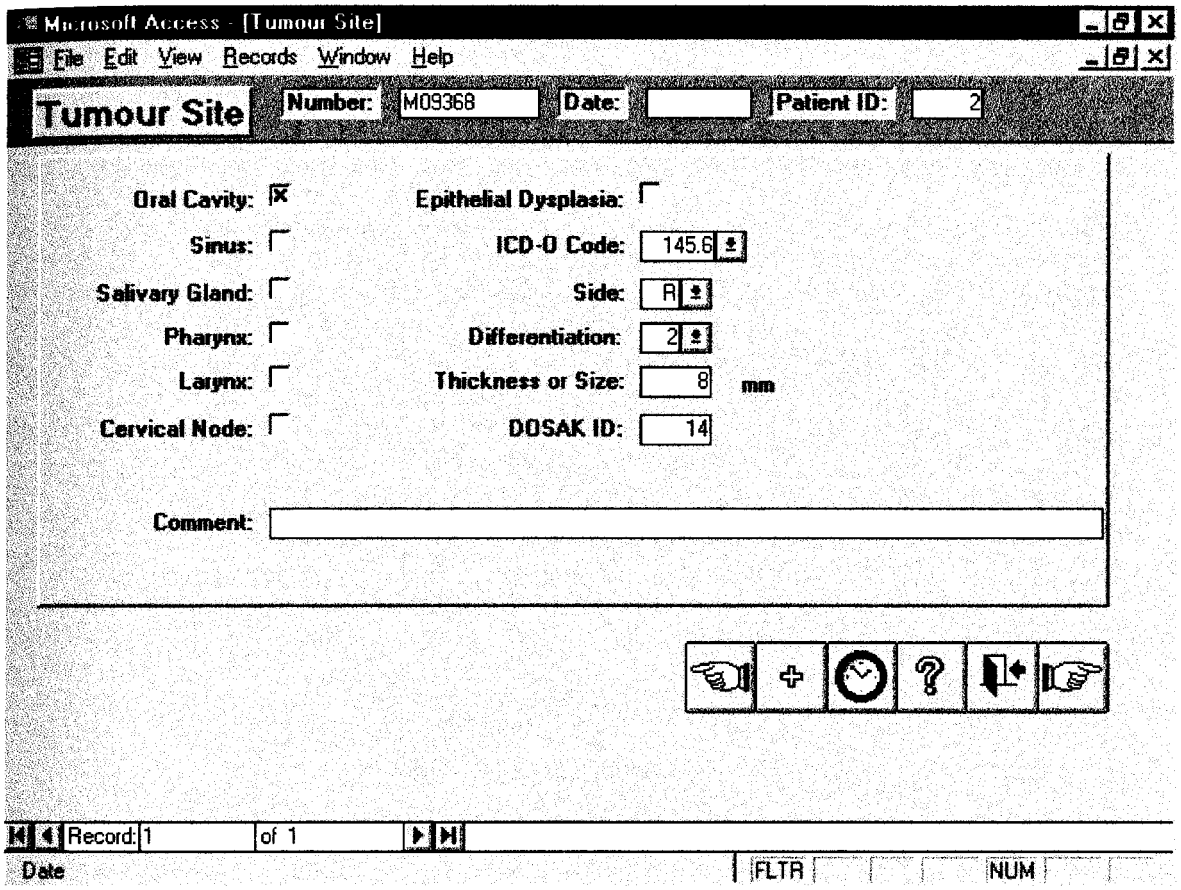
Figure13



Patient Details Form.

This is the main data entry form and is used for the recording of demographic and hospital data. In addition, all the other database forms and functions can be accessed from this single form.

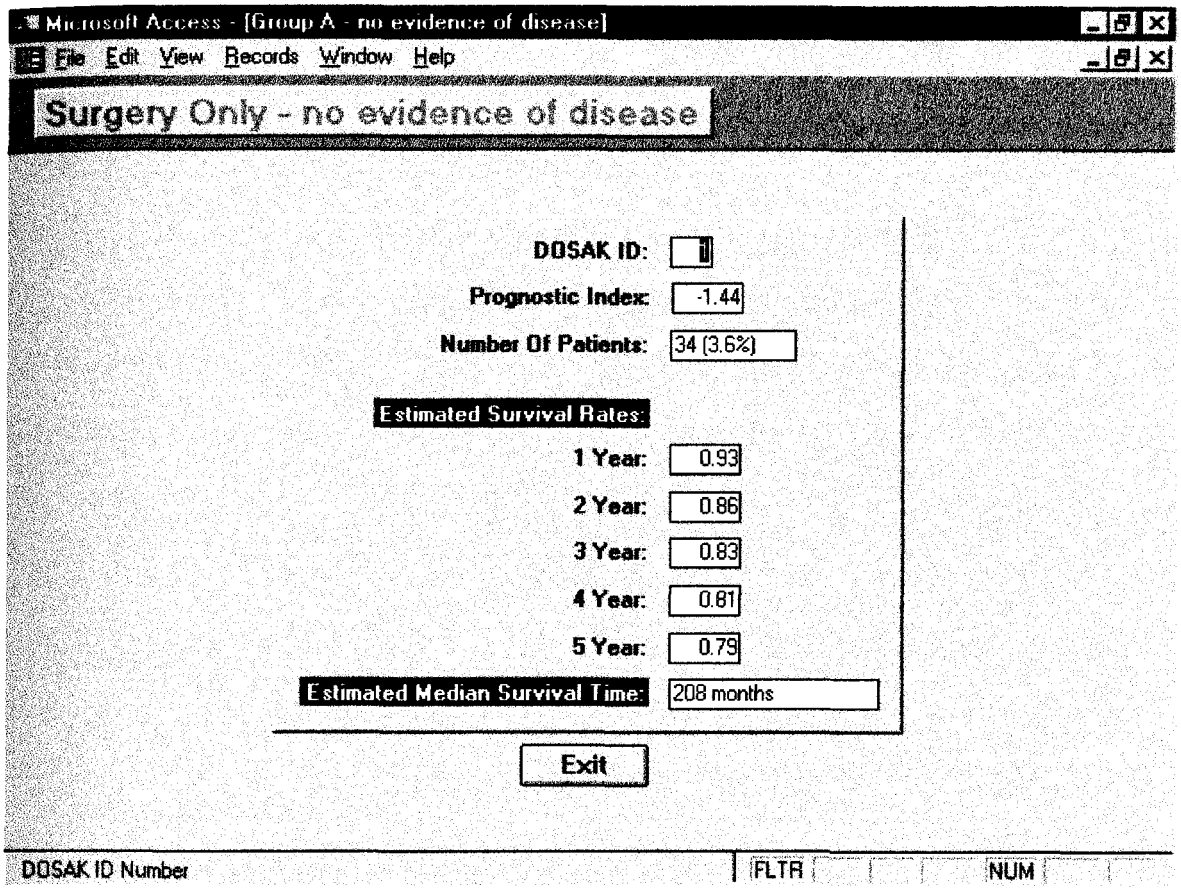
Figure 14



Tumour Site Form.

This form is for recording details of the site and pathological differentiation of the tumour. It is also used to access the DOSAK survival database enabling patient survival prediction (see Figure 15).

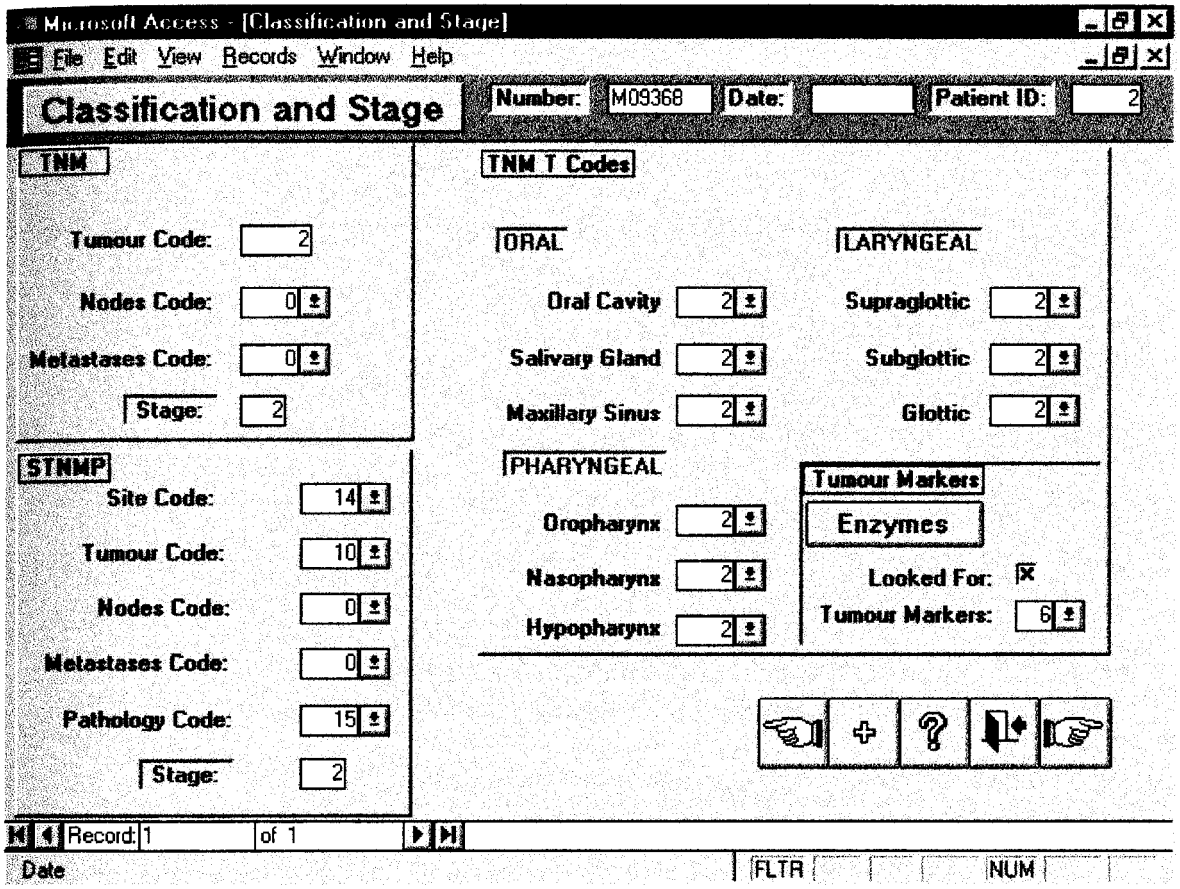
Figure 15



DOSAK Form.

By incorporating the DOSAK survival data directly into the database it is possible to estimate an individual patients' survival based on 5 pre-treatment parameters and the degree of tumour clearance following surgical and non-surgical treatments.

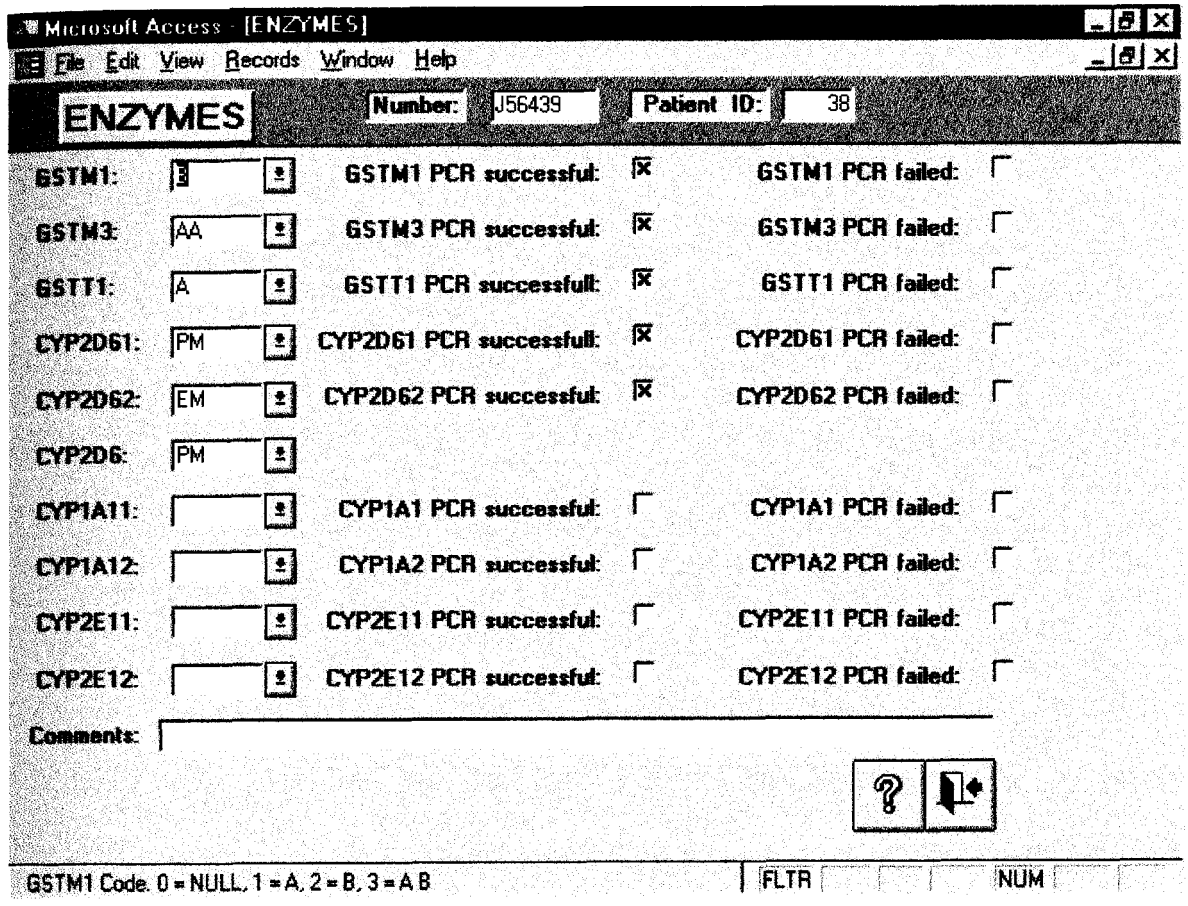
Figure 16



Classification and Stage Form.

This form automatically calculates the appropriate tumour stage from TNM and **STNMP** data. It is also the entry point for accessing patient detoxification enzyme data (see Figure 17).

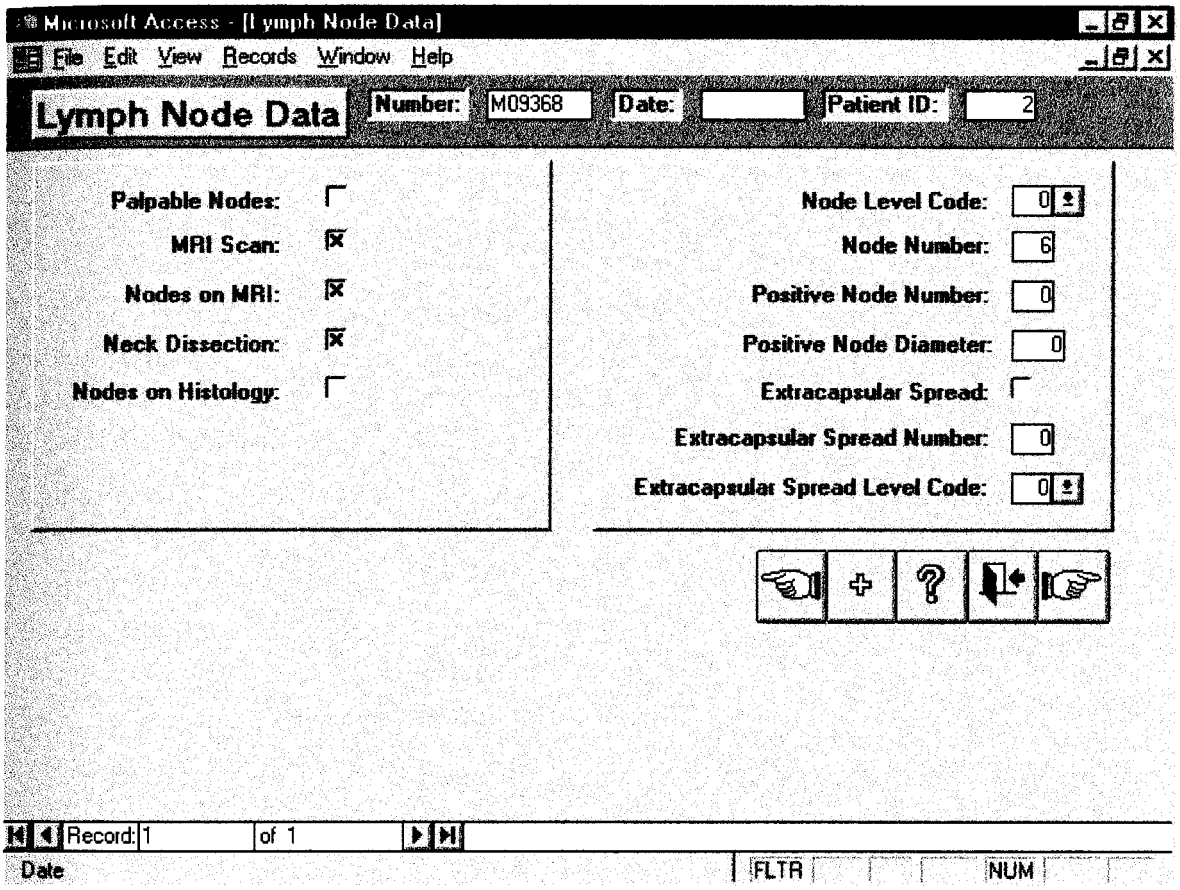
Figure 17



Detoxification Enzyme Data Form.

This form is used to record details of the patient's detoxification enzyme genotype and PCR data.

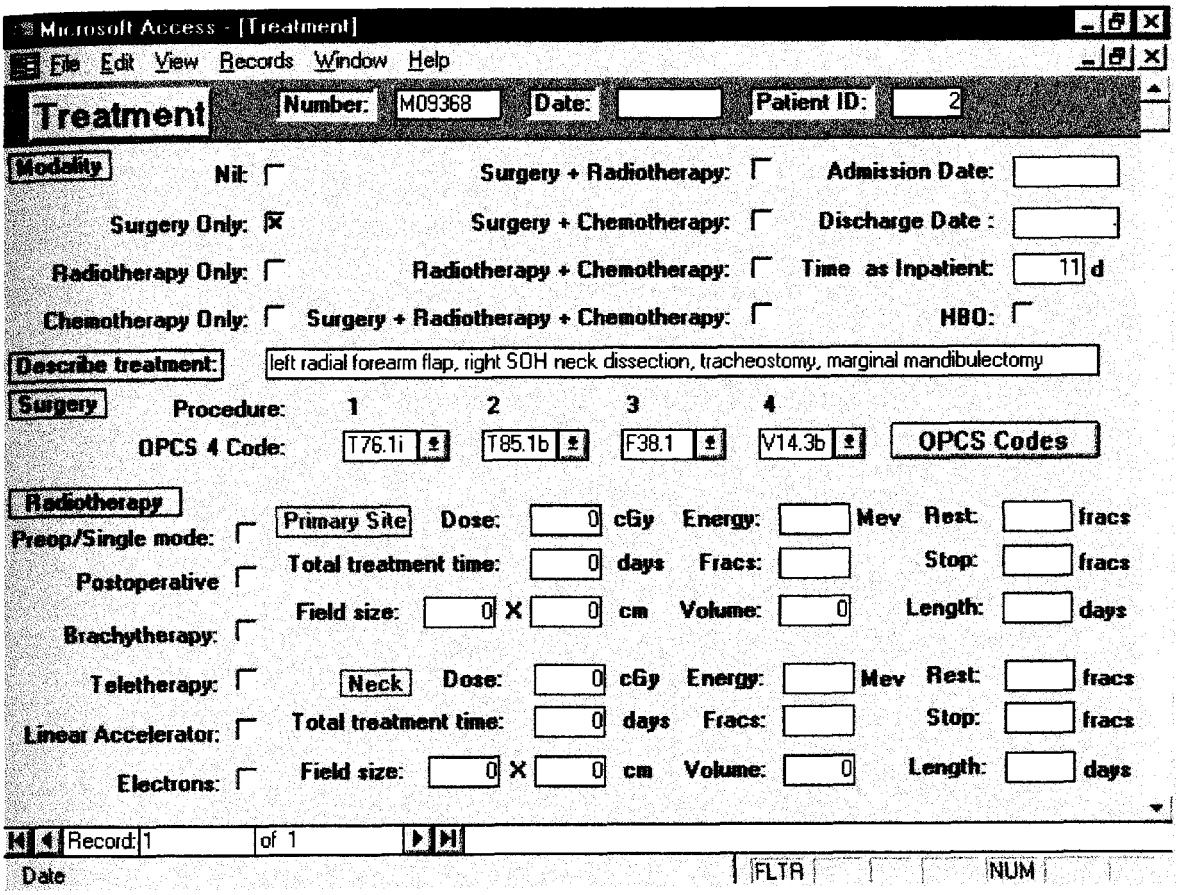
Figure 18



Lymph Node Data Form.

In this section data is entered concerning the preoperative clinical and imaging findings with respect to the presence of cervical lymph nodes. From this data the program can analyse the false negative and false positive rates for assessing nodal status as well as the association between nodal disease extent and recurrence and survival data.

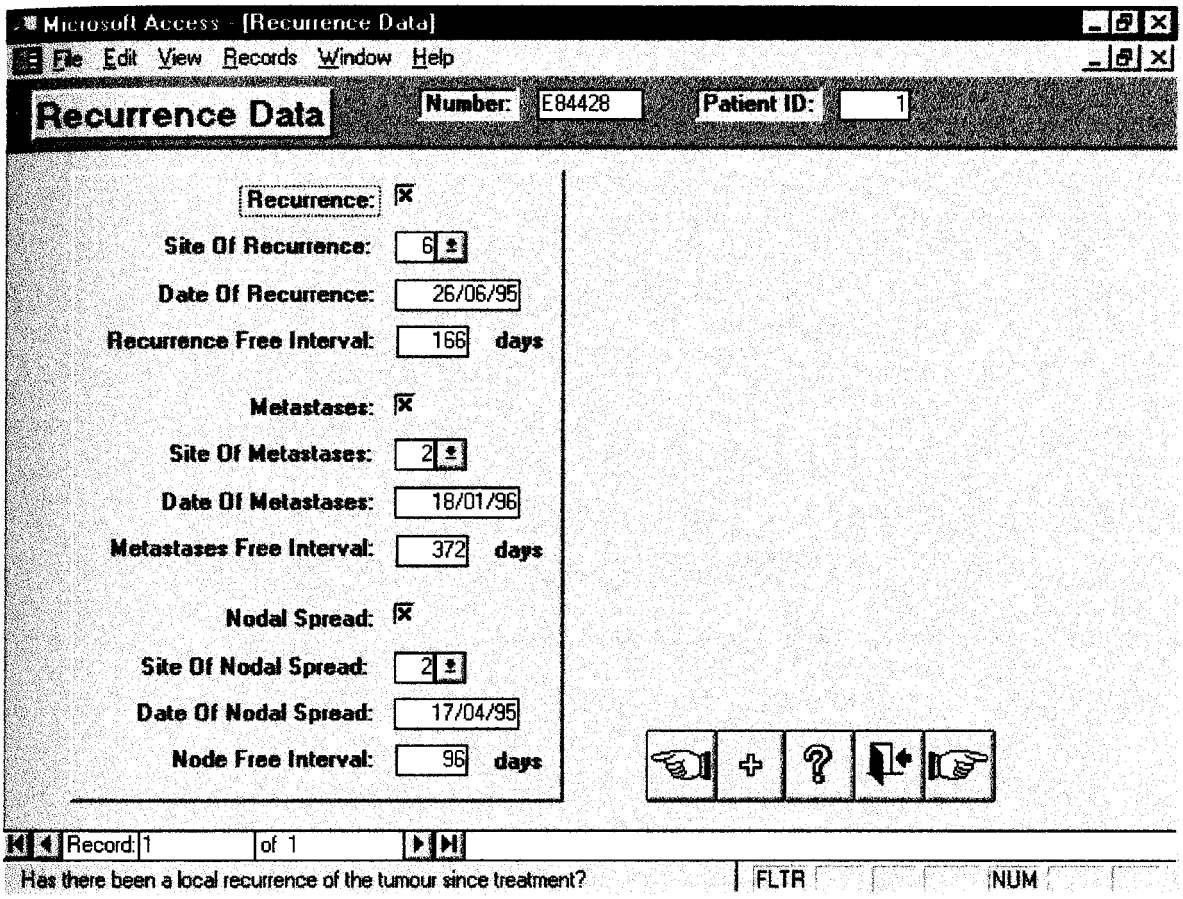
Figure 19



Treatment Form.

All surgery, radiotherapy and chemotherapy **data** is entered on this form. The chemotherapy **data** section is not shown in this screen shot as it appears off screen at the bottom and is accessed by scrolling down the page.

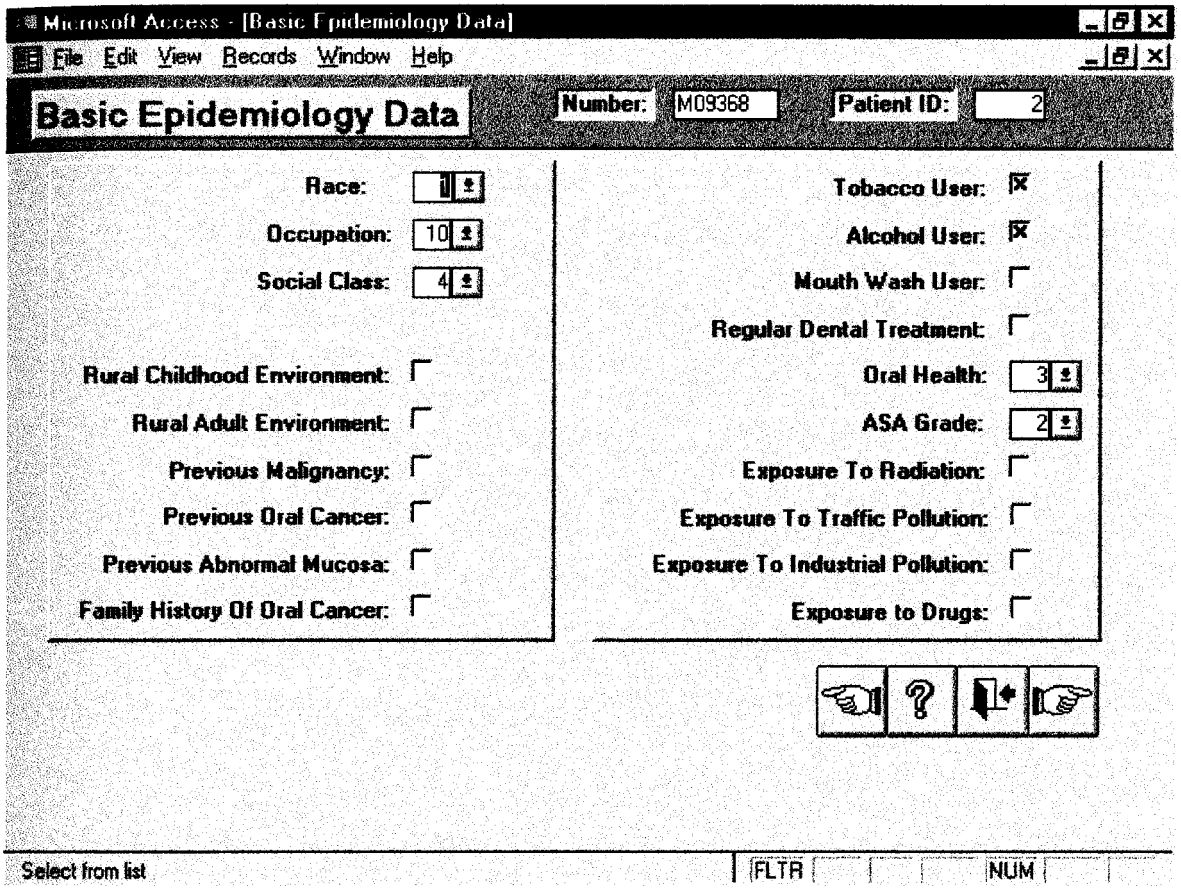
Figure 20



Recurrence Data Form.

The site and timing of local recurrences and metastasis to cervical lymph nodes and the rest **of** the body are recorded on this form. Event delays are automatically calculated from entered dates.

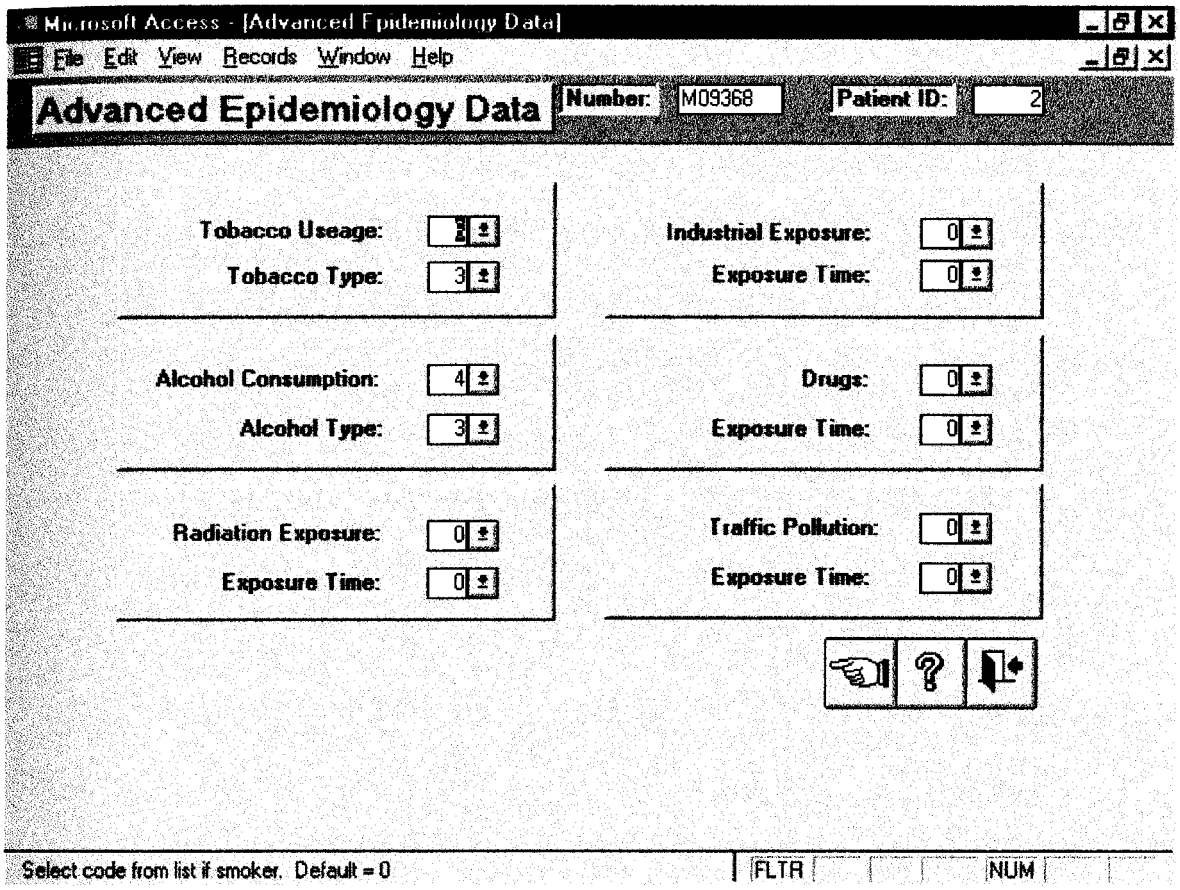
Figure 21



Basic Epidemiology Data Form.

Data concerning risk factor exposure together with oral and general health is entered here.

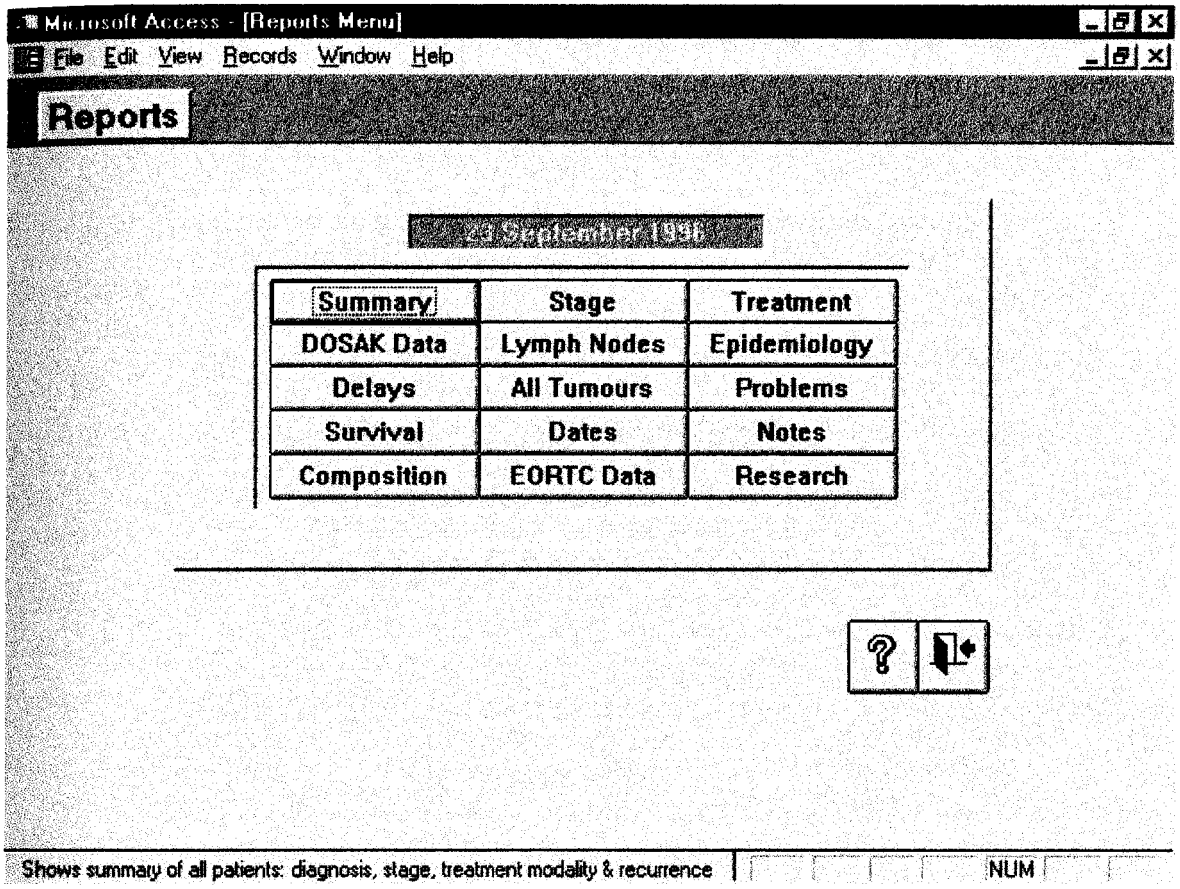
Figure 22



Advanced Epidemiology Data Form.

This form is used to augment the data entered on the Basic Epidemiology form. The type and amount of tobacco and alcohol consumption together with duration **of** exposure to environmental pollutants are recorded here.

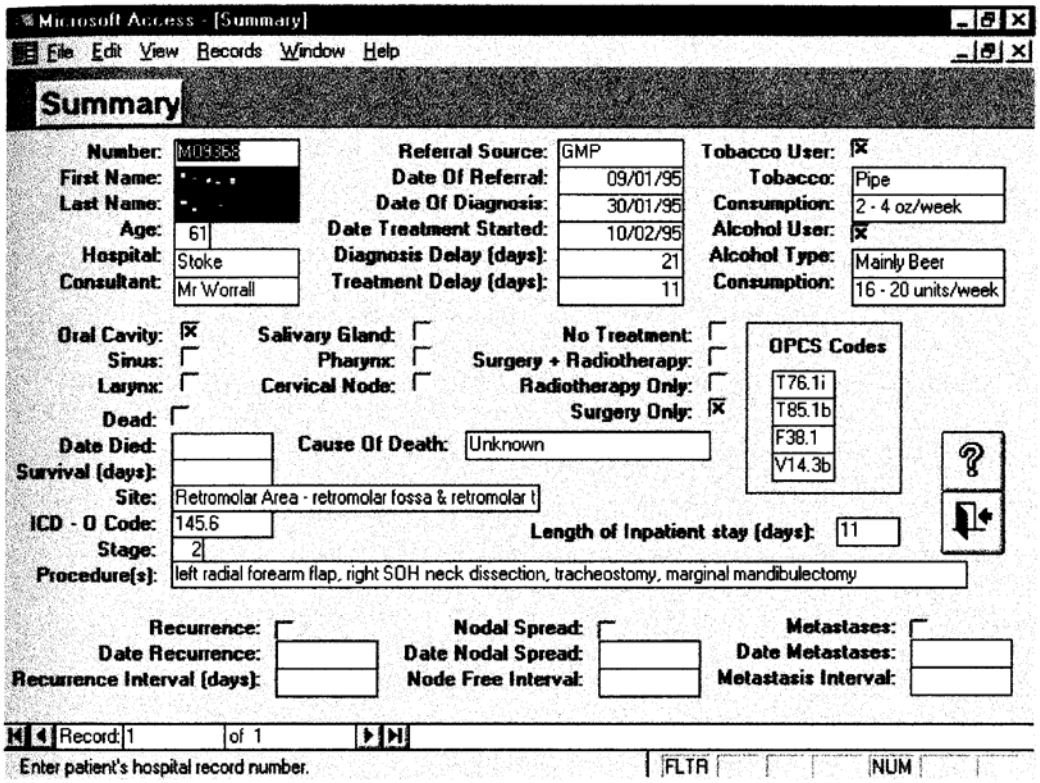
Figure 23



Reports Menu.

This screen provides access to several dozen pre-set database searches and queries. Each of the command buttons shown opens up a secondary menu from which the searches are launched. Custom searching and querying **is** performed using Access 2 filters and SQL **queries.**

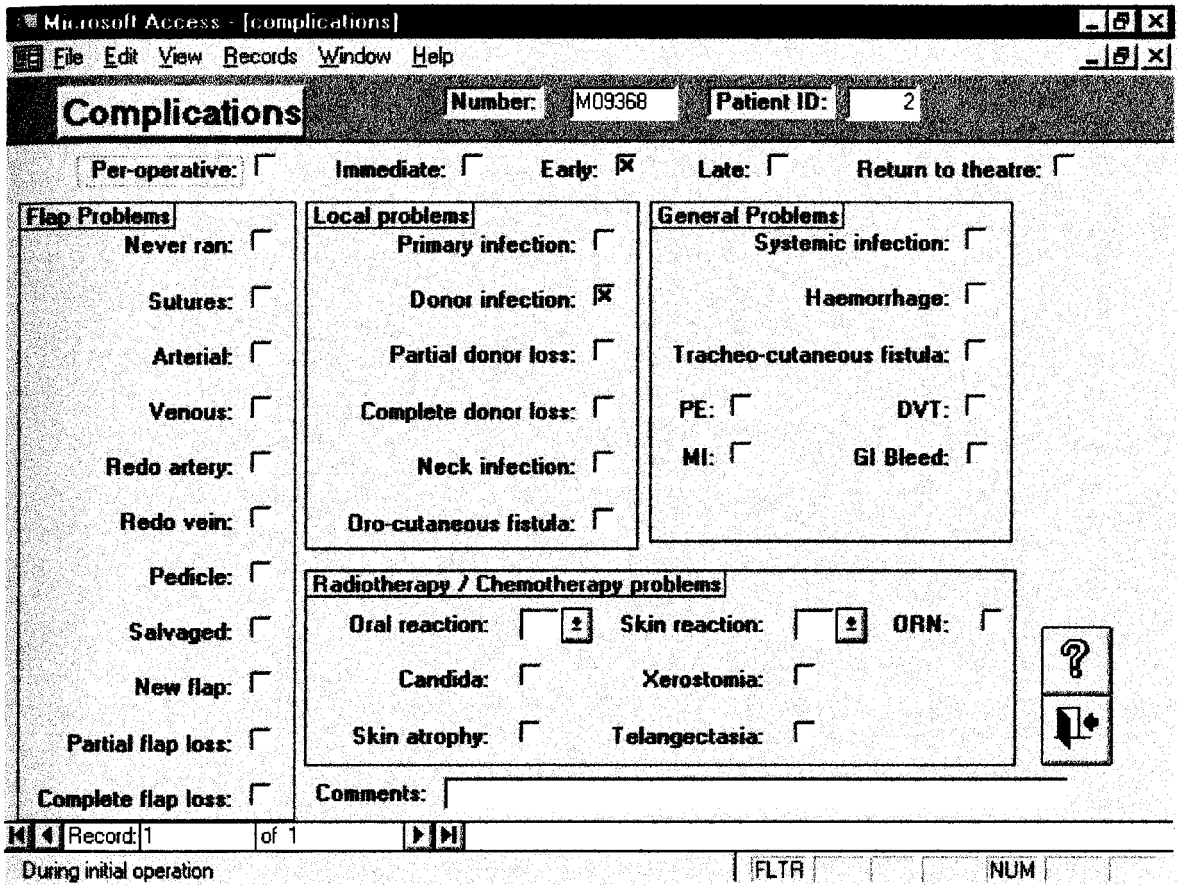
Figure 24



Summary Form.

The patient summary can be accessed from the Patient Details form or the Reports menu. It gives a single screen overview of all the important data concerning the chosen patient and is automatically updated as new or changed data is added.

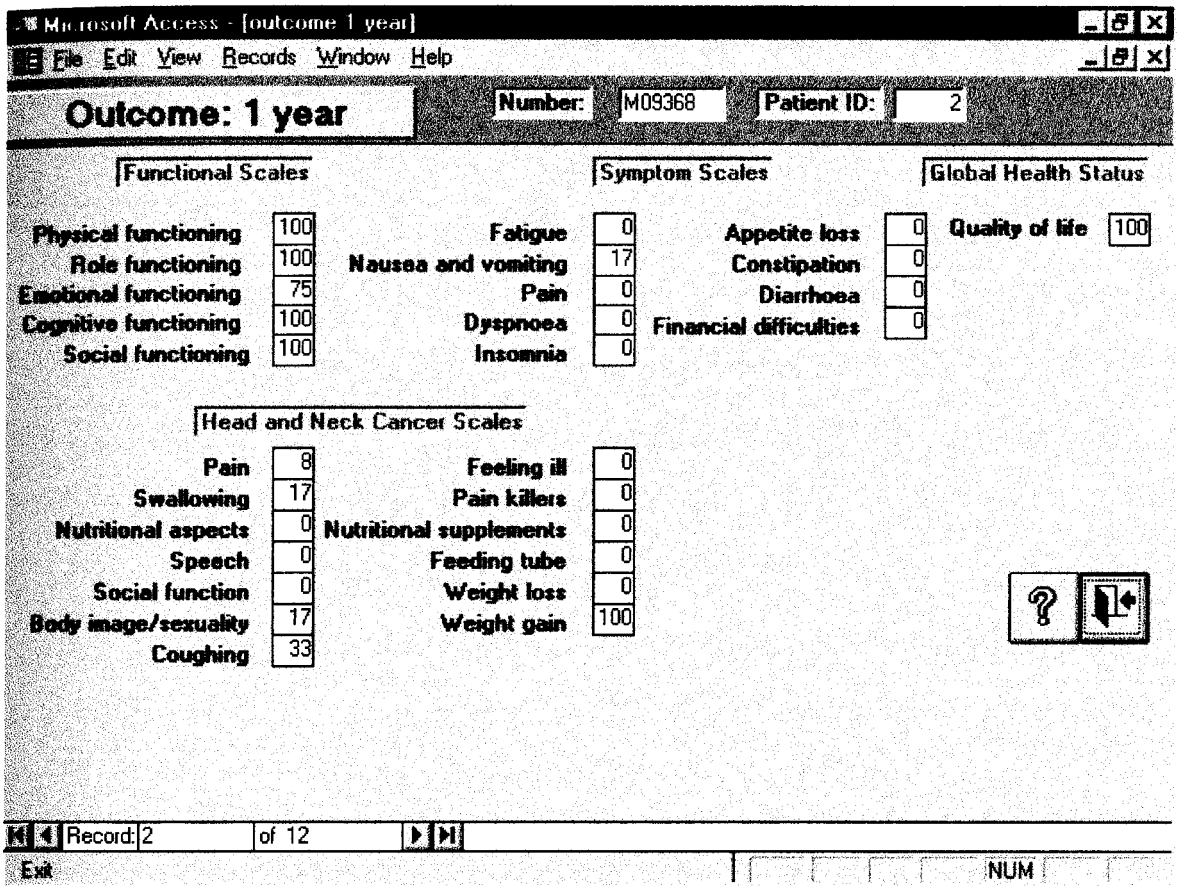
Figure 25



Complications Form.

Complication data **is** entered into this form from the Patient Details form and can be read from the Reports Menu. Data entered here can be used for compiling individual and unit morbidity statistics and as a tool for auditing treatment complications.

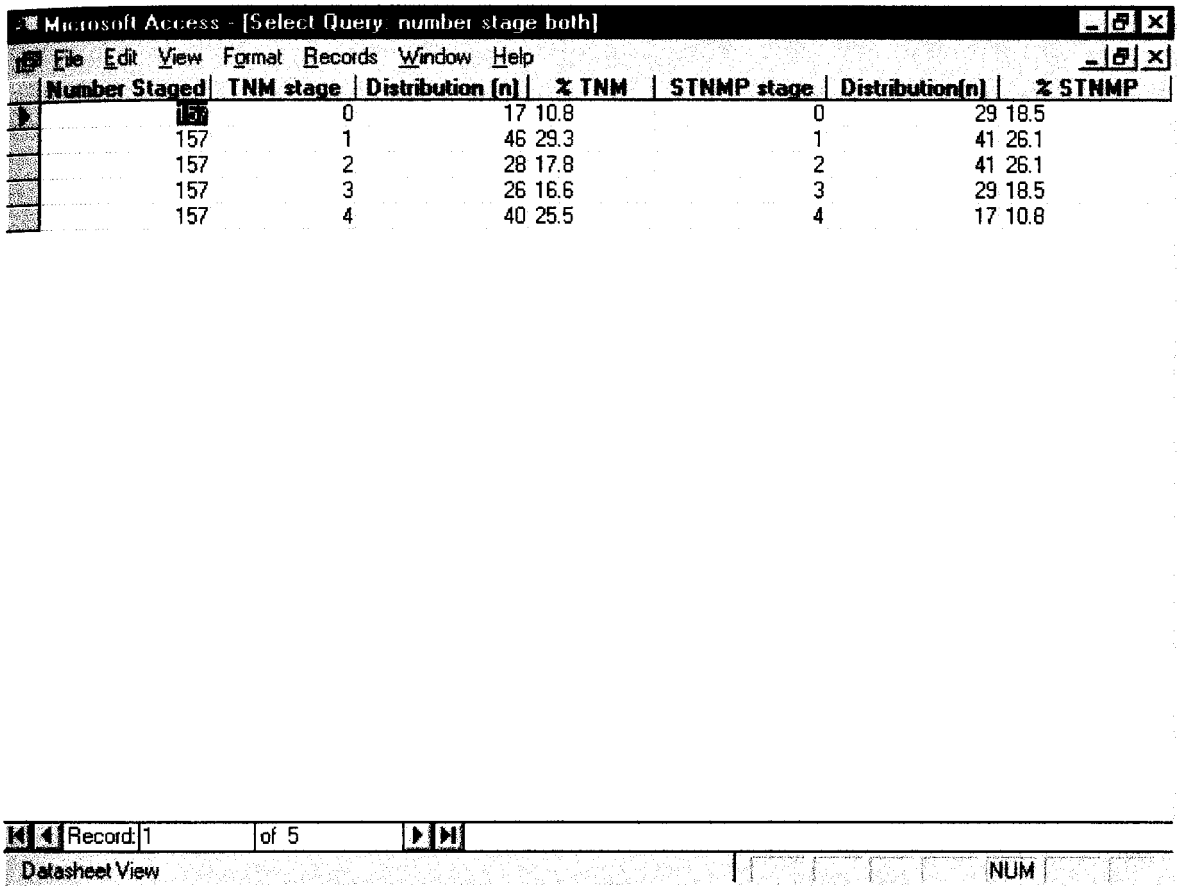
Figure 26



1 Year outcome Form.

Quality of life data **is** assessed using the EORTC QLQ-C30 and H&N 35 questionnaires. This form shows the EORTC **data** at 1 year post treatment.

Figure 27



TNM and STNMP Stages at Presentation.

This is an example of one of the many pre-set database queries available. Here, the tumour stages and distribution of all patients at presentation is calculated. This data is extremely useful for comparing patient populations between different units.

2.2 Patients and Samples

From January 1st 1995 and following the approval of the local research and ethics committee, all patients attending the head and neck oncology clinic at the North Staffordshire Hospital, Stoke-on-Trent, UK with a histologically proven diagnosis of oral squamous cell carcinoma (ICD 9 codes 140 – 141 and 143 - 145 (WHO, 1977; Johnson *et al.*, 1993) were eligible for recruitment into the study. As a result of locally agreed management protocols all patients with oral carcinoma in North Staffordshire (population – 480,000) presenting for treatment are managed *via* this clinic. Each patient is fully assessed on a joint clinic following initial histological diagnosis prior to commencing treatment and at frequent intervals post-treatment until disease-free for a minimum of 5 years or death. Data including tobacco and alcohol consumption history, tumour stage at presentation, tumour differentiation at presentation, treatment, primary site recurrence, cervical lymph node metastases, systemic metastases and survival times were recorded for each patient and entered onto a standard proforma and subsequently onto the computer database.

Non-smokers and non-drinkers had never smoked or consumed alcohol respectively. Tobacco consumption was assessed in number of cigarettes smoked per day or ounces of tobacco smoked per week and alcohol consumption was assessed as the number of alcohol units consumed per week. One unit = 10ml of alcohol and equates to ½ pint beer, one glass of wine or a single measure of spirits. Ever drinkers and smokers had done so for a minimum of 15 years (mean 47 years). Patients were classified as heavy drinkers and smokers if they consumed more than 20 units of alcohol/week and over 10 cigarettes/day or over 4 **oz** tobacco/week.

All tumours were staged according to the TNM system (UICC,1987). Patients with carcinoma *in situ* or severe epithelial dysplasia were staged as TNM 0. Local recurrence, nodal metastases and survival times were taken from the date of initial tumour diagnosis.

All patients were treated according to agreed protocols. For patients undergoing surgery with curative intent, if microscopic resection margins were anywhere less than 5mm postoperative external beam adjuvant radiotherapy was delivered to the primary site. Where more than 2 cervical lymph nodes contained deposits of metastatic squamous cell carcinoma or there was histological evidence of extracapsular spread of disease in a single cervical lymph node, the neck was treated with external beam adjuvant radiotherapy. Patients given external beam radiotherapy with curative intent as their primary treatment modality received 5500 cGy predominantly in 20 fractions over **28** days unless side effects forced a treatment rest.

Between January 1st 1995 and July 1st 1997, 159 patients were eligible for study entry. Thirty-seven patients either declined their consent to participate or were inadvertently missed. Seventy-four patients were entered on initial diagnosis and **48** patients were entered post-treatment. From January 1st 1997 patients were also recruited from The Leeds Dental Institute, Leeds, UK as part of a multi-centre collaboration. Approval for this arm of the study was obtained from the United Leeds Teaching Hospitals Research and Ethics Committee. The same patient proformas and computer database were used at both centres. An earlier version of the current database was developed and trialed in Leeds while I was a senior registrar to monitor patients with oral cancer attending the Leeds Dental Institute (Worrall and Corrigan,1995).

A previous study (Webster and Worrall, 1995) compared the demography, management, tumour stage, and outcome of patients in the two centres The male:female

ratios were 1.7:1 and 2:1 with mean ages at first presentation of 64.3 years and 63.7 years in the Leeds and Stoke groups respectively. Further data on the two groups is shown in tables 18 - 21.

Table 18

	Leeds	Stoke
Lip	3.5%	3.2%
Tongue	29.3%	30.2%
Alveolus	19.8%	9.5%
Floor of mouth	30.2%	22.2%
Other	17.2%	32%

Comparison of the tumour sites at presentation between patients with oral squamous cell carcinoma in Leeds (n = 116) and Stoke (n = 63).

Table 19

	Leeds	Stoke
0	0	3.1%
1	32.7%	32.8%
2	31.9%	14.1%
3	12.9%	25%
4	22.4%	25%

Comparison of the tumour TNM stages at presentation between patients with oral squamous cell carcinoma in Leeds (n = 116) and Stoke (n = 63).

Table 20

	Leeds	Stoke
Surgery only	32.7%	33.3%
Radiotherapy only	13.7%	14.3%
Surgery + radiotherapy	50%	41.9%
Radiotherapy + chemotherapy	0.9%	9.5%
Chemotherapy only	0.9%	1%

Comparison of the treatments received between patients with oral squamous cell carcinoma in Leeds (n = 116) and Stoke (n = 63).

Table 21

	Leeds	Stoke
Primary site	403	403
Cervical lymph nodes	299	376

Comparison of the mean recurrence delays at the primary site and in the cervical lymph nodes between patients with oral squamous cell carcinoma in Leeds (n = 116) and Stoke (n = 63).
Figures are days from initial diagnosis to first recurrence

The similarity of the Leeds and Stoke cancer patients was felt to be sufficient to justify amalgamating them into a single study sample. Twenty-one Leeds patients all with newly diagnosed oral carcinomas were eligible for inclusion. Complete clinical data sufficient for study entry was available for 95 Stoke and 11 Leeds patients. There were thus 106 patients (100% white Caucasians, mean age 63 years) on whom full clinical data was available enabling complete data analysis. All patients were unrelated except 2 who were father and son. There were 65 males (mean age 61 years) and 41 females (mean age 67 years). The median follow-up time was 1.7 years (mean 2.7 years, range 27 days – 9.3 years).

As a result of extensive previous work involving detoxification enzyme polymorphisms, Professor Strange's laboratory at the North Staffordshire Hospital has recruited a large patient control group, which has been used extensively in previous research projects and is being continually expanded (Heagerty *et* al.,1994; Warwick *et* al.,1994a; Warwick *et* al.,1994b; Elexpuru-Camiruaga *et* al.,1995; Lear *et* al.,1996. Detoxification data were available for over 700 patients attending the North Staffordshire Hospital for the treatment of non-malignant conditions. North Staffordshire has previously

been noted for its stable population base and low rate of immigration and emigration into the area (Mackay and Cole, 1984).

Following obtaining informed patient consent and preparation of a suitable venepuncture site with an alcohol-impregnated swab, 5ml of venous blood was withdrawn into a glass blood collection tube containing EDTA as an anticoagulant. The tubes were then placed in individual 50ml plastic centrifuge tubes (Falcon) and kept at -20° C until required. The samples from the Leeds patients were collected personally and transported on *dry* ice to the laboratory at the North Staffordshire Hospital where all subsequent investigations and data analysis was performed.

After initial data analysis in July 1997, evidence emerged for a possible association between *CYP2D6* polymorphisms and oral cancer susceptibility, implying a role for CYP2D6 metabolism in the oral mucosa. However, it was difficult to reconcile these findings with the knowledge that CYP2D6 was held to be primarily an hepatic enzyme **(Ingleman-Sundberg, 1995)** and previous workers had failed to find evidence of CYP2D6 expression in normal human gingiva (Zhou *et al.*, 1996). Therefore, evidence of CYP2D6 enzyme activity in specimens of normal oral mucosa was sought using immunohistochemistry.

Specimens of normal oral mucosa for immunohistochemical study were obtained from surgical waste generated by patients undergoing surgical procedures for non-malignant oral conditions at the North Staffordshire Hospital. Tissue samples were fixed in 10% phosphate buffered formalin and processed to paraffin wax (Hilley ***et al.***, 1988).

Statistical analysis

This is an exploratory project in which the genes studied were selected because of their presumed biological activities rather than for any statistical considerations. The results presented are considered therefore, as hypothesis setting and needing confirmation in further independent studies. Any possible multiple testing influences are thus felt to be negligble.

Data were manipulated and analysed using a personal computer running Access 2 ™ (Microsoft), Stata 5 ™ (Stata Corporation), Excel 7 ™ (Microsoft), and Kwikstat 4 ™ (TexaSoft).

χ^2 analysis was used to examine for detoxification enzyme allele homogeneity between cases and controls and between various sub-groups within the case group. Yates' χ^2 was used where appropriate.

Cox proportional hazards regression analysis was used to determine which factors, both alone and in combination, significantly influenced oral cancer outcome (Christensen, **1987).**

The log-rank test was used in preference to the Cox proportional hazards model where there were more than 2 data items within a group and the lowest coded value could not reliably be predicted to carry the lowest hazard. Hence, the log-rank test was used to analyse the GSTM1 data set because it contained 4 variables and the lowest coded item (1) represented the *GSTM1* ***null*** allele, which could not reliably be predicted to have the lowest hazard. In contrast, the log-rank test was not necessary in order to analyse the TNM stage data set even though it contained 5 data items, because the lowest coded value (0), which represented TNM stage 0 (carcinoma ***in-situ)*** could be reasonably expected to carry the lowest hazard.

2.3 Health and Safety Considerations

The extraction of genomic DNA from patient's blood samples and the subsequent electrophoretic analysis of specific PCR products has numerous health and safety implications. Before undertaking any supervised or unsupervised laboratory bench work I received thorough instruction on the relevant Health and Safety guidelines from the Head of Department, Professor R Strange. There are several important issues relating to materials and methods used during the study:

- Organic solvents. Phenol is toxic by ingestion, inhalation and skin contact. It can cause burns and is carcinogenic and mutagenic. Chloroform is toxic by ingestion, inhalation and skin contact. It causes skin and eye irritation, drowsiness or unconsciousness and may be carcinogenic. Whenever Phenol or Chloroform was used they were handled in a class-2 safety cabinet and I always wore rubber gloves and eye protection
- Ethidium bromide is a mutagen and carcinogen. This material was always handled with extreme care in a class-2 safety cabinet while wearing rubber gloves and eye protection
- Ultraviolet (UV) radiation from the transilluminator used to observe the DNA fragments in agarose gels is carcinogenic and can cause skin carcinomas and blindness. Whenever the transilluminator was used UV safe protective face shields and eye protection was used.

In addition to the above specific safety measures, standard laboratory Health & Safety policies such as no mouth pipetting and the wearing of protective laboratory coats and gloves at all times were strictly adhered to.

Throughout the remainder of this chapter I have given extensive details of all of the laboratory methodologies used in order to enable readers to repeat the procedures described if desired.

2.4 DNA Extraction

DNA was extracted from patient's whole blood samples by the phenol/chloroform method. Blood samples were transferred to a class-2 safety cabinet from storage at -20°C and allowed to thaw. Thawing always took place with the glass blood collecting tube still sealed within its original Falcon Hepatitis-risk protective tube as glass fracture is not an uncommon event during thawing. After complete thawing the blood samples were subsequently transferred to new, labelled Falcon tubes. Stock cold lysis buffer (0.32M sucrose, 10mM Tris-HC1 pH 7.5, 5 mM magnesium chloride and 1% (wh) Triton-X100) was then added to a total volume of 35ml and the contents mixed by inversion. Samples were then centrifuged at 2000rpm for 10 minutes. The supernatant was then immediately decanted into 10% bleach and discarded, taking care to leave the leukocyte nuclei in the bottom of the Falcon tube. The nuclear pellet was then re-suspended and vortexed in cold SE buffer (0.75M sodium chloride containing 0.024M disodiumethylene diamine tetraacetic acid pH 8.0). 0.17ml of 5% (w/v) sodium dodecyl sulphate (SDS) containing 2mg/ml of proteinase K were then added and the reaction mixture left to incubate overnight at 37° C. Using individual Pasteur pipettes each sample was transferred to a separate, labelled 10ml screw-top glass tube containing 1.5ml of phenol containing 0. 1% (wh) 8-hydroxyquinolone saturated with 20mM Tris-HC1 pH *8.0).* The caps were screwed on firmly and each glass tube placed

in a Falcon tube with screw cap and centrifuged at 2000 rpm for 10 minutes. After centrifuging, the viscous aqueous top layer of the reaction mixture was carefully aspirated with a Pasteur pipette and transferred into **a** labelled 10 ml glass screw-top tube to which was added 1.5ml of ch1oroform:isoamyl alcohol (24:1). The tube caps were screwed on firmly and each glass tube placed in a Falcon tube with screw cap and centrifuged at 2000 rpm for 5 minutes. The viscous aqueous top layer of the reaction mixture was again pipetted off into a 10 ml glass screw-top tube and the chloroform:isoamyl alcohol extraction repeated. The viscous aqueous top layer of the reaction mixture was pipetted into a final labelled glass 10ml screw-top tube to which was added 0. 15ml of 3M sodium acetate pH 5.2 and 3.3ml of ethanol. Following gentle mixing by inversion the **DNA** precipitated out and was gently removed from the reaction vessel using a glass hook. The **DNA** was transferred to a labelled 0.5ml plastic Eppendorf tube and re-suspended in 0. 1ml of sterile water. Each individual patient's extracted **DNA** was then kept in these Eppendorf tubes in a 4°C fridge until required. All phenol and chloroform waste was carefully discarded into appropriate waste containers in a class-2 cabinet.

2.5 Determination of *GSTM1* Genotype

Polymorphism at the ***GSTM1*** locus is based on 3 alleles, ***GSTM1*** *0, ***GSTM1 *A*** and ***GSTM1****B*. ***GSTM1 *0*** is deleted resulting in individuals homozygous for this allele expressing no GSTM1 protein ***GSTM1 *A*** and ***GSTM1*** *B are identical apart from a single base pair difference in exon 7. Although individuals who are homozygous for the ***GSTM1*** *0 allele (GSTM1 null genotype) can be reliably identified by PCR because of their failure to amplie **for** example, the exon 4-5 region of the ***GSTM1*** gene, (Heagerty *et al.*, 1994) because the full extent of the ***GSTM1*** *0 deletion has yet to be determined it is difficult to distinguish the heterozygote genotypes ***GSTM1 *O/GSTM1****A* and ***GSTM1 *O/GSTM1****B* from the ***GSTM1****A* and ***GSTM1 *B*** homozygous genotypes respectively (Strange and Fryer, 1997).

In order to fully describe the ***GSTM1*** genotype for patients in this study the amplification refractory mutation system (ARMS), described by Fryer *et al.*, 1993 was used. The technique uses primers specific to exon 4-5, intron 6 and ***GSTM1 *A*** and ***GSTM1 *B*** on exon ***7.*** ß-globin primers were used as an internal control. The normal ***GSTM1*** *A and ***GSTM1*** *B primer sequences were identical apart from an A-G substitution at the 3' end of the ***GSTM1 *A*** primer which both improved primer binding specificity and also introduced an HaeII restriction enzyme cutting site (Fryer *et al.*, 1993). The Exon 7 A and B specific primers differed only in their 3' terminal bases (Figure ***28).*** Thus the ***GSTM1*** *null* individuals would amplie the control ß-globin DNA but not the target DNA and the ***GSTM1 *A/GSTM1****A* and ***GSTM1 *B/GSTM1****B* individuals would be distinguished from the ***GSTM1 *A/GSTM1****B* individuals.

Figure 28

Primer	Sequence
Exon 4 forward	5'-CTGCCCTACTTGATTGATGGG-3'
Exon 5 reverse	5'-CTGGATTGTAGCAGATCATGC-3'
Intron 6 forward	5'-GCTTCACGTGTTATGAAGGTTC-3'
Exon 7 (A-specific)	5'-TTGGGAAGGCGTCCAAGC**G**C-3'
Normal *GSTM1*A* sequence for comparison	5'-TTGGGAAGGCGTCCAAGC**AC**-3'
Exon 7 (B-specific)	5'-TTGGGAAGGCGTCCAAGCA**G**-3'
B-globin (PC03)	5'-ACACAACTGTGTTCACTAGC-3'
B-globin (PC04)	5'-CAACTTCATCCACGTTCACC-3'

Primer sequences used in the ARMS method to determine *GSTMI* genotype highlighting the differences at the 3' ends of the normal *GSTMI *A* sequence, A specific exon 7 primer and B specific exon 7 primer.

PCR

For each patient, 2 new 0.5ml plastic microcentrifuge tubes were labelled "A' and "B". Two 0.5 ml microcentrifuge tubes were labelled as "Positive A" and "Positive B" and a further 2 0.5ml microcentrifuge tubes were labelled as "Negative A" and "Negative B" These 4 tubes were used for the positive and negative controls respectively. Two 1.5ml microcentrifuge tubes were labelled as **"A** stock" and "B stock". Stock solutions were made up and aliquoted into the appropriate stock tubes as detailed below.

Component	Volume A stock	Volume B stock
Sterile water	216µl	216µl
10x Taq buffer (including Mg^{++})	100µl	100ml
Exon 4/5 primers (2x5µM)	200µl	200µl
Intron 6 primer (5µM)	100µl	100µl
A-specific primer (5µM)	100µl	-
B-specific primer (5µM)	-	100µl
β-globin primer (PC03,5µM)	100µl	100µl
β-globin primer (PC04,5µM)	100µl	100µl
dNTP's (4x5µM)	40µl	40µl
Taq polymerase	4µl	4µl

After mixing each stock tube by inversion 48µl of A stock were aliquoted into each of the 0.5ml "A'tubes and 48µl of B stock were aliquoted into each of the 0.5ml "B" tubes. 1.5µl of sterile water were aliquoted into both of the negative control tubes and 1.5µl of known *GSTM1 *A* and *GSTM2 *B* DNA were aliquoted into the positive **A** control and positive B control tubes respectively. 1.5µl of each patient's test DNA were aliquoted into the "A" and "B" 0.5ml microcentrifuge tubes. Two drops (50µl) of light paraffin oil were added to each tube to prevent evaporation, which were then centrifuged at 12,000 rpm for 20 seconds. All tubes were then placed randomly in a thermal cycler (Omnigene, Hybaid Ltd) which was pre-programmed to deliver the following PCR cycle parameters:

Temperature (^{0}C)	Time (seconds)	Number of cycles	Effect
94	150	1	Initial denaturation
94	45	5	Denaturation
60	60	5	Primer annealing
72	60	5	Primer extension
94	30	30	Denaturation
58	30	30	Primer annealing
72	45	30	Primer extension
40	5	1	Stop reaction

Agarose gel electrophoresis

Fresh agarose gels were poured for each batch of tests and the technique described below was used to make gels for all of the genotype determinations performed throughout this study. Using an electrical balance, 1.2 g of agarose were placed into a clean, sterile 50ml conical flask to which was added 3ml of 5x TBE buffer (54g Tris base, 27.58 Boric Acid, 20ml 0.5M EDTA pH 8.0 made up to a total volume of 800ml with distilled water) and **27** ml of sterile water. The flask neck was lightly plugged with tissue paper and heated in a microwave oven until the agarose was fully dissolved and a clear, precipitate

free solution produced. This technique required constant vigilance and patience in order to produce a gel of the required concentration (in this case **4%** w/v). Over-heating lead to excessive evaporation and/or boiling over and under-heating produced a poor quality gel laden with precipitate resulting in a gel with poor resolving characteristics. Once a satisfactory solution was produced the flask was cooled to 60°C under running water and transferred to a class-2 safety cabinet. Using full Health and Safety precautions, 1µl of ethidium bromide (10mg/ml) was added and the flask contents mixed by gentle agitation. The agarose was then poured into a pre-prepared plastic electrophoresis tray and allowed to set for 30 minutes.

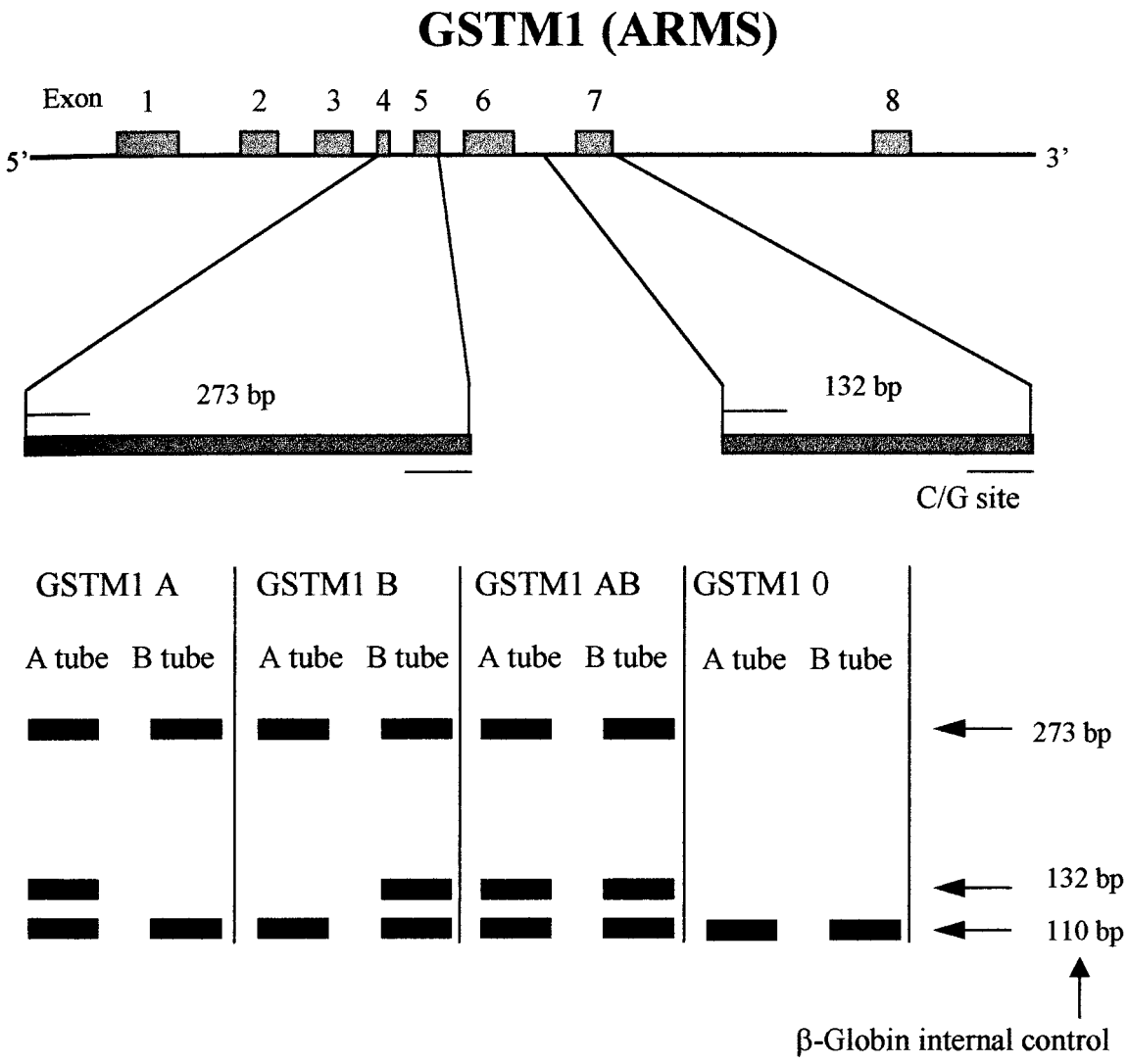
While the agarose gel was setting, 8µl of loading buffer (30mg of 0.15% w/v bromophenol blue, 30mg of 0.15% w/v xylene cyanole, 6ml of 30% w/v glycerol, 8ml 5x TBE, 6ml sterile water) were added to each of the thawed PCR tubes and all tubes centrifuged at 12,000 rpm for 20 seconds.

When set, the combs and blanking tape were removed from the agarose gel/tray which was placed in an electrophoresis tank and covered to a depth of 2mm with 0.5x TBE containing 5µl of ethidium bromide. 7µl of each sample (test **DNA,** positive and negative controls) were then loaded into separate wells on the gel and a note made of the orientation and location of each sample for future reference. The end well on each gel was loaded with 7µl of previously prepared molecular weight markers (1µl pBR322 HaeII1 digest [Sigma: approx. concentration 500-1000µg/ml], 10µl 0.5x TBE, 8µl loading buffer).

The electrophoresis tank lid was replaced and the gel run at 120 volts for 40 minutes. Slight adjustments to the running time were made on each "run" depending on the position of the leading blue bands. On completion of electrophoretic separation the gel

was carefully placed onto a *UV* transilluminator, and using full Health and Safety precautions photographed onto type 667 Polaroid film using a Wratten 23A filter at f4.7 for ¼ of a second. A diagrammatic representation of the GSTM1 ARMS method is shown in Figure 29.

Figure 29



Diagrammatic representation of the expected banding pattern seen on agarose gel electrophoresis using the Amplification Refractory Mutation System for the determination of *GSTM1* genotype.

2.6 Determination of *GSTM3* Genotype

The 2 ***GSTM3*** alleles, ***GSTM3*A*** and ***GSTM3*B*** have been identified. They differ by a **3** base pair deletion in intron 6 of ***GSTM3****B.* PCR amplification across exons 6 and 7 followed by restriction enzyme digestion with Mnl 1 will identify individuals who are ***GSTM3*A/GSTM3*A, GSTM3*A/GSTM3*B*** or ***GSTM3*B/GSTM3*B.*** The Mnl 1 enzyme **digests** the 273 base pair PCR product of the ***GSTM3*A*** allele into ***3*** fragments of 137, 125 and 11 base pair fragments and the 270 base pair PCR product of the ***GSTM3****B* allele into 3 fragments of 134, 125 and 11 base pairs. The ***GSTM3*A*** allele 137 base pair fragment is subsequently digested by Mnl 1 into 2 fragments of 86 and 51 base pairs whereas the ***GSTM3*B*** allele 134 base pair fragment lacks an Mnl 1 restriction site and **is** not cut further. The primer sequences used for ***GSTM3*** determination are shown in Figure 30.

Figure 30

Primer	Sequence
Exon 6 forward	5'-CCTCAGTACTTGGAAGAGCT-5'
Exon 7 reverse	5'-CACATGAAAGCCTTCAGGTT-3'

Primers used to determine *GSTM3* genotype by amplification across exons 6 and 7

PCR

New 0.5ml plastic microcentrifuge tubes were labelled for each test DNA sample. one new 0.5ml microcentrifuge tubes was labelled **as** "Positive Control" and another was labelled as "Negative Control". A 1.5ml microcentrifuge tubes was labelled as "Stock" The stock solution was made up as detailed below:

Component	Volume
Sterile water	616µl
10x Taq buffer (including Mg^{++})	100µl
Exon 6 primer (5µM)	100µl
Exon 7 primer (5µM)	100µl
dNTP's (4x5µM)	40µl
Taq polymerase	4µl

After mixing the stock tube by inversion 48µl of stock solution were aliquoted into each of the 0.5ml tubes. 1.5 µl of sterile water were aliquoted into the negative control tube and 1.5 µl of known *GSTM3*A/GSTM3*A* DNA were aliquoted into the positive control tube. 1.5 µl of each patient's test DNA were aliquoted into the individually labelled 0.5ml microcentrifuge tubes. Two drops (50µl) of light paraffin oil were added to each tube to prevent evaporation, which were then centrifuged at 12,000 rpm for 20 seconds. All tubes were then placed randomly in a thermal cycler (Omnigene, Hybaid Ltd) which was pre-programmed to deliver the following PCR cycle parameters:

Temperature (^{0}C)	Time (seconds)	Number of cycles	Effect
94	180	1	Initial denaturation
94	45	35	Denaturation
58	30	35	Primer annealing
72	45	35	Primer extension
94	45	1	Denaturation
58	30	1	Primer annealing
72	480	1	Primer extension

Mnl 1 Restriction Enzyme Digestion

A 0.5ml microcentrifuge tube was labelled "Mnl 1 Stock" to which was added the following components:

Component	Volume
Sterile water	160µl
Mnl 1 10x restriction enzyme buffer	40µl
Mnl 1 restriction enzyme	5µl
Bovine Serum Albumin (BSA)	8µl

New 0.5µl microcentrifuge tubes were labelled for each patient and the positive control. Ten microlitres of the relevant PCR product and 10µl of Mnl 1 stock were aliquoted into each microcentrifuge tube which were then centrifuged at 12,000 rpm for 20 seconds and subsequently incubated at 37°C for 18 hours.

Agarose gel electrophoresis

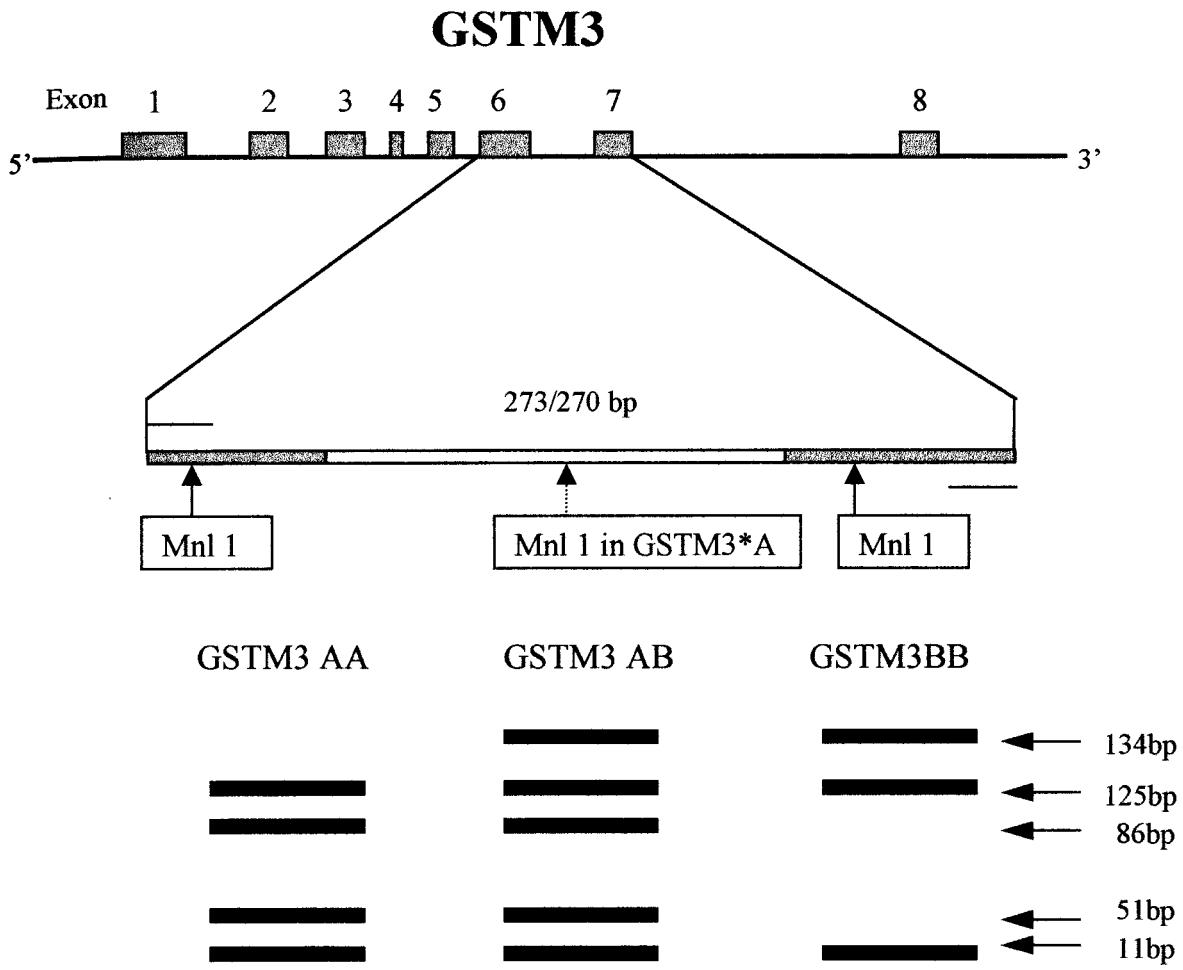
A 4% agarose gel was mixed and poured according to the protocol previously described for *GSTMI* electrophoresis. Two microlitres of loading buffer (30mg of 0.15% w/v bromophenol blue, 30mg of 0.15% w/v xylene cyanole, 6ml of 30% w/v glycerol, 8ml 5x TBE, 6ml sterile water) were aliquoted into each of the test microcentrifuge tubes (8µl into the negative control) and all tubes centrifuged at 12,000 rpm for 20 seconds.

When set, the combs and blanking tape were removed from the agarose gel/tray which was placed in an electrophoresis tank and covered to a depth of 2mm with 0.5x TBE containing 5µl of ethidium bromide. Seven microlitres of each sample (test DNA, positive and negative controls) were then loaded into separate wells on the gel and a note made of the orientation and location of each sample for future reference. The end well on each gel was loaded with 7µl of previously prepared molecular weight markers (1µl pBR322

Haell1 digest [Sigma: approx. concentration 500-1000μg/ml], 10μl 0.5x TBE, 8μl loading buffer).

The electrophoresis tank lid was replaced and the gel run at 120 volts for 45 minutes. Slight adjustments to the running time were made on each "run" depending on the position of the leading blue bands. On completion of electrophoretic separation the gel was carefully placed onto a UV transilluminator, and using full Health and Safety precautions photographed onto type 667 Polaroid film using a Wratten 23A filter at f4.7 for ¼ of a second. A diagrammatic representation of the expected banding pattern for *GSTM3* following Mnl 1 digestion is shown in Figure 31.

Figure 31



Diagrammatic representation of the expected banding pattern seen on agarose gel electrophoresis for *GSTM3* following Mnl 1 digestion.

2.7 Determination of *GSTP1* Genotype

The *GSTP1*B* allele differs from the *GSTP1*A* allele as a consequence of the exon 5 A-G transition at position 1578 in GSTP1*B. The two alleles can be identified following digestion with the restriction enzyme Alw 261. Following PCR with exon 5 specific primers (Figure 32) to produce a 176 base pair DNA fragment, *GSTP1*A* is resistant to digestion by Alw 261 whereas *GSTP1*B* is digested to produce 2 DNA fragments of 91 and 85 base pairs.

Figure 32

Primer	Sequence
Exon 5 forward	5'-ACCCCAGGGCTCTATGGGAA-3'
Exon 5 reverse	5'-TGAGGGCACAAGAAGCCCCT-3'

Primers used to determine *GSTP1* genotype by amplification across exon 5.

PCR

New 0.5ml plastic microcentrifuge tubes were labelled for each test **DNA** sample. One new 0.5ml microcentrifuge tubes was labelled as "Positive Control" and another was labelled as "Negative Control". A 1.5ml microcentrifuge tubes was labelled as "Stock" The stock solution was made up as detailed below:

Component	Volume
Sterile water	616µl
10x Taq buffer (including Mg^{++})	100µl
Exon 5 forward primer (5µM)	100µl
Exon 5 reverse primer (5µM)	100µl
dNTP's (4x5µM)	40µl
Taq polymerase	4µl

After mixing the stock tube by inversion 48μl of stock solution were aliquoted into each of the 0.5ml tubes. 1.5μl of sterile water were aliquoted into the negative control tube and 1.5μl of known *GSTP1*B/GSTP1*B* DNA were aliquoted into the positive control tube. 1.5μl of each patient's test DNA were aliquoted into the individually labelled 0.5ml microcentrifuge tubes. Two drops (50μl) of light paraffin oil were added to each tube to prevent evaporation, which were then centrifuged at 12,000 rpm for 20 seconds. All tubes were then placed randomly in a thermal cycler (Omnigene, Hybaid Ltd) which was pre-programmed to deliver the following PCR cycle parameters:

Temperature (^{0}C)	Time (seconds)	Number of cycles	Effect
94	300	1	Initial denaturation
94	30	30	Denaturation
55	30	30	Primer annealing
72	30	30	Primer extension
94	30	1	Denaturation
55	30	1	Primer annealing
72	300	1	Primer extension

Awl 261 restriction enzyme digestion

A 0.5ml microcentrifuge tube was labelled "Awl 261 Stock" to which was added the following components:

Component	Volume
Sterile water	80μl
Awl 26110x restriction enzyme buffer	20μl
Awl 261 restriction enzyme	5μl
Bovine Serum Albumin (BSA)	10μl

New 0.5μl microcentrifuge tubes were labelled for each patient and the positive control. Ten microlitres of the relevant PCR product and 5μl of Awl 261 stock were aliquoted into each microcentrifuge tube which were then centrifuged at 12,000 rpm for 20 seconds and subsequently incubated at 37°C for 18 hours.

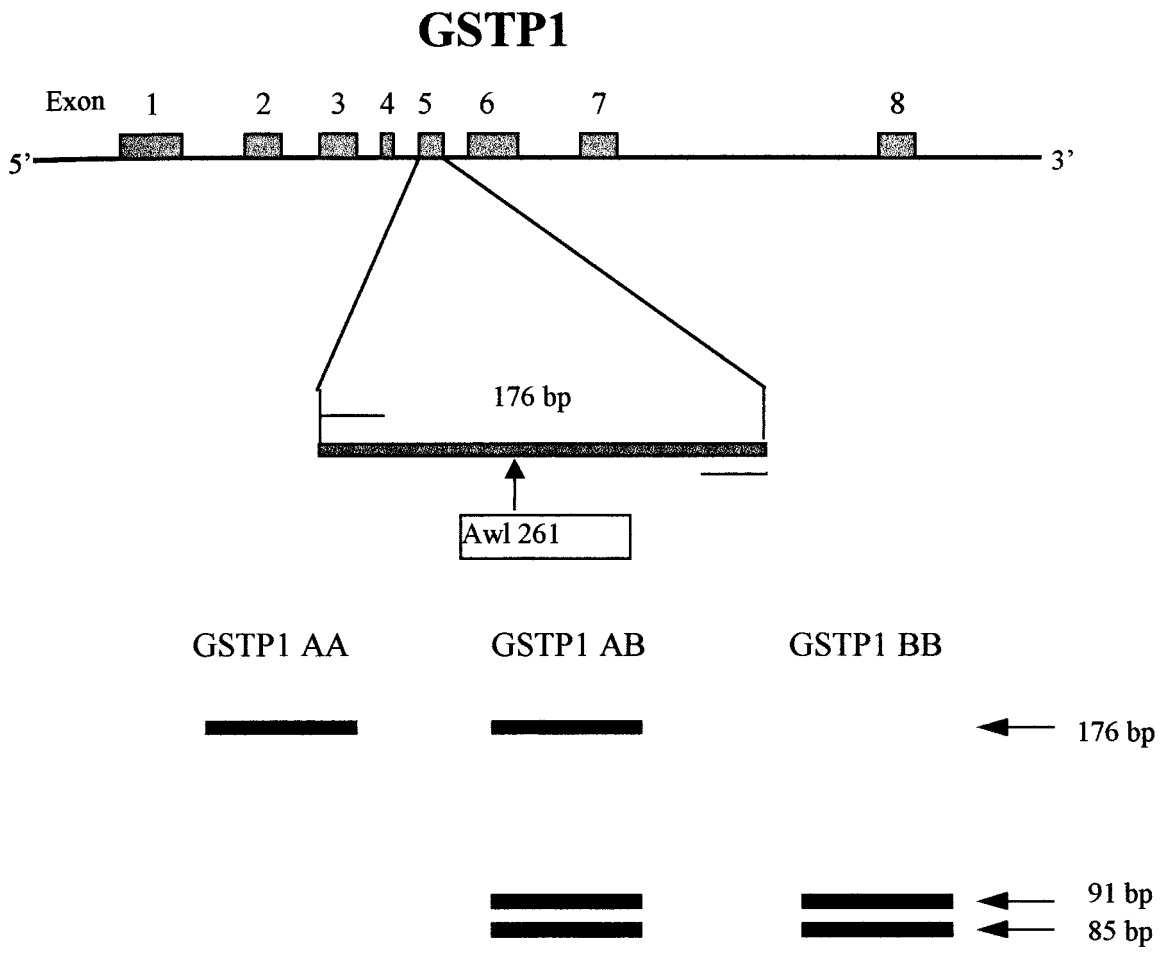
Agarose gel electrophoresis

A 4% agarose gel was mixed and poured according to the protocol previously described for *GSTMI* electrophoresis. Two microlitres of loading buffer (30mg of 0.15% w/v bromophenol blue, 30mg of 0.15% w/v xylene cyanole, 6ml of 30% w/v glycerol, 8ml 5x TBE, 6ml sterile water) were aliquoted into each of the test microcentrifuge tubes (8µl into the negative control) and all tubes centrifuged at 12,000 rpm for 20 seconds.

When set, the combs and blanking tape were removed from the agarose gel/tray which was placed in an electrophoresis tank and covered to a depth of 2mm with 0.5x TBE containing 5µl of ethidium bromide. Seven microlitres of each sample (test DNA, positive and negative controls) were then loaded into separate wells on the gel and a note made of the orientation and location of each sample for future reference. The end well on each gel was loaded with 7µl of previously prepared molecular weight markers (1µl pBR322 HaeII1 digest [Sigma: approx. concentration 500-1000µg/ml], 10µl 0.5x TBE, 8µl loading buffer).

The electrophoresis tank lid was replaced and the gel run at 120 volts for 45 minutes. Slight adjustments to the running time were made on each "run" depending on the position of the leading blue bands. On completion of electrophoretic separation the gel was carefully placed onto a *UV* transilluminator, and using full Health and Safety precautions photographed onto type 667 Polaroid film using a Wratten 23A filter at f4.7 for ¼ of a second. A diagrammatic representation of the expected banding pattern for *GSTM3* following Awl 261 digestion is shown in Figure 33.

Figure 33



Diagrammatic representation of the expected banding pattern seen on agarose gel electrophoresis for *GSTP1* following Awl 261 digestion.

2.8 Determination of *GSTT1* Genotype

A *null* polymorphism resulting from homozygosity for *GSTT1*0* has been identified at the *GSTT1* locus (Pemble ***et al.,*** 1994). PCR amplification using the specific primers (Figure 34) described by Pemble ***et al.*** (1994) distinguishes individuals with at least one copy of the wild type allele *(GSTT1*A)* from those with the *null* polymorphism by the production of a 480 base pair DNA fragment in the latter but not the former.

Figure 34

Primer	Sequence
Forward	5'-TTCCTTACTGGTCCTCACATCTC-3'
Reverse	5'-TCACCGGATCATGGCCAGCA-3'

Primers used to determine *GSTT1* genotype.

PCR

New 0.5ml plastic microcentrifuge tubes were labelled for each test DNA sample. One new 0.5ml microcentrifuge tubes was labelled as "Positive Control" and another was labelled as "Negative Control". A 1.5ml microcentrifuge tubes was labelled as "Stock" The stock solution was made up as detailed below:

Component	Volume
Sterile water	416µl
10x Taq buffer (including Mg^{++})	100µl
GSTT1 forward primer (5µM)	100µl
GSTT1 reverse primer (5µM)	100µl
β-globin primer (KM38,5µM)	100µl
β-globin primer (GH20, 5µM)	100µl
dNTP's (4x5µM)	40µl
Taq polymerase	4µl

After mixing the stock tube by inversion 48µl of stock solution were aliquoted into each of the 0.5ml tubes. 1.5µl of sterile water were aliquoted into the negative control tube and 1.5µl of known *GSTT1*A/GSTT1*A* DNA were aliquoted into the positive control tube. 1.5 µl of each patient's test DNA were aliquoted into the individually labelled 0.5ml microcentrifuge tubes. Two drops (50µl) of light paraffin oil were added to each tube to prevent evaporation, which were then centrifuged at 12,000 rpm for 20 seconds. All tubes were then placed randomly in a thermal cycler (Omnigene, Hybaid Ltd) which was pre-programmed to deliver the following PCR cycle parameters:

Temperature (^{0}C)	Time (seconds)	Number of cycles	Effect
94	150	1	Initial denaturation
94	60	39	Denaturation
58	60	39	Primer annealing
72	60	39	Primer extension
94	60	1	Denaturation
58	60	1	Primer annealing
72	450	1	Primer extension

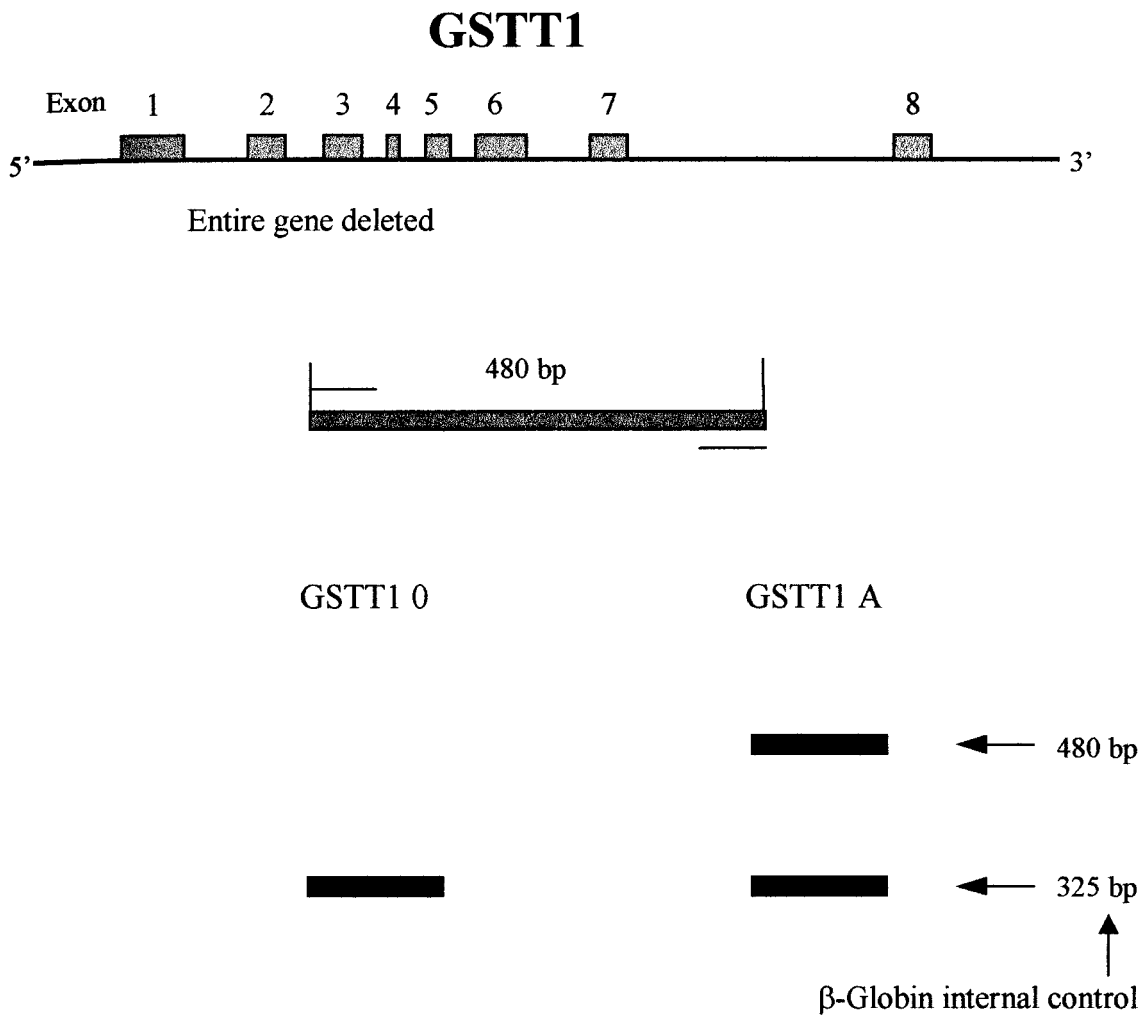
Agarose gel electrophoresis

A 2% agarose gel was mixed and poured according to the protocol previously described for *GSTMI* electrophoresis except that 0.6g agarose was used rather than 1.2g. Four microlitres of loading buffer (30mg of 0. 15% w/v bromophenol blue, 30mg of 0. 15% w/v xylene cyanole, 6ml of 30% w/v glycerol, 8ml 5x TBE, 6ml sterile water) were aliquoted into each of the test microcentrifuge tubes which were then centrifuged at 12,000 rpm for 20 seconds.

When set, the combs and blanking tape were removed from the agarose gel/tray which was placed in an electrophoresis tank and covered to a depth of 2mm with 0.5x TBE containing 5µl of ethidium bromide. Five microlitres of each sample (test DNA, positive and negative controls) were then loaded into separate wells on the gel and a note made of the orientation and location of each sample for future reference. The end well on each gel was loaded with 7µl of previously prepared molecular weight markers (1µl pBR322 HaeII1 digest [Sigma: approx. concentration 500-1000µg/ml], 10µl 0.5x TBE, 8µl loading buffer).

The electrophoresis tank lid was replaced and the gel run at 120 volts for 25 minutes. Slight adjustments to the running time were made on each "run" depending on the position of the leading blue bands. On completion of electrophoretic separation the gel was carefully placed onto a W transilluminator, and using full Health and Safety precautions photographed onto type 667 Polaroid film using a Wratten 23A filter at f4.7 for ¼ of a second. A diagrammatic representation of the expected banding pattern for *GSTM3* following Awl 261 digestion is shown in Figure 35.

Figure 35



Diagrammatic representation of the expected banding pattern seen on agarose gel electrophoresis for *GSTT1*.

2.9 Determination of *CYP2D6* Genotype

There are over 53 allelic variants of *CYP2D6* but only 3 are common (Marez *et al.*, **1997).** PCR methodology was used to identify the 2 commonest polymorphisms at the *CYP2D6* locus. These are termed *CYP2D6 A* and *CYP2D6 B.* With respect to both alleles individuals may be classified as extensive metabolisers (homozygous wild type), poor metabolisers (homozygous *null)* or heterozygotes. These genotypes are referred to as *EM,* PM and *HET* respectively. Because of their mode of inheritance and the possible allele combinations that result from it, specific combinations of *CYP2D6 A* and *CYP2D6 B* polymorphisms combine to produce an overall CYP2D6 phenotype (Figure 36).

Figure 36

***CYP2D6 A* genotype**	***CYP2D6 B* genotype**	**CYP2D6 phenotype**
EM	*EM*	EM
HET	*EM*	HET
EM	*HET*	HET
HET	*HET*	PM
PM	*HET*	PM
HET	*PM*	PM
PM	*PM*	PM

Determination of overall CYP2D6 phenotype by various combinations of *CYP2D6* A and B genotypes.

The *CYP2D6* **A** polymorphism results from a base pair deletion in exon 5. PCR across exon 5 and intron 5 using specific primers (Figure 37) yields a 270 base pair DNA fragment that in extensive metabolisers is cut by the restriction enzyme Hpa II into 2 fragments of 188 and 82 base pairs. The exon 5 base pair deletion introduces a second Hpa II restriction site such that in *CYP2D6* **A** *PM* individuals the 188 base pair fragment is further cut into 2 fragments of 168 and 20 base pairs.

The *CYP2D6* B polymorphism results from a point mutation at the intron 3 exon 4 boundary that produces a subsequent splice site defect. PCR across intron 3 and exon 4 using specific primers (Figure 38) yields a 334 base pair **DNA** fragment that in extensive metabolisers is cut by the restriction enzyme Bstnl into 2 fragments of 229 and 105 base pairs. The intron 3/exon 4 mutation results in loss of the normal Bstnl restriction site. Consequently *CYP2D6* B PM individuals will still have a single 334 base pair band following digestion with Bstnl .

Figure 37

Primer	Sequence
Exon 5 forward	5'-GATGAGCTGCTAACTGAGCCC-3'
Intron 5 reverse	5'-CCGAGAGCATACTCGGGAC-3'

Primer sequences used to identify *CYP2D6* A polymorphisms

Figure 38

Primer	Sequence
Exon 3 forward	5'-GCCTTCGCCAACCACTCCG-3'
Intron 4 reverse	5'-AAATCCTGCTCTTCCGAGGC-3'

Primer sequences used to identify *CYP2D6* B polymorphisms

CYP2D6 A PCR

New 0.5ml plastic microcentrifuge tubes were labelled for each test DNA sample. One new 0.5ml microcentrifuge tubes was labelled as "Positive Control" and another was labelled as "Negative Control". **A** 1.5ml microcentrifuge tubes was labelled as "Stock" The stock solution was made up as detailed below:

Component	Volume
Sterile water	616µl
10x Taq buffer (including Mg^{++})	100µl
Exon 5 forward primer (5µM)	100µl
Intron 5 reverse primer (5µM)	100µl
dNTP's (4x5µM)	40µl
Taq polymerase	4µl

After mixing the stock tube by inversion 48µl of stock solution were aliquoted into each of the 0.5ml tubes. 1.5 µl of sterile water were aliquoted into the negative control tube and 1.5 µl of known *CYP2D6* A PMDNA were aliquoted into the positive control tube. 1.5 µl of each patient's test DNA were aliquoted into the individually labelled 0.5ml microcentrifuge tubes. Two drops (50µl) of light paraffin oil were added to each tube to prevent evaporation, which were then centrifuged at 12,000 rpm for 20 seconds. All tubes were then placed randomly in a thermal cycler (Omnigene, Hybaid Ltd) which was pre-programmed to deliver the following PCR cycle parameters:

Temperature (^{0}C)	Time (seconds)	Number of cycles	Effect
94	180	1	Initial denaturation
94	30	34	Denaturation
60	60	34	Primer annealing
72	60	34	Primer extension
94	30	1	Denaturation
60	60	1	Primer annealing
72	480	1	Primer extension

HPA II restriction enzyme digestion

A 0.5ml microcentrifuge tube was labelled "Hpa II Stock" to which was added the following components:

Component	Volume
Sterile water	160µl
Hpa II 10x restriction enzyme buffer	40µl
Hpa II restriction enzyme	5µl

New 0.5µl microcentrifuge tubes were labelled for each patient and the positive control. Ten microlitres of the relevant PCR product and 10µl of Hpa II stock were aliquoted into each microcentrifuge tube, which were then centrifuged at 12,000 rpm for 20 seconds and subsequently incubated at 37°C for 18 hours.

Agarose Gel Electrophoresis

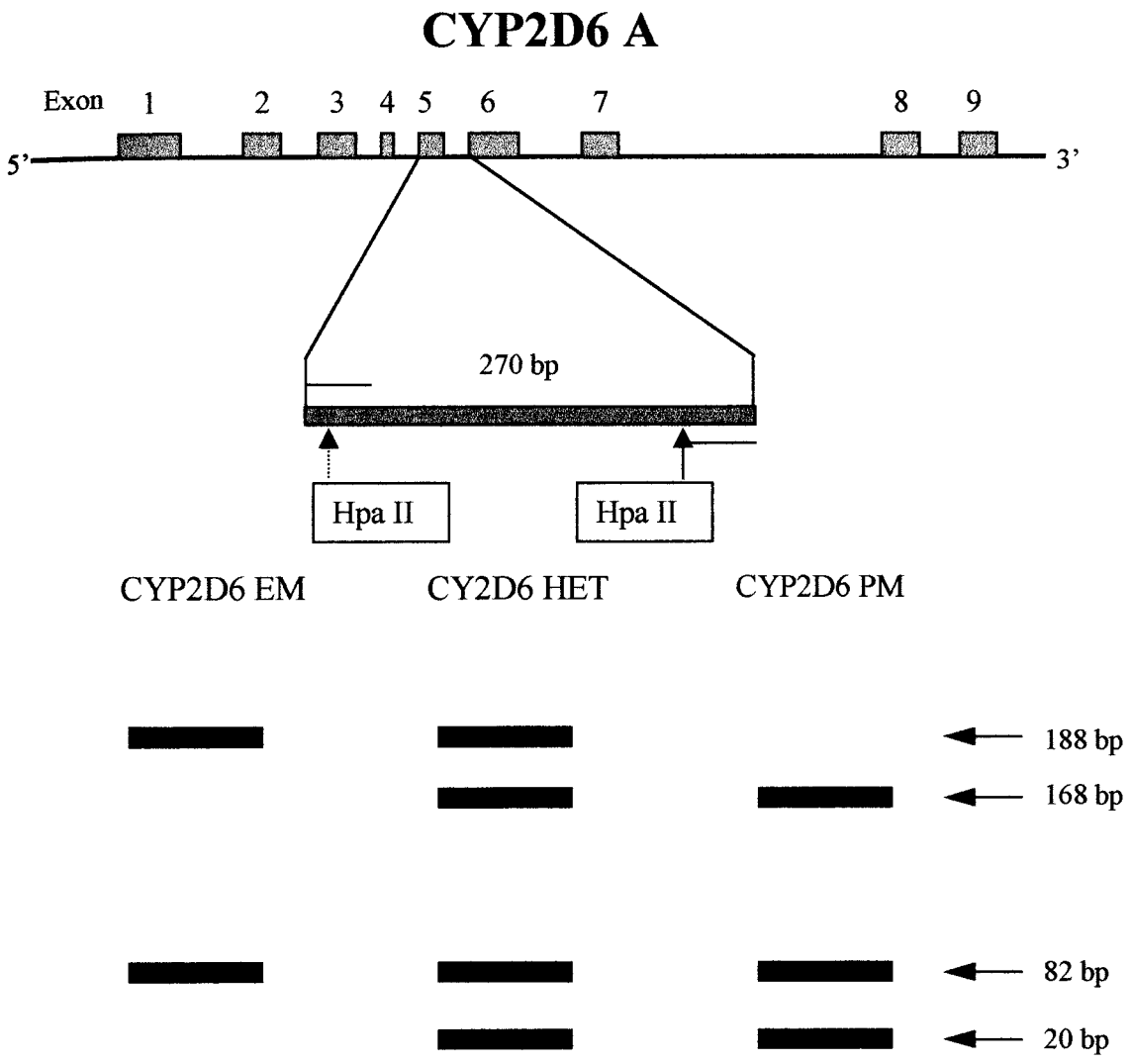
A **4%** agarose gel was mixed and poured according to the protocol previously described for *GSTMZ* electrophoresis. Eight microlitres of loading buffer (30mg of 0.15% w/v bromophenol blue, 30mg of 0.15% w/v xylene cyanole, 6ml of 30% w/v glycerol, 8ml 5x TBE, 6ml sterile water) were aliquoted into each of the test microcentrifuge tubes (20pl into the negative control) and all tubes centrifuged at 12,000 rpm for 20 seconds.

When set, the combs and blanking tape were removed from the agarose gel/tray which was placed in an electrophoresis tank and covered to a depth of 2mm with 0.5x TBE containing 5µl of ethidium bromide. Seven microlitres of each sample (test DNA, positive and negative controls) were then loaded into separate wells on the gel and a note made of the orientation and location of each sample for future reference. The end well on each gel was loaded with 7µl of previously prepared molecular weight markers (1µl pBR322

Haell1 digest [Sigma: approx. concentration 500-1000pg/ml], 10µl 0.5x TBE, 8µl loading buffer).

The electrophoresis tank lid was replaced and the gel run at 120 volts for **90** minutes. Slight adjustments to the running time were made on each "run" depending on the position of the leading blue bands. On completion of electrophoretic separation the gel was carefully placed onto a *UV* transilluminator, and using full Health and Safety precautions photographed onto type 667 Polaroid film using a Wratten **23A** filter at f4.7 for ¼ of a second. **A** diagrammatic representation of the expected banding pattern for *CYP2D6* **A** following Hpa II digestion is shown in Figure **39.**

Figure 39



Diagrammatic representation of the expected banding pattern seen on agarose gel electrophoresis for *CYP2D6* A following Hpa II restriction enzyme digestion.

CP2D6 B PCR

New 0.5ml plastic microcentrifuge tubes were labelled for each test DNA sample. One new 0.5ml microcentrifuge tubes was labelled as "Positive Control" and another was labelled as "Negative Control". A 1.5ml microcentrifuge tubes was labelled as "Stock" The stock solution was made up as detailed below:

Component	Volume
Sterile water	616µl
10x Taq buffer (including Mg^{++})	100µl
Exon 3 forward primer (5µM)	100µl
Intron 4 reverse primer (5µM)	100µl
dNTP's (4x5µM)	40µl
Taq polymerase	4µl

After mixing the stock tube by inversion 48µl of stock solution were aliquoted into each of the 0.5ml tubes. 1.5 µl of sterile water were aliquoted into the negative control tube and 1.5 µl of known *CYP2D6* B EMDNA were aliquoted into the positive control tube. 1.5 µl of each patient's test DNA were aliquoted into the individually labelled 0.5ml microcentrifuge tubes. Two drops (50µl) of light paraffin oil were added to each tube to prevent evaporation, which were then centrifuged at 12,000 rpm for 20 seconds. All tubes were then placed randomly in a thermal cycler (Omnigene, Hybaid Ltd) which was pre-programmed to deliver the following PCR cycle parameters:

Temperature (^{0}C)	Time (seconds)	Number of cycles	Effect
94	180	1	Initial denaturation
94	30	34	Denaturation
60	60	34	Primer annealing
72	60	34	Primer extension
94	30	1	Denaturation
60	60	1	Primer annealing
72	480	1	Primer extension

Bstn I restriction enzyme digestion

A 0.5ml microcentrifuge tube was labelled "Bstnl Stock" to which was added the following components:

Component	Volume
Sterile water	160µl
Bstn1 10x restriction enzyme buffer	40µl
Bstn1 restriction enzyme	5µl
Bovine Serum Albumin	10µl

New 0.5µl microcentrifuge tubes were labelled for each patient and the positive control. Ten microlitres of the relevant PCR product and 10µl of Bstnl stock were aliquoted into each microcentrifuge tube, which were then centrifuged at 12,000 rpm for 20 seconds and subsequently incubated at 60°C for 18 hours.

Agarose gel electrophoresis

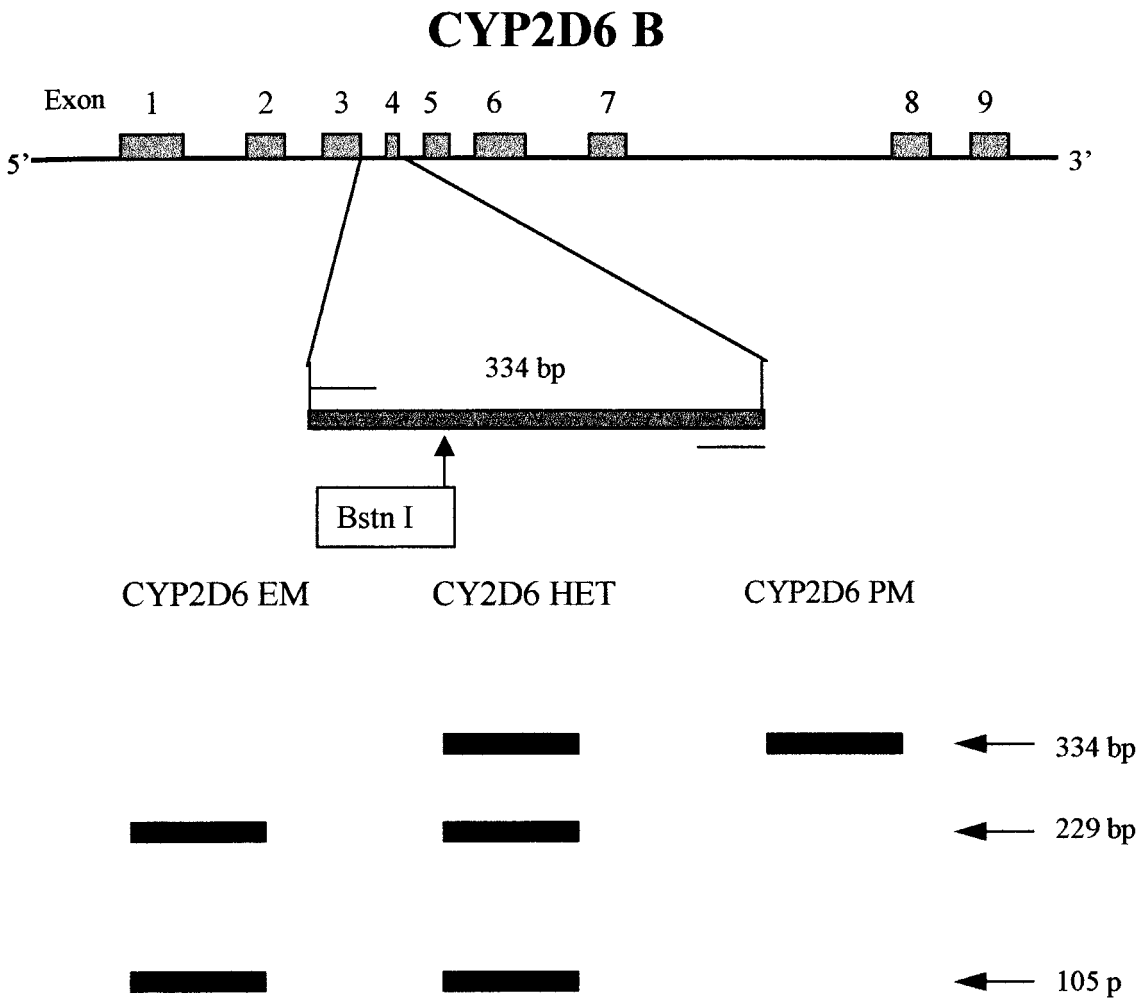
A 2% agarose gel was mixed and poured according to the protocol previously described for *GSTTZ* electrophoresis. Eight microlitres of loading buffer (30mg of 0. 15% w/v bromophenol blue, 30mg of 0. 15% w/v xylene cyanole, 6ml of 30% w/v glycerol, 8ml 5x TBE, 6ml sterile water) were aliquoted into each of the test microcentrifuge tubes (20µl into the negative control) and all tubes centrifuged at 12,000 rpm for 20 seconds.

When set, the combs and blanking tape were removed from the agarose gel/tray which was placed in an electrophoresis tank and covered to a depth of 2mm with 0.5x TBE containing 5µl of ethidium bromide. Five microlitres of each sample (test **DNA,** positive and negative controls) were then loaded into separate wells on the gel and a note made of the orientation and location of each sample for future reference. The end well on each gel was loaded with 7µl of previously prepared molecular weight markers (1µl pBR322

HaeII1 digest [Sigma: approx. concentration 500-1000μg/ml], 10μl 0.5x TBE, 8μl loading buffer).

The electrophoresis tank lid was replaced and the gel run at 85 volts for 35 minutes. Slight adjustments to the running time were made on each "run" depending on the position of the leading blue bands. On completion of electrophoretic separation the gel was carefully placed onto a *UV* transilluminator, and using full Health and Safety precautions photographed onto type 667 Polaroid film using a Wratten **23A** filter at f4.7 for ¼ of a second. **A** diagrammatic representation of the expected banding pattern for *CYP2D6* B following Bstnl digestion is shown in Figure 40.

Figure 40



Diagrammatic representation of the expected banding pattern seen on agarose gel electrophoresis for *CYP2D6* B following Bstn1 restriction enzyme digestion.

2.10 Determination of *CYP1A1* Genotype

Two variant *CYP1A1* alleles (exon 7 Ile-Val and 3' flankingregion MspI mutations were detected using PCR and restriction enzyme digestion.

Following PCR using exon 7 specific primers (Figure 41) a 322 base pair DNA fragmentis produced. Subsequent digestion with the restriction enzyme NcoI splits the restriction fragment into 3 bands of 232,70 and 20 base pairs. In the mutated polymorphsm with the valine substitution Nco 1 digestion produces 2 restriction fragments of 252 and 70 base pairs. The banding pattern seen in heterozygotes is a combination of the wild type and mutated alleles.

Following PCR with *CYP1A1* MspI specific primers (Figure **42),** individuals who have the 3' MspI mutation develop an MspI restriction site resulting in the digestion by MspI of the 340 base pair PCR product into 2 restriction fragments of 200 and 140 base pairs. Individuals with the m1/m1 genotype do not have the MspI restriction site and MspI does not digest the 340 base pair PCR product. Heterozygotes have a combination of the m1/m1 and *m2/m2* fragments

Figure 41

Primer	Sequence
Exon 7 forward	5'-AAAGGCTGGGTCCACCCTCT-3'
Exon 7 reverse	5'-AAAGACCTCCCAGCGGGCCA-3'

Primers used to determine *CYP1A1 Ile-Val* genotype by amplification across exon 7 (*CYP1A1-1*)

Figure 42

Primer	Sequence
Non-coding forward (NCF)	5'-CAGTGAAGAGGTGTAGCCGCT-3'
Non-coding reverse (NCR)	5'-TAGGAGTCTTGTCTCATGCCT-3'

Primers used to determine *CYP1A1 Msp1* genotype (*CYP1A1-2*)

CYP1A1 Ile-Val (CIPIA1-1) PCR

New 0.5ml plastic microcentrifuge tubes were labelled for each test DNA sample. One new 0.5ml microcentrifuge tubes was labelled as "Positive Control" and another was labelled as "Negative Control". A 1.5ml microcentrifuge tubes was labelled as "Stock" The stock solution was made up as detailed below:

Component	Volume
Sterile water	616µl
10x Taq buffer (including Mg^{++})	100µl
Exon 7 forward primer (5µM)	100µl
Exon 7 reverse primer (5µM)	100µl
dNTP's (4x5µM)	40µl
Taq polymerase	4µl

After mixing the stock tube by inversion 48µl of stock solution were aliquoted into each of the 0.5ml tubes. 1.5µl of sterile water were aliquoted into the negative control tube and 1.5µl of known *CYP1A1 m1/m2* DNA were aliquoted into the positive control tube. 1.5µl of each patient's test DNA were aliquoted into the individually labelled 0.5ml microcentrifuge tubes. Two drops (50µl) of light paraffin oil were added to each tube to prevent evaporation, which were then centrifuged at 12,000 rpm for 20 seconds. All tubes were then placed randomly in a thermal cycler (Omnigene, Hybaid Ltd) which was pre-programmed to deliver the following PCR cycle parameters:

Temperature (^{0}C)	Time (seconds)	Number of cycles	Effect
94	180	1	Initial denaturation
94	60	30	Denaturation
58	60	30	Primer annealing
72	60	30	Primer extension

Ncol restriction enzyme digestion

A 0.5ml microcentrifuge tube was labelled "Ncol Stock" to which was added the following components:

Component	Volume
Sterile water	160µl
Nco1 10x restriction enzyme buffer	40µl
Nco1 restriction enzyme	5µl

New 0.5µl microcentrifuge tubes were labelled for each patient and the positive control. Ten microlitres of the relevant PCR product and 1.25µl of Ncol stock were aliquoted into each microcentrifuge tube which were then centrifuged at 12,000 rpm for 20 seconds and subsequently incubated at 37°C for 18 hours.

Agarose gel electrophoresis

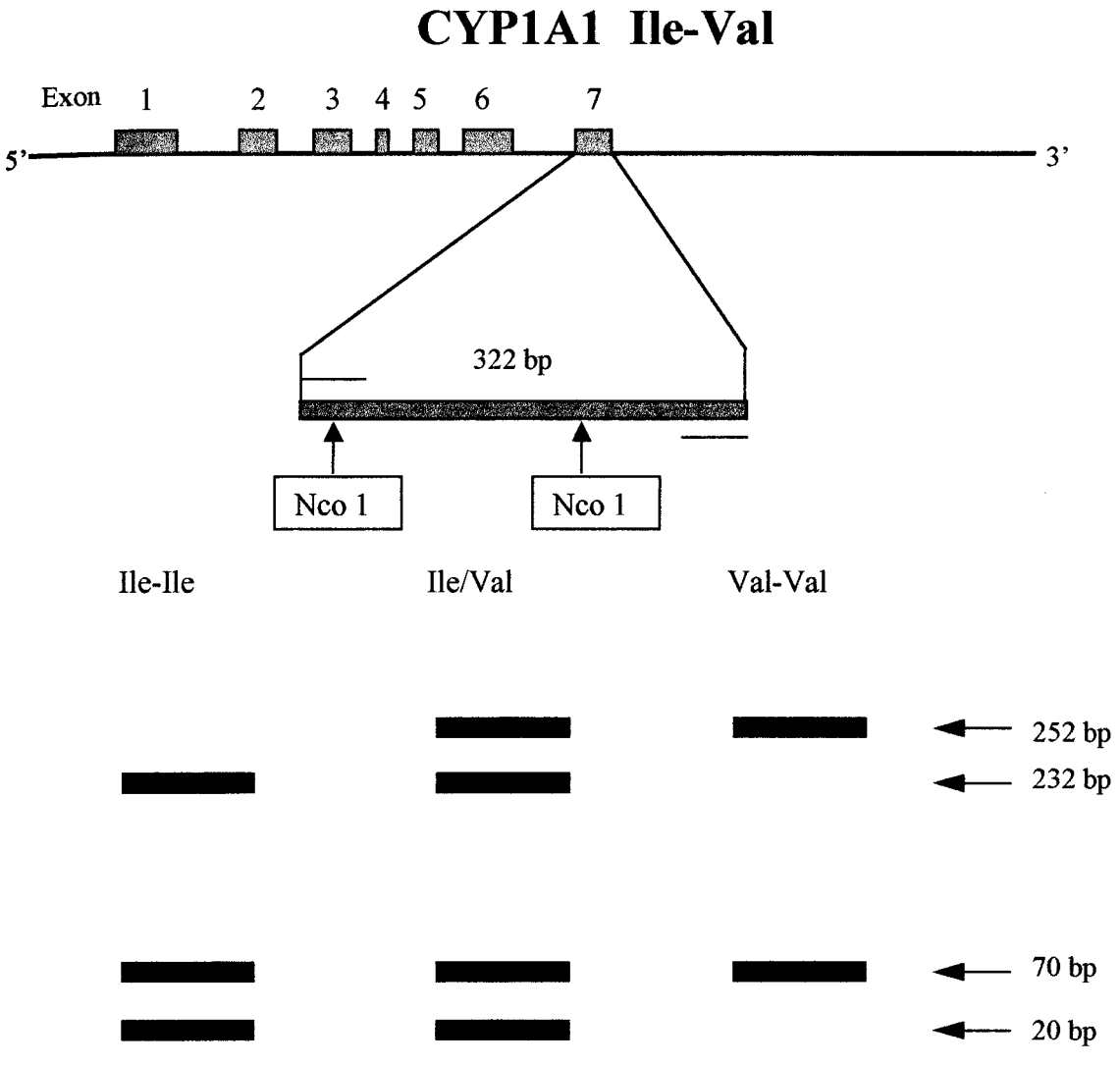
A 4% agarose gel was mixed and poured according to the protocol previously described for *GSTMI* electrophoresis. Two microlitres of loading buffer (30mg of 0.15% w/v bromophenol blue, 30mg of 0.15% w/v xylene cyanole, 6ml of 30% w/v glycerol, 8ml 5x TBE, 6ml sterile water) were aliquoted into each of the test microcentrifuge tubes (8µl into the negative control) and all tubes centrifuged at 12,000 rpm for 20 seconds.

When set, the combs and blanking tape were removed from the agarose gel/tray which was placed in an electrophoresis tank and covered to a depth of 2mm with 0.5x TBE

containing 5μl of ethidium bromide. Seven microlitres of each sample (test DNA, positive and negative controls) were then loaded into separate wells on the gel and a note made of the orientation and location of each sample for future reference. The end well on each gel was loaded with 7μl of previously prepared molecular weight markers (1y1 pBR322 HaeII1 digest [Sigma: approx. concentration 500-1000μg/ml], 10μl 0.5x TBE, 8μl loading buffer).

The electrophoresis tank lid was replaced and the gel run at 120 volts for 60 minutes. Slight adjustments to the running time were made on each "run" depending on the position of the leading blue bands. On completion of electrophoretic separation the gel was carefully placed onto a *UV* transilluminator, and using full Health and Safety precautions photographed onto type **667** Polaroid film using a Wratten 23A filter at f4.7 for ¼ of a second. A diagrammatic representation of the expected banding pattern for *CYP1A1-1* following Ncol digestion is shown in Figure 43.

Figure 43



Diagrammatic representation of the expected banding pattern seen on agarose gel electrophoresis for *CYP1A1 Ile-Val* (*CYP1A1-1*) following Nco1 restriction enzyme digestion.

CYP1A1 MspI (CYP1A1-2) PCR

New 0.5ml plastic microcentrifuge tubes were labelled for each test DNA sample. One new 0.5ml microcentrifuge tubes was labelled as "Positive Control" and another was labelled as "Negative Control". A 1.5ml microcentrifuge tubes was labelled as "Stock" The stock solution was made up as detailed below:

Component	Volume
Sterile water	616µl
10x Taq buffer (including Mg^{++})	100µl
Non-coding forward primer (5µM)	100µl
Non-coding reverse primer (5µM)	100µl
dNTP's (4x5µM)	40µl
Taq polymerase	4µl

After mixing the stock tube by inversion 48µl of stock solution were aliquoted into each of the 0.5ml tubes. 1.5 µl of sterile water were aliquoted into the negative control tube and 1.5µl of known *CYP1A1 m2/m2* DNA were aliquoted into the positive control tube. 1.5µl of each patient's test DNA were aliquoted into the individually labelled 0.5ml microcentrifuge tubes. Two drops (50µl) of light paraffin oil were added to each tube to prevent evaporation, which were then centrifuged at 12,000 rpm for 20 seconds. All tubes were then placed randomly in a thermal cycler (Omnigene, Hybaid Ltd) which was pre-programmed to deliver the following PCR cycle parameters:

Temperature (^{0}C)	Time (seconds)	Number of cycles	Effect
94	180	1	Initial denaturation
94	45	5	Denaturation
66	60	5	Primer annealing
72	60	5	Primer extension
94	30	30	Denaturation
64	30	30	Primer annealing
72	45	30	Primer extension
72	360	1	Primer extension
40	5	1	Stop reaction

Mspl restriction enzyme digestion

A 0.5ml microcentrifuge tube was labelled "Mspl Stock" to which was added the following components:

Component	Volume
Msp1 10x restriction enzyme buffer	20µl
Msp1 restriction enzyme	5µl

New 0.5µl microcentrifuge tubes were labelled for each patient and the positive control. Ten microlitres of the relevant PCR product and 1.25µl of Mspl stock were aliquoted into each microcentrifuge tube which were then centrifuged at 12,000 rpm for 20 seconds and subsequently incubated at 37°C for 18 hours.

Agarose gel electrophoresis

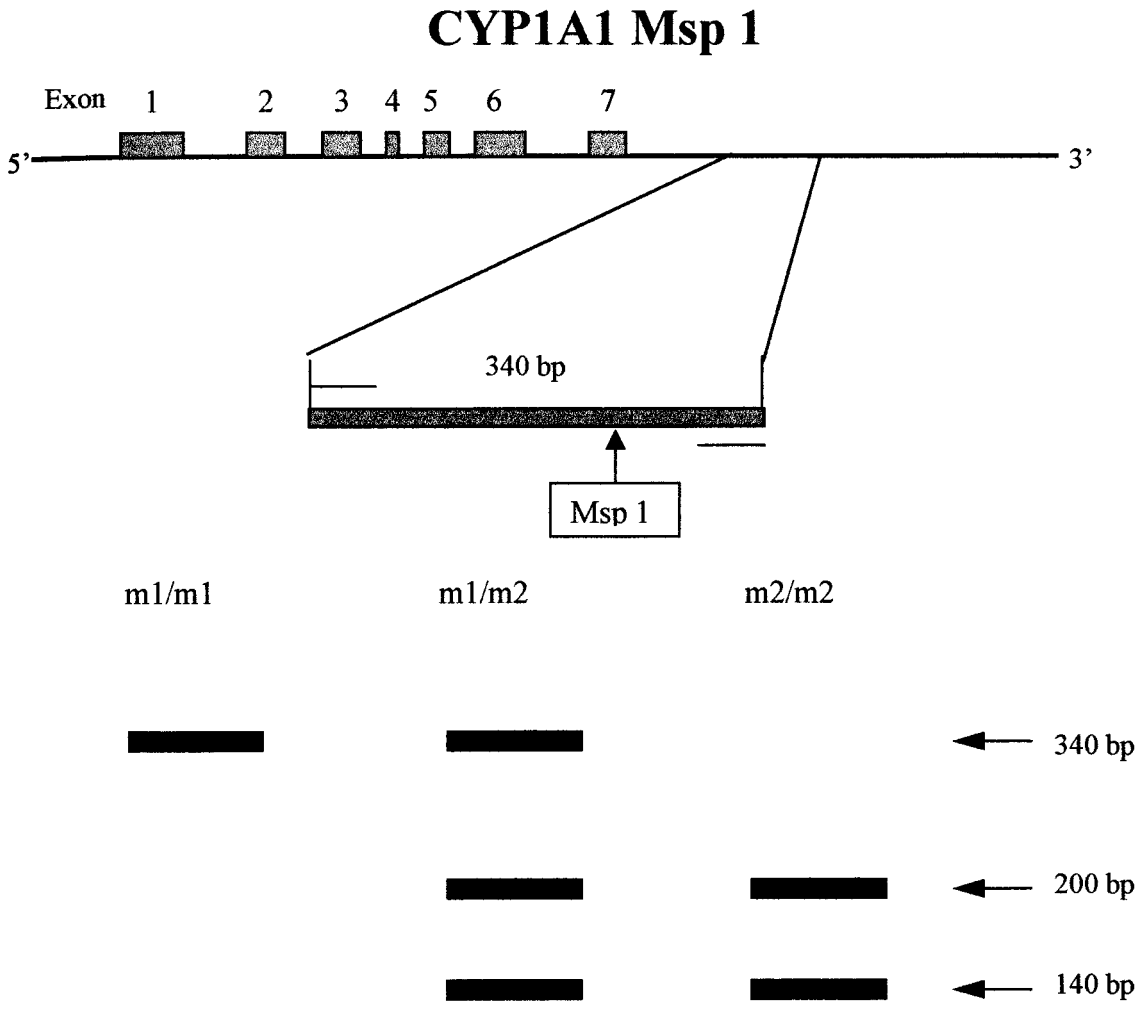
A 2% agarose gel was mixed and poured according to the protocol previously described for *GSTT1* electrophoresis. Two microlitres of loading buffer (30mg of 0. **15%** w/v bromophenol blue, 30mg of 0. 15% w/v xylene cyanole, 6ml of 30% w/v glycerol, 8ml 5x TBE, 6ml sterile water) were aliquoted into each of the test microcentrifuge tubes (**8µl** into the negative control) and all tubes centrifuged at 12,000 rpm for 20 seconds.

When set, the combs and blanking tape were removed from the agarose gel/tray which was placed in an electrophoresis tank and covered to a depth of 2mm with 0.5x TBE containing 5µl of ethidium bromide. Five microlitres of each sample (test DNA, positive and negative controls) were then loaded into separate wells on the gel and a note made of the orientation and location of each sample for future reference. The end well on each gel was loaded with 7µl of previously prepared molecular weight markers (1µl pBR322

Haell1 digest [Sigma: approx. concentration 500-1000μg/ml], 10μl 0.5x TBE, 8μl loading buffer).

The electrophoresis tank lid was replaced and the gel run at 120 volts for **30** minutes. Slight adjustments to the running time were made on each "run" depending on the position of the leading blue bands. On completion of electrophoretic separation the gel was carefully placed onto a UV transilluminator, and using full Health and Safety precautions photographed onto type 667 Polaroid film using a Wratten **23A** filter at f4.7 for ¼ of a second. **A** diagrammatic representation of the expected banding pattern for *CYP1A1-1* following Ncol digestion is shown in Figure 44.

Figure 44



Diagrammatic representation of the expected banding pattern seen on agarose gel electrophoresis for *CYP1A1 Msp1* (*CYP1A1-2*) following Msp1 restriction enzyme digestion.

2.11 Immunohistochemical Detection of CYP2D6

Serial 5pm sections were cut from the normal oral mucosa specimens, mounted on glass microscope slides and dried overnight in a 37°C incubator and placed in a 56°C incubator for 30 minutes. The sections were then de-waxed in xylene for 3 minutes and taken up through graded alcohols to industrial methylated spirit (IMS). Endogenous peroxidase activity was blocked by immersion in 0.3% hydrogen peroxide 10 minutes followed by washing in running tap water for 20 minutes. The specimens were then microwaved at 10 minutes on high power followed by 10 minutes on medium power in a solution of 10 mM citrate buffer, pH 6.0 and allowed to cool to room temperature before being mounted onto cover plates and loaded into an auto-stainer (The Cadenza, Shandon Scientific Ltd, UK). Specimens were then incubated in normal swine serum diluted **1:5** with TRIS-buffered saline (TBS) pH 7.6 (8g NaCl, 6g TRIS in 10 litres H_20) for 20 minutes and washed with TBS for 5 minutes. Sections were incubated for 60 minutes at 20°C with a rabbit polyclonal antiserum (Hand ***et al.,*** 1996) specific for the human CYP2D6 subunit (generous gift from Professor C R Wolf, Imperial Cancer Research Fund, Scotland) diluted 1:75. Normal human liver and kidney sections were used as a positive control and an oral mucosal section not exposed to the primary antibody served as a negative control. All sections were then exposed to a biotinylated sheep anti-rabbit antiserum (Dakopatts, Denmark) diluted 1:100 for 30 minutes and washed in TBS for 5 minutes. Streptavidin conjugated horseradish peroxidase (Binding Site, UK) diluted 1:75 was added and the specimens incubated for 30 minutes before washing for 5 minutes in TBS Peroxidase activity was developed using a 10mg tablet of diaminobenzidine-

tetrahydrochloride (Sigma, UK), 30 cm^3 TBS and 15 cm^3 0.3% H_2O_2 for 5 minutes, counterstained with haematoxylin, dehydrated and assessed by standard light microscopy.

2.12 Problems and Errors

With increasing experience the majority of DNA extractions, PCR's and electrophoreses worked satisfactorily. However, there were several occasions, especially early in the study when problems were encountered with all aspects of the methodology.

DNA **extraction**

Insufficient or absent DNA harvest following Phenol/Chloroform extraction was most commonly due to having taken less than the desired 4ml **of** venous blood and/or allowing the blood sample to clot in the collecting bottle. These events commonly coincided, as it was occasionally difficult to perform adequate venepuncture on certain patients, especially those who were elderly, frail or dehydrated.

PCR

It rapidly became apparent that successful PCR requires a methodical approach with strict attention to detail. Any deviation from the specific protocol, either intentional or unintentional, usually resulted in PCR failure. This most commonly occurred while making up the various PCR stock mixtures due to failure to add dNTP's, allele specific primers or Taq polymerase. Occasionally, despite all reagents being correctly added the PCR would still fail because one or more reagents had been aliquoted from stock that had undergone numerous freeze-thaw cycles resulting in partial or complete loss of activity. These errors resulted in failure of all the batched PCR reactions. Failure of individual PCR's within a batch of otherwise successful reactions also occurred. It was noted that occasionally, blood samples had been collected in blood bottles containing Lithium

Heparin as the anticoagulant rather than EDTA. The Lithium Heparin samples produced good DNA harvests but the anticoagulant commonly inhibited the PCR reaction. Wherever possible repeat blood samples using EDTA tubes were taken. Simple, avoidable errors such as failing to add the PCR stock to the sample tubes also resulted in individual PCR failures.

In a technique as exquisitely sensitive as PCR, contamination by, and subsequent amplification of "foreign" DNA is a constant danger. Both general and specific techniques were employed to minimise the risk of contamination. The laboratory containing the thermal cyclers where the PCR was actually performed was physically removed from those where the PCR stock solutions were made up and electrophoresis of final PCR product undertaken in order to reduce as far as possible the amount of extraneous DNA in the PCR laboratory. New sterile reaction vessels were used for each PCR step and new sterile pipette tips were used to aliquot reagents and samples.

Electrophoresis

The electrophoresis process ***per se*** was usually trouble free, although occasionally the gels would run faster or slower than expected. The main determinant of successful electrophoresis and subsequent *UV* visualisation and Polaroid photography was the quality **of** the agarose gel. **As** described previously all gels were made and poured individually, commercial gels were never employed. Making the 2% gels was fairly straightforward and the electrophoreses requiring 2% gels usually ran correctly first time and produced good quality Polaroid photographs on which the individual lanes were well resolved. However, the 4% gels were more problematical. The main difficulty was ensuring complete

dissolution of the agarose in the water. Not infrequently the gels would "boil-over'' in the microwave oven or were subject to excessive evaporation, both scenarios resulting in a gel **of** >4% concentration. Gels with inadequate agarose dissolution or those of >4% concentration were of poor quality, containing collections of undissolved agarose, air bubbles and streaks. The subsequent Polaroid photographs were often indistinct with poor detail resolution.

As experience increased the above errors were minimised and the number of samples requiring repeat PCR reduced.

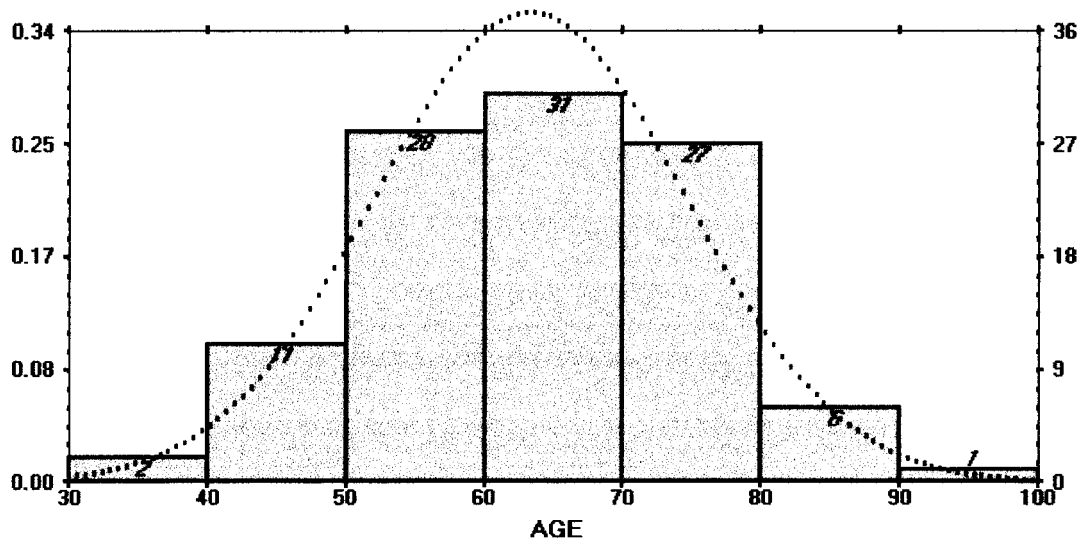
Chapter 3

RESULTS

3.1 Patient Demographics

Patient age at initial tumour diagnosis was normally distributed (Figure. 45). The mean age was 63.3 years and the median age was 63.5 years (range. 31 – 90 years, SD = 11.3). 54 patients (51%) were younger than or the same as the median age and 52 patients (49%) were older than the median age at initial tumour diagnosis. For the purposes of this study, patients aged 63 years or younger were termed "young" and patients over 63 years were termed "old".

Figure 45



Histogram and distribution curve describing the patient age in years of the study population at initial tumour diagnosis. Figures in italics are age group counts (n = 106).

Over one third of patients presented with late stage tumours (TNM stage 3 or 4) (Table. 22). There was no association between gender and tumour stage at presentation ($\chi^2_1 = 2.64, p = 0.619$).

Table 22

Tumour stage	Males	Females	All
0	2 (3.1)	2 (4.9)	4 (3.8)
1	30 (46.1)	15 (36.6)	45 (42.4)
2	11 (16.9)	9 (21.9)	20 (18.9)
3	6 (9.2)	7 (17.1)	13 (12.3)
4	16 (24.6)	8 (19.5)	24 (22.6)

TNM tumour stage distribution (%) at initial presentation by gender (n = 106)

Over **15%** of patients were non-drinkers and non-smokers and over 20% were heavy drinkers and heavy smokers (Table 23). Heavy drinking and heavy smoking was more common in males than in females ($\chi^2_1 = 14.57, p<0.001$) and in young than in old patients ($\chi^2_1 = 12.46, p<0.001$).

Table 23

	Non-drinkers + Non-smokers	Heavy drinkers + Heavy smokers
All (n = 106)	16 (15.1)	22 (20.7)
Males (n = 65)	4 (6.1)	20 (30.8)
Females (n = (41)	12 (29.3)	2 (4.9)
Young (n = 54)	3 (5.5)	18 (33.3)
Old (n = 52)	13 (25)	4 (7.7)

Tobacco and alcohol consumption (%) by sex and age

3.2 Oral Cancer Susceptibility

100/106 patients were fully informative for the *GSTM1* genotype, 100/106 patients were fully informative for the *GSTM3* genotype, 99/106 patients were fully informative for the *GSTT1* genotype, 100/106 patients were fully informative for the GSTP1 genotype, 100/106 patients were fully informative for the *CYP2D6* genotype, 91/106 patients were fully informative for the *CYP1A1-1* genotype and 93/106 patients were fully informative for the *CYP1A1-2* genotype.

Following χ^2 analysis of the frequencies of the GSTM1, GSTM3, GSTT1, GSTP1, CYP2D6 and CYP1A1 phenotypes in the oral cancer patients and in the North Staffordshire controls, only the CYP2D6 phenotype was found to be significantly different (Tables 24 and 25).

CYP2D6 genotype frequencies in the controls were similar to those previously reported in Caucasians resident in North Staffordshire (Warwick *et al.*, 1994a; Elexpuru-Camiruaga *et al.*, 1995; Lear *et al.*, 1996). The frequency of the wild type EM allele was 0.79 and of the two mutant alleles combined was 0.21 (Equation 1). Assuming Hardy-Weinberg equilibrium (Equation 2), these frequencies gave the expected genotype frequencies for *CYP2D6 EM*, HET and *PM* (Equation 3).

Equation 1.

EM allele frequency = *EM* frequency + 0.5 *HET* frequency.

$\therefore$ EM allele frequency = 0.63 + 0.16 = 0.79 (corrected to 2 decimal places).

Mutant allele frequency = *PM* frequency + 0.5 HET frequency.

$\therefore$ Mutant allele frequency = 0.05 + 0.16 = 0.21 (corrected to 2 decimal places).

Equation 2. (Hardy-Weinberg equation).

This equation, discovered independently in **1908** by Wilhelm Weinberg, a German physician, and Godfrey Harold Hardy, a British mathematician, states that in a large, random-mating population, the proportion of dominant and recessive genes present tends to remain constant from generation to generation unless outside forces act to change it (Internet **1**). Consider an adult population having **2** allelic genes *a* and *b* of frequencies p and q respectively. If the population is assumed to mate randomly, then the proportion of the genotypes *AA*, *ab* and *BB* in the first generation after mating is: $p^2 + 2pq + q^2 = 1$ (Internet **2**). For North Staffordshire control patients p = **0.79** and q = **0.21.**

$\therefore 0.6241 + 2(0.79 \times 0.21) + 0.0441 = 1$

Equation 3

Expected wild type homozygous allele frequency ***(EM)*** $= 0.79^2$, = **0.6241** = **62.4%.**

Observed *EM* frequency = **63%.**

Expected mutant homozygous allele frequency *(PM)* $= 0.21^2$, $= 0.21^2$ = **0.0441** = **4.4%.**

Observed PM frequency = **4.5%.**

Expected heterozygote allele frequency ***(HET)*** = **2(0.79x0.21)** = **0.3318,** = **33%.**

Observed ***HET*** frequency = **32.5%.**

The *CYP2D6 PM* frequency was significantly higher in the oral cancer patients than in the controls (χ^2_1 = **10.56, p = 0.0012, OR** = 3.178, 95% CI = **1.55 – 6.5**).

Apart from the *CYP2D6* polymorphisms there were no significant differences in phenotype frequencies between the young and old patient groups (Tables **26** and **27**). The mean age at presentation of the patients with the *CYP2D6* ***EM*** genotype (**60.4** years, SD =

10.9) was lower than that of the patients with the *CYP2D6* PM and *CYP2D6 HET* genotypes (66.6 years, **SD** = 13.7 and 66.8 years, SD = 10.1 respectively). The Newman-Keuls test demonstrated that this difference was significant at the 5% level.

The difference seen with the *CYP2D6* genotype was mainly due to the *EM* frequency being significantly lower in the old patients ($\chi^2_1 = 8.67, p = 0.003$). The low EM allele frequency in the old patient group is reflected in the observation that both the *HET* and the PM allele frequencies are correspondingly higher than in the young patient group ($\chi^2_1 = 3.78, p = 0.052$ and $\chi^2_1 = 2.96, p = 0.085$ respectively).

Young males accounted for 46.7% of the *CYP2D6* EM genotypes and old females accounted for 46.1% of the *CYP2D6* PM genotypes ($\chi^2_1 = 5.17, p = 0.023$).

No significant differences were found in any phenotype frequencies between males and females (Tables 28 and 29). Even though the *CYP2D6* PM allele frequency in females was over twice that in males this difference was not significant ($\chi^2_1 = 1.08, p = 0.222$).

No significant differences were found in any phenotype frequencies between non-drinkers and non-smokers and heavy drinkers and heavy smokers (Tables 30 and 31). The much higher *CYP2D6* EM frequency in the heavy smoking and drinking group compared to the non-smoking and non-drinking group failed to reach significance ($\chi^2 = 1.51, p = 0.140$). Similarly, the high *CYP2D6* PM frequency observed in the non-drinkers and non-smokers was not significantly different to that in the heavy drinkers and heavy smokers ($\chi^2 = 1.06, p = 0.303$).

93.7% of heavy smokers and heavy drinkers were young males with the *CYP2D6 EM* genotype. 67% of the *CYP2D6 EM* heavy drinkers and heavy smokers were *GSTM1 null* and 94% were *GSTM3 AA*. No association was found between detoxification enzyme genotype and tumour differentiation or TNM stage at presentation.

Table 24

	A	AA	B	A/B	BB	Null	EM	HET	PM
GSTM1	186		94	26		397			
(n = 703)	(26.5)		(13.4)	(3.7)		(56.5)			
GSTM3		221		64	15				
(n = 300)		(73.7)		(21.3)	(5)				
GSTT1	486					113			
(n = 599)	(81.1)					(18.9)			
GSTP1		73		61	22				
(n = 156)		(46.8)		(39.1)	(14.1)				
CYP2D6							294	152	21
(n = 467)							(63)	(32.5)	(4.5)
CYP1A1-1							572	94	7
(n = 673)							(85)	(14)	(1)
CYP1A1-2							561	119	4
(n = 684)							(82)	(17.4)	(0.6)

Detoxification enzyme phenotype frequencies (%) in North Staffordshire controls.

Table 25

	A	AA	B	A/B	BB	Null	EM	HET	PM	χ^2	*p*
GSTM1	27		17	5		51				1.7	0.638
(n = 100)	(27)		(17)	(5)		(51)				(DF = 3)	
GSTM3		75		21	4					0.18	0.914
(n = 100)		(75)		(21)	(4)					(DF = 2)	
GSTT1	74					25				2.19	0.14
(n = 99)	(74.7)					(25.3)				(DF = 1)	
GSTP1		37		51	12					3.54	0.172
(n = 100)		(37)		(51)	(12)					(DF = 2)	
CYP2D6							60	27	13	10.85	0.005
(n = 100)							(60)	(27)	(13)	(DF = 2)	
CYP1A1-1							77	14	0	1.06	0.588
(n = 91)							(84.6)	(15.4)	(0)	(DF = 2)	
CYP1A1-2							79	12	2	3.67	0.161
(n = 93)							(84.9)	(12.9)	(2.2)	(DF = 2)	

Detoxification enzyme phenotype frequencies (%) in oral cancer patients.

p = level of significance derived from χ^2 crosstabulation calculations comparing oral cancer patients with the North Staffordshire controls. DF = degrees of freedom.

Table 26

	A	AA	B	A/B	BB	Null	EM	HET	PM
GSTM1	12		8	3		29			
(n = 52)	(23.1)		(15.4)	(5.8)		(55.8)			
GSTM3		40		11	1				
(n = 52)		(76.9)		(21.1)	(1.9)				
GSTT1	37					13			
(n = 50)	(74)					(26)			
GSTP1		20		27	6				
(n = 53)		(37.7)		(50.9)	(11.3)				
CYP2D6							39	10	4
(n = 53)							(73.6)	(18.9)	(7.5)
CYP1A1-1							40	8	0
(n = 48)							(83.3)	(16.7)	
CYP1A1-2							42	7	0
(n = 49)							(85.7)	(14.3)	

Detoxification enzyme phenotype frequencies (%) in young oral cancer patients (age < = 63 years).

Table 27

	A	AA	B	A/B	BB	Null	EM	HET	PM	χ^2	*p*
GSTM1	15		9	2		22				1.40	0.707
(n = 48)	(31.2)		(18.7)	(4.2)		(45.8)				(DF = 3)	
GSTM3		35		10	3					1.22	0.543
(n = 48)		(72.9)		(20.8)	(6.2)					(DF = 2)	
GSTT1	37					12				0.03	0.863
(n = 49)	(75.5)					(24.5)				(DF = 1)	
GSTP1		17		24	6					0.06	0.97
(n = 47)		(36.2)		(51.1)	(12.8)					(DF = 2)	
CYP2D6							21	17	9	8.81	0.013
(n = 47)							(44.7)	(36.2)	(19.1)	(DF = 2)	
CYP1A1-1							37	6	0	0.13	0.720
(n = 43)							(86.1)	(13.9)		(DF = 1)	
CYP1A1-2							37	5	2	2.39	0.304
(n = 44)							(84.1)	(11.4)	(4.5)	(DF = 2)	

Detoxification enzyme phenotype frequencies (%) in old oral cancer patients (age > 63 years).

p = level of significance derived from χ^2 crosstabulation calculations comparing young oral cancer patients with old oral cancer patients.
DF = degrees of freedom.

Table 28

	A	AA	B	A/B	BB	Null	EM	HET	PM
GSTM1	17		11	1		33			
(n = 62)	(27.4)		(17.7)	(1.6)		(53.2)			
GSTM3		49		9	3				
(n = 61)		(80.3)		(14.7)	(4.9)				
GSTT1	46					15			
(n = 61)	(75.4)					(24.6)			
GSTP1		22		33	6				
(n = 61)		(36.1)		(54.1)	(9.8)				
CYP2D6							40	17	6
(n = 63)							(63.5)	(27)	(9.5)
CYP1A1-1							48	8	0
(n = 56)							(85.7)	(14.3)	
CYP1A1-2							48	8	2
(n = 58)							(82.7)	(13.8)	(3.5)

Detoxification enzyme phenotype frequencies (%) in male oral cancer patients.

Table 29

	A	AA	B	A/B	BB	Null	EM	HET	PM	χ^2	*p*
GSTM1	10		6	4		18				3.97	0.266
(n = 38)	(26.3)		(15.8)	(10.5)		(47.4)				(DF = 3)	
GSTM3		26		12	1					3.83	0.149
(n = 39)		(66.7)		(30.8)	(2.6)					(DF = 2)	
GSTT1	28					10				0.04	0.848
(n = 38)	(73.7)					(26.3)				(DF = 1)	
GSTP1		15		18	6					0.94	0.625
(n = 39)		(38.5)		(46.1)	(15.4)					(DF = 2)	
CYP2D6							20	10	7	1.93	0.382
(n = 37)							(54.1)	(27)	(18.9)	(DF = 2)	
CYP1A1-1							29	6	0	0.14	0.713
(n = 35)							(82.9)	(17.1)		(DF = 2)	
CYP1A1-2							31	4	0	1.39	0.50
(n = 35)							(88.6)	(11.4)		(DF = 2)	

Detoxification enzyme phenotype frequencies (%) in female oral cancer patients.

p = level of significance derived from χ^2 crosstabulation calculations comparing male oral cancer patients with female oral cancer patients. DF = degrees of freedom.

Table 30

	A	AA	B	A/B	BB	Null	EM	HET	PM
GSTM1	2		2	2		9			
(n = 15)	(13.3)		(13.3)	(13.3)		(60)			
GSTM3		11		4	1				
(n = 16)		(68.7)		(25)	(6.3)				
GSTT1	11					3			
(n = 14)	(78.6)					(21.4)			
GSTP1		6		7	2				
(n = 15)		(40)		(46.7)	(13.3)				
CYP2D6							8	4	2
(n = 14)							(57.1)	(28.6)	(14.3)
CYP1A1-1							13	1	0
(n = 14)							(92.9)	(7.1)	
CYP1A1-2							12	1	1
(n = 14)							(85.7)	(7.1)	(7.1)

Detoxification enzyme phenotype frequencies (%) in non-drinkers and non-smokers

Table 31

	A	AA	B	A/B	BB	Null	EM	HET	PM	χ^2	*p*
GSTM1	5		3	0		13				3.30	0.348
(n = 21)	(23.8)		(14.3)			(61.9)				(DF = 3)	
GSTM3		19		3	0					2.39	0.304
(n = 22)		(86.4)		(13.6)						(DF = 2)	
GSTT1	14					7				0.58	0.445
(n = 21)	(66.6)					(33.3)				(DF = 1)	
GSTP1		7		12	2					0.40	0.817
(n = 21)		(33.3)		(57.1)	(9.5)					(DF = 2)	
CYP2D6							18	3	1	2.68	0.263
(n = 22)							(81.8)	(13.6)	(4.5)	(DF = 2)	
CYP1A1-1							16	4	0	1.09	0.298
(n = 20)							(80)	(20)		(DF = 1)	
CYP1A1-2							17	3	0	1.86	0.395
(n = 20)							(85)	(15)		(DF =2)	

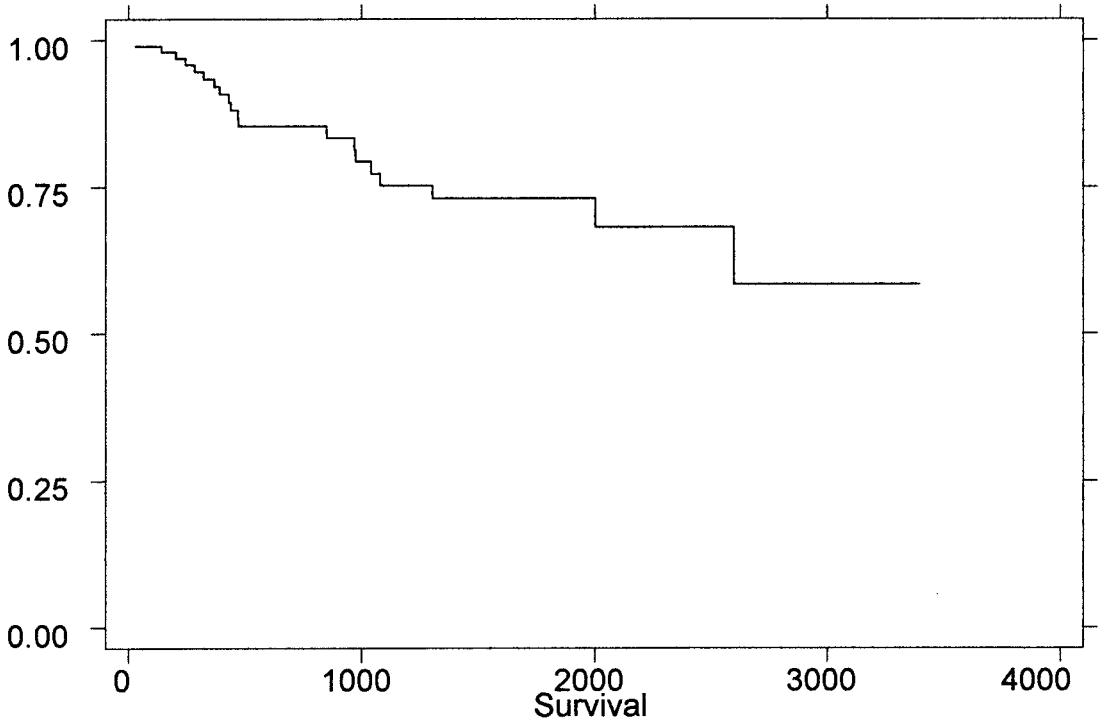
Detoxification enzyme phenotype frequencies (%) in heavy drinkers and heavy smokers.

p = level of significance derived from χ^2 crosstabulation calculations comparing non-drinkers and non-smokers with heavy drinkers and heavy smokers. DF = degrees of freedom.

3.3 Oral Cancer Outcome

During the study period 13 patients (12.2%) developed a recurrence at the primary site, 21 patients (19.8%) developed cervical lymph node metastases and 20 patients (18.9%) died. In **8** patients (40%) the cause of death was either directly or indirectly related to their oral cancer. The mean group survival time was 2511 days (Figure. **46).**

Figure 46



Kaplan-Meier survival estimate for oral cancer group (n = 106).

Survival is time to death in days from date of diagnosis.

Patient age, tumour differentiation, tumour stage and the development of a local recurrence or cervical lymph node metastasis were all independent poor prognostic factors (Table 32).

Table 32

Risk Factor	Cox hazards Model	Hazard Ratio	χ^2	P	95% confidence interval
Sex	Male v Female	0.97		0.961	0.39 – 2.39
Age	Total	1.04		0.040	1.00 – 1.09
	Old v Young	3.30		0.021	1.20 – 9.10
Differentiation	Total *		15.01	0.005	
	Well v Other	0.40		0.111	0.13 – 1.23
	Poor v Other	5.31		0.001	2.05 – 13.80
TNM stage	Total	1.69		0.005	1.17 – 2.47
Recurrence	Yes v No	4.36		0.001	1.79 – 10.57
Nodes	Yes v No	5.12		< 0.001	2.09 –12.55
Tobacco	Total *		1.43	0.699	
	Ever v Never	1.10		0.834	0.44 – 2.77
	Heavy v Nil	1.44		0.423	0.59 – 3.48
Alcohol	Total *		7.14	0.067	
	Ever v Never	1.31		0.582	0.50 – 3.41
	Heavy v Nil	1.32		0.554	0.52 – 3.32
GSTM1	Total *		2.23	0.527	
	Null v *A* + *B* + *AB*	0.95		0.911	0.38 – 2.35
	A v *Null* + *B* + *AB*	0.55		0.338	0.16 – 1.88
	B v *Null* + *A* + *AB*	1.94		0.183	0.73 – 5.17
	AB v *Null* + *A* + *B*	0.80		0.832	0.11 – 6.10
GSTT1	*Null* v *A*	1.54		0.491	0.45 – 5.27
GSTM3	Total *		0.47	0.791	
	AA v *AB* + *BB*	1.46		0.501	0.47 – 4.37
	AB v *AA* + *BB*	0.68		0.536	0.20 – 2.32
	BB v *AA* + *AB*	0.81		0.840	0.11 – 6.13
GSTP1	Total *		1.21	0.546	
	AA v *AB* + *BB*	0.67		0.439	0.24 – 1.85
	AB v *AA* + *BB*	1.68		0.277	0.66 – 4.27
	BB v *AA* + *AB*	0.69		0.617	0.16 – 2.98
CYP2D6	Total *		0.51	0.774	
	EM v *HET* + *PM*	0.76		0.548	0.31 – 1.84
	HET v *EM* + *PM*	1.39		0.478	0.56 – 3.43
	PM v *EM* + *HET*	0.92		0.911	0.21 – 3.98
CYP1A1-1	Total *		1.29	0.256	
	EM v *HET* + *PM*	0.52		0.265	0.17 – 1.63
	HET v *EM* + *PM*	1.90		0.265	0.61 – 5.91
	PM v *EM* + *HET*				
CYP1A1-2	Total *		2.33	0.311	
	EM v *HET* + *PM*	1.65		0.501	0.38 – 7.14
	HET v *EM* + *PM*	0.33		0.281	0.04 – 2.47
	PM v *EM* + *HET*	2.91		0.305	0.38 – 22.45

The log-rank test (*) and Cox proportional hazards model of all potential independent risk factors for death.

Tumour stage at diagnosis and the development of cervical lymph node metastases were the strongest predictors of poor survival after correcting for the influence of age, differentiation and local recurrence (Table 33).

Table 33

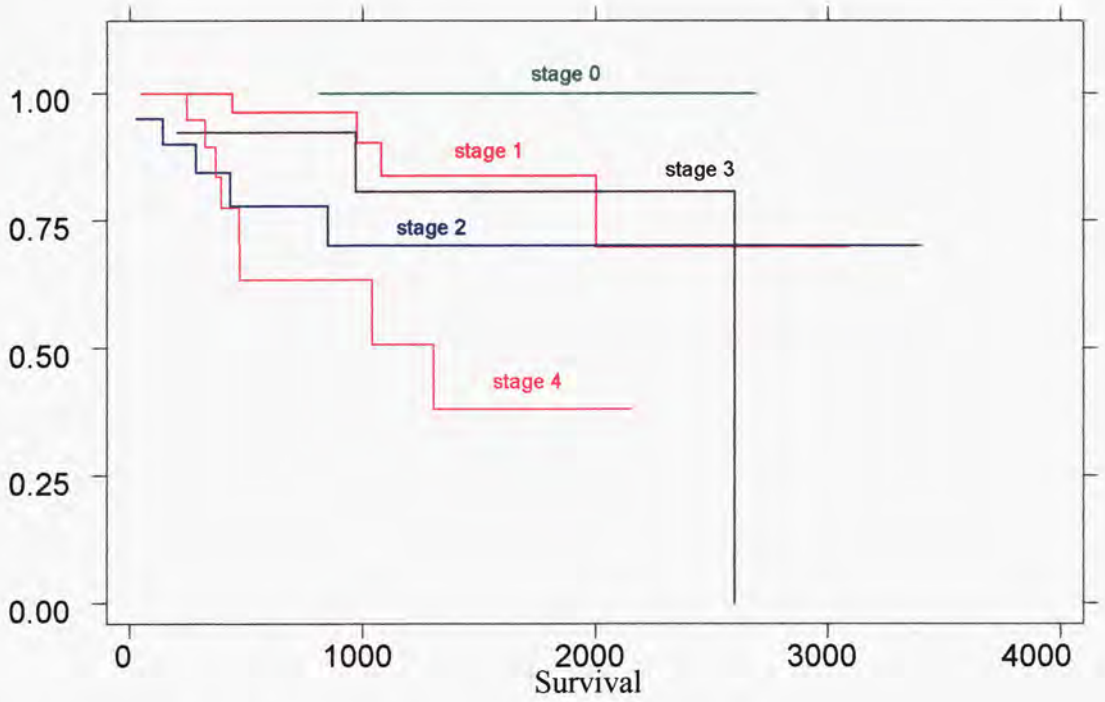
Risk Factor	Hazard ratio	*p*	95 % confidence interval
TNM stage	1.61	0.037	1.03 – 2.50
Cervical nodes	3.16	0.057	0.97 – 10.35
Age	2.34	0.151	0.73 – 7.45
Recurrence	2.08	0.25	0.60 – 7.21
Differentiation	1.03	0.95	0.36 – 2.91

Cox proportional hazards model of the most significant combined risk factors for death.

Mantel-Haenszel comparison of the Kaplan-Meier survival estimates by TNM stage (Figure 47) confirmed the Cox regression model analysis model that tumour stage at diagnosis was a significant prognostic factor for survival ($\chi^2_4 = 10.64, p = 0.032$).

There were no significant differences in any of the detoxification enzyme phenotype frequencies between survivors and non-survivors (Tables 34 and 35).

Figure 47



Kaplan-Meier survival estimates by TNM stage at initial diagnosis.

Survival is time to death in days from date of diagnosis.

Of all the potential risk factors for local recurrence, only cervical lymph node metastasis, tumour differentiation, alcohol consumption and the *GSTP1 AB* genotype were significant (Table 36). Cox analysis of the combined effects of these 4 risk factors demonstrated that the presence of lymph node metastases and the consumption of alcoholic beverages were the most powerful predictors for local recurrence (Table 37). Stage 4 tumours recurred earlier than lower stage tumours (Figure 48).

Table 34

	A	AA	B	A/B	BB	Null	EM	HET	PM
GSTM1	24		11	4		42			
(n = 81)	(29.6)		(13.6)	(4.9)		(51.8)			
GSTM3		59		18	3				
(n = 80)		(73.7)		(22.5)	(3.7)				
GSTT1	57					22			
(n = 79)	(72.2)					(27.8)			
GSTP1		32		39	10				
(n = 81)		(39.5)		(48.1)	(12.3)				
CYP2D6							50	8	11
(n = 80)							(62.5)	(23.7)	(13.7)
CYP1A1-1							63	10	0
(n = 73)							(86.3)	(13.7)	
CYP1A1-2							61	11	1
(n = 73)							(83.5)	(15.1)	(1.4)

Detoxification enzyme phenotype frequencies (%) in survivors.

Table 35

	A	AA	B	A/B	BB	Null	EM	HET	PM	χ^2	*p*
GSTM1	3		6	1		9				4.09	0.253
(n = 19)	(15.6)		(31.6)	(5.3)		(47.4)				(DF = 3)	
GSTM3		16		3	1					0.57	0.750
(n = 20)		(80)		(15)	(5)					(DF = 2)	
GSTT1	17					3				1.40	0.238
(n = 20)	(85)					(15)				(DF = 1)	
GSTP1		5		12	2					1.45	0.486
(n = 19)		(26.3)		(63.2)	(10.5)					(DF = 2)	
CYP2D6							10	8	2	2.15	0.341
(n = 20)							(50)	(40)	(10)	(DF = 2)	
CYP1A1-1							14	4	0	0.81	0.370
(n = 18)							(77.8)	(22.2)		(DF = 1)	
CYP1A1-2							18	1	1	2.27	0.322
(n = 20)							(90)	(5)	(5)	(DF = 2)	

Detoxification enzyme phenotype frequencies (%) in non-survivors.

p = level of significance derived from χ^2 crosstabulation calculations comparing survivors and non-survivors.
DF = degrees of freedom.

Table 36

Risk Factor	Cox hazards Model	Hazard Ratio	χ^2	p	95% confidence interval
Sex	Male v Female	0.70		0.562	0.217 – 2.29
Age	Total	1.01		0.794	0.96 – 1.06
	Old v Young	1.33		0.607	0.45 – 3.96
Differentiation	Total *		10.71	0.005	
	Well v Other	0.15		0.038	0.15 – 0.89
	Poor v Other	4.66		0.012	1.40 – 15.52
TNM stage	Total	1.50		0.069	0.97 – 2.33
Nodes	Yes v No	4.62		0.006	1.55 – 13.79
Tobacco	Total *		1.91	0.592	
	Ever v Never	0.36		0.187	0.80 – 1.64
	Heavy v Nil	1.55		0.437	0.51 – 4.65
Alcohol	Total *		8.76	0.033	
	Ever v Never	0.26		0.198	0.34 – 2.01
	Heavy v Nil	2.98		0.051	0.99 – 8.93
GSTM1	Total *		1.40	0.705	
	Null v *A* + *B* + *AB*	0.71		0.554	0.22 – 2.23
	A v *Null* + *B* + *AB*	1.43		0.562	0.43 – 4.78
	B v *Null* + *A* + *AB*	1.49		0.546	0.40 – 5.53
	AB v *Null* + *A* + *B*				
GSTT1	*Null* v *A*	.934		0.918	0.25 – 3.43
GSTM3	Total *		2.31	0.315	
	AA v *AB* + *BB*	4.16		0.171	0.54 – 32.0
	AB v *AA* + *BB*	0.35		0.307	0.05 – 2.66
	BB v *AA* + *AB*				
GSTP1	Total *		5.62	0.063	
	AA v *AB* + *BB*	0.37		0.196	0.08 – 1.68
	AB v *AA* + *BB*	5.0		0.038	1.10 – 22.86
	BB v *AA* + *AB*				
CYP2D6	Total *		1.0	0.607	
	EM v *HET* + *PM*	0.58		0.324	0.19 – 1.72
	HET v *EM* + *PM*	1.57		0.433	0.51 – 4.8
	PM v *EM* + *HET*	1.37		0.683	0.30 – 6.20
CYP1A1-1	Total *		1.94	0.163	
	EM v *HET* + *PM*	0.40		0.178	0.10 – 1.52
	HET v *EM* + *PM*	2.50		0.178	0.66 – 9.57
	PM v *EM* + *HET*				
CYP1A1-2	Total *		0.54	0.763	
	EM v *HET* + *PM*	0.81		0.783	0.18 – 3.67
	HET v *EM* + *PM*	1.51		0.591	0.33 – 6.86
	PM v *EM* + *HET*				

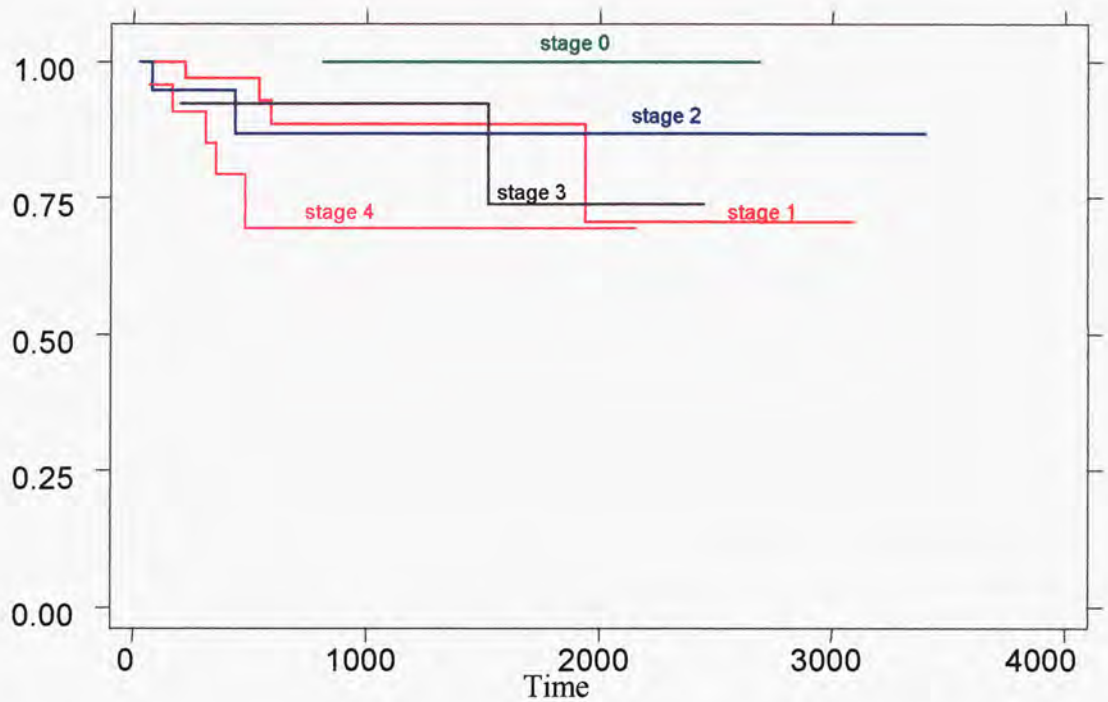
The log-rank test (*) and Cox proportional hazards model of all potential independent risk factors for local recurrence.

Table 37

Risk Factor	Hazard Ratio	*p*	95% confidence interval
Cervical nodes	6.20	0.011	1.51 – 25.33
Alcohol	2.02	0.049	1.0 – 4.07
GSTP1 AB	3.94	0.098	0.79 – 19.49
Differentiation	1.76	0.208	0.73 – 4.24

Cox proportional hazards model of the most significant combined risk factors for local recurrence.

Figure 48



Kaplan-Meier survival estimates for local recurrence by TNM stage at initial diagnosis.

Time is time to first recurrence in days from date of diagnosis.

GSTP1 was the only detoxification enzyme genotype shown to have a possible association with local recurrence. The GSTP1 phenotype frequency differences between patients who did, and who did not develop a local recurrence, approached significance at the 5% level (Tables 38 and 39). This was due to the higher GSTP1 AB phenotype frequency in the patients developing local recurrence (Yate's $\chi^2_1 = 4.33, p = 0.038$).

Table 38

	A	AA	B	A/B	BB	Null	EM	HET	PM
GSTM1	23		14	5		46			
(n = 88)	(26.1)		(15.9)	(5.7)		(52.3)			
GSTM3		63		20	4				
(n = 87)		(72.4)		(23)	(4.6)				
GSTT1	64					22			
(n = 86)	(74.4)					(25.6)			
GSTP1		35		41	12				
(n = 88)		(39.8)		(46.6)	(13.6)				
CYP2D6							54	22	11
(n = 87)							(62.1)	(25.3)	(12.6)
CYP1A1-1							68	11	0
(n = 79)							(86.1)	(13.9)	
CYP1A1-2							68	10	2
(n = 80)							(85)	(12.5)	(2.5)

Detoxification enzyme phenotype frequencies (%) in patients without local recurrence.

Table 39

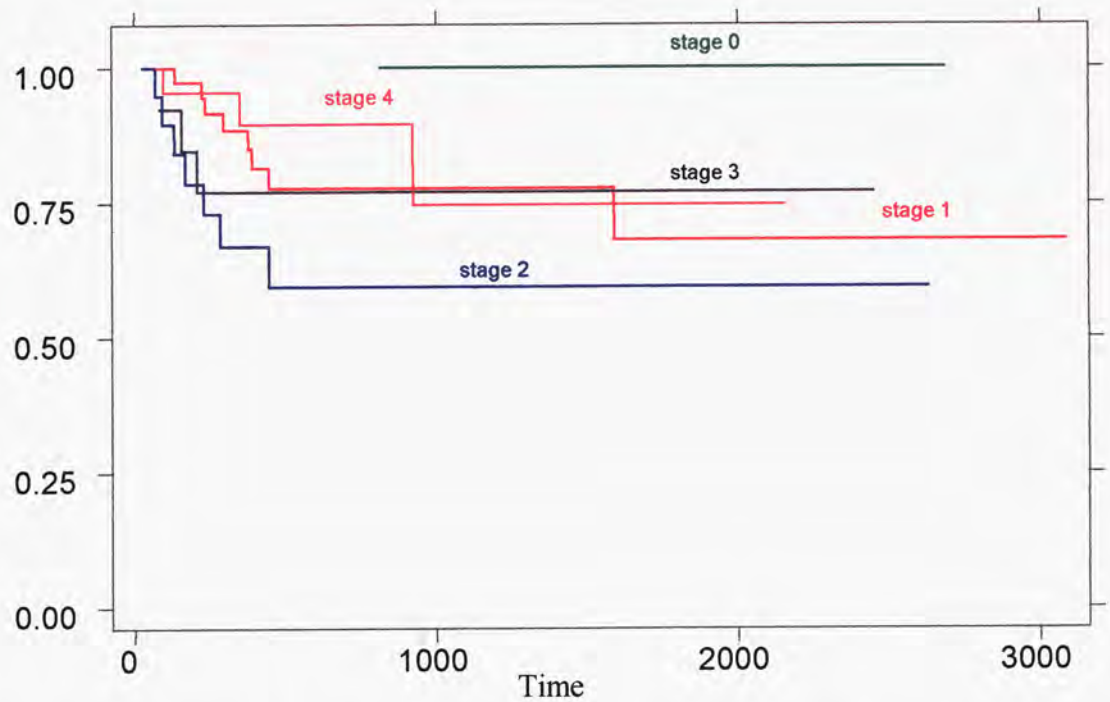
	A	AA	B	A/B	BB	Null	EM	HET	PM	χ^2	*p*
GSTM1	4		3	0		5				1.63	0.653
(n = 12)	(38.5)		(23)			(38.5)				(DF = 3)	
GSTM3		12		1	0					2.45	0.294
(n = 13)		(92.3)		(7.7)						(DF = 2)	
GSTT1	10					3				0.04	0.846
(n = 13)	(76.9)					(23.1)				(DF = 1)	
GSTP1		2		10	0					5.95	0.052
(n = 12)		(16.7)		(83.3)						(DF = 2)	
CYP2D6							6	5	2	1.27	0.53
(n = 13)							(46.1)	(38.5)	(15.4)	(DF = 2)	
CYP1A1-1							9	3	0	0.98	0.322
(n = 12)							(75)	(25)		(DF = 1)	
CYP1A1-2							11	2	0	0.40	0.820
(n = 13)							(84.6)	(15.4)		(DF = 2)	

Detoxification enzyme phenotype frequencies (%) in patients with local recurrence.

p = level of significance derived from χ^2 crosstabulation calculations comparing patients without nodal metastases with patients who developed nodal metastases. DF = degrees of freedom.

The Cox hazards model did not identify overall TNM stage at presentation as being a significant prognostic factor in the development of cervical node metastasis. However, TNM stage at presentation did influence the subsequent development of cervical lymph node metastases (Figure 49).

Figure 49



Kaplan-Meier survival plots of time to first cervical lymph node metastasis by TNM stage at initial diagnosis.

Time is time in days to first lymph node metastasis from initial diagnosis.

Local recurrence, poor tumour differentiation and the *CYP2D6* EM and *HET* genotypes were the only independent risk factors found to be significantly associated with the development of cervical lymph node metastasis (Table 40). Of these 4 risk factors only the *CYP2D6* EM genotype remained a significant prognostic factor after correcting for their combined effect (Table 41). Similarly, after correcting for the effects of age, sex, tobacco and alcohol the *CYP2D6* EM genotype was a significant prognostic factor for the development of cervical lymph node metastasis ($p = 0.006, HR = 0.24, 95\%\ \text{CI} = 0.09 - 0.66$). The *CYP2D6* EM genotype conferred a significantly reduced risk for cervical node metastasis, i.e. the *CYP2D6* genotype appears to be protective with respect to the development of cervical lymph node metastasis in this patient population.

The individual contributions of each of the **3** *CYP2D6* genotypes to the risk of developing cervical node metastasis is shown graphically in the Kaplan-Meier survival plot (Figure 50). Patients with the PM and HET genotypes developed nodal metastases significantly earlier and more frequently than those with the EM genotype. The Kaplan-Meier plot suggests that the PM and HET genotypes carry an almost identical risk for the development of cervical node metastasis, indicating that the increased risk is mediated by the loss of a single normal allele.

The *GSTP1BB* genotype was also positively associated with the development **of** cervical node metastases, which approached significance at the 5% level (Table 40).

Table 40

Risk Factor	Cox hazards Model	Hazard Ratio	χ^2	P	95% confidence interval
Sex	Male v Female	0.83		0.692	0.33 – 2.06
Age	Total	1.02		0.206	0.98 – 1.07
	Old v Young	2.32		0.070	0.93 – 5.74
Differentiation	Total *		10.68	0.005	
	Well v Other	0.20		0.011	0.06 – 0.69
	Poor v Other	3.28		0.022	1.19 – 9.04
TNM stage	Total	0.99		0.967	0.71 – 1.39
Recurrence	Yes v No	3.29		0.010	1.33 – 8.16
Tobacco	Total *		4.89	0.180	
	Ever v Never	1.03		0.946	0.42 – 2.56
	Heavy v Nil	0.57		0.228	0.23 – 1.41
Alcohol	Total *		4.03	0.259	
	Ever v Never	1.79		0.206	0.72 – 4.46
	Heavy v Nil	0.96		0.934	0.37 – 2.48
GSTM1	Total *		4.22	0.239	
	Null v *A* + *B* + *AB*	0.89		0.796	0.36 – 2.19
	A v *Null* + *B* + *AB*	0.70		0.530	0.23 – 2.19
	B v *Null* + *A* + *AB*	2.38		0.079	0.90 – 6.28
	AB v *Null* + *A* + *B*				
GSTT1	*Null* v *A*	1.12		0.751	0.40 – 3.58
GSTM3	Total *		1.19	0.552	
	AA v *AB* + *BB*	1.86		0.322	0.54 – 6.34
	AB v *AA* + *BB*	0.46		0.293	0.11 – 1.97
	BB v *AA* + *AB*	0.97		0.980	0.13 – 7.29
GSTP1	Total *		4.07	0.131	
	AA v *AB* + *BB*	0.62		0.362	0.22 – 1.72
	AB v *AA* + *BB*	0.84		0.698	0.34 – 2.06
	BB v *AA* + *AB*	2.69		0.057	0.97 – 7.49
CYP2D6	Total *		10.61	0.005	
	EM v *HET* + *PM*	0.23		0.003	0.09 – 0.60
	HET v *EM* + *PM*	3.0		0.014	1.15 – 7.21
	PM v *EM* + *HET*	2.21		0.158	0.73 – 6.65
CYP1A1-1	Total *		0.04	0.834	
	EM v *HET* + *PM*	0.88		0.834	0.25 – 3.01
	HET v *EM* + *PM*	1.14		0.834	0.33 – 3.93
	PM v *EM* + *HET*				
CYP1A1-2	Total *		1.52	0.468	
	EM v *HET* + *PM*	1.64		0.506	0.38 – 7.10
	HET v *EM* + *PM*	0.35		0.314	0.05 – 2.65
	PM v *EM* + *HET*	2.03		0.492	0.27 – 15.29

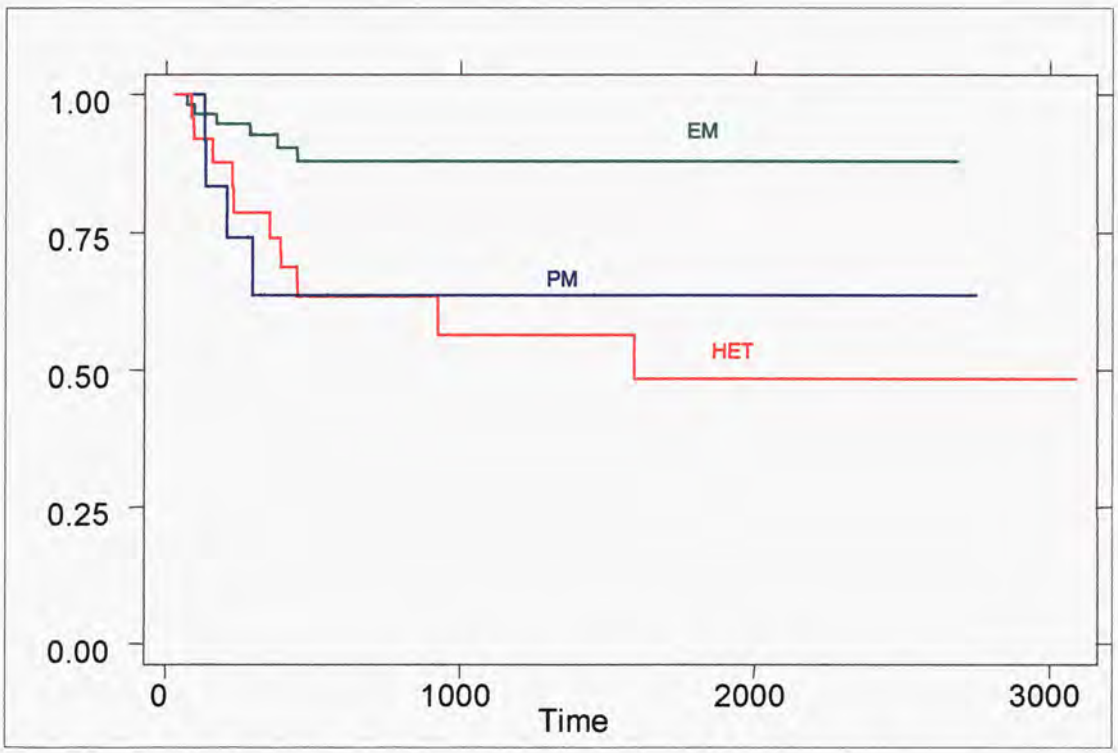
The log-rank test (*) and Cox proportional hazards model of all potential independent risk factors for cervical lymph node metastasis.

Table 41

Risk Factor	Hazard Ratio	p	95% confidence interval
CYP2D6 EM	0.18	0.015	0.05 – 0.7
CYP2D6 HET	0.87	0.814	0.26 – 2.86
Poor differentiation	2.11	0.225	0.63 – 7.07
Recurrence	1.92	0.246	0.64 – 5.78

Cox proportional hazards model of the combined effect of the most significant independent risk factors for cervical lymph node metastasis.

Figure 50



Kaplan-Meier survival plots of time to first cervical lymph node metastasis by CYP2D6 phenotype.

Time is time in days to first lymph node metastasis from initial diagnosis.

Mantel-Haenszel comparison of the Kaplan-Meier survival estimates for cervical node metastasis by *CYP2D6* genotype (Figure 50) demonstrated a statistically significant difference between the 3 genotypes ($\chi^2_1 = 1.08, p = 0.005$) confirming the results of the log rank test (Table 40).

There was a significant difference between CYP2D6 phenotype frequencies in patients who did and did not develop a nodal metastasis during the study period (Tables 42 and 43). This was as a result of the EM frequency in patients developing nodal metastases being significantly lower than in those with no nodal disease (Yates' $\chi^2_1 = 7.88, p = 0.005$). The high PM phenotype frequency (20%) in patients with nodal metastases was not significantly different to that seen in patients with no nodal metastases almost certainly due to the small numbers involved ($\chi^2_1 = 1.08, p = 0.30$).

An enlarged photograph of a representative *CYP2D6B* gel illustrating the EM, HET and PM bands is shown in Figure 51.

Table 42

	A	AA	B	A/B	BB	Null	EM	HET	PM
GSTM1	23		11	5		42			
(n = 81)	(28.4)		(13.6)	(6.2)		(51.8)			
GSTM3		58		19	3				
(n = 80)		(72.5)		(23.7)	(3.7)				
GSTT1	58					21			
(n = 79)	(73.4)					(26.6)			
GSTP1		32		42	7				
(n = 81)		(39.5)		(51.8)	(8.6)				
CYP2D6							54	17	9
(n = 80)							(67.5)	(21.2)	(11.2)
CYP1A1-1							61	11	0
(n = 72)							(84.7)	(15.3)	
CYP1A1-2							61	11	1
(n = 73)							(83.5)	(15.1)	(1.4)

Detoxification enzyme phenotype frequencies (%) in patients without cervical lymph node metastases.

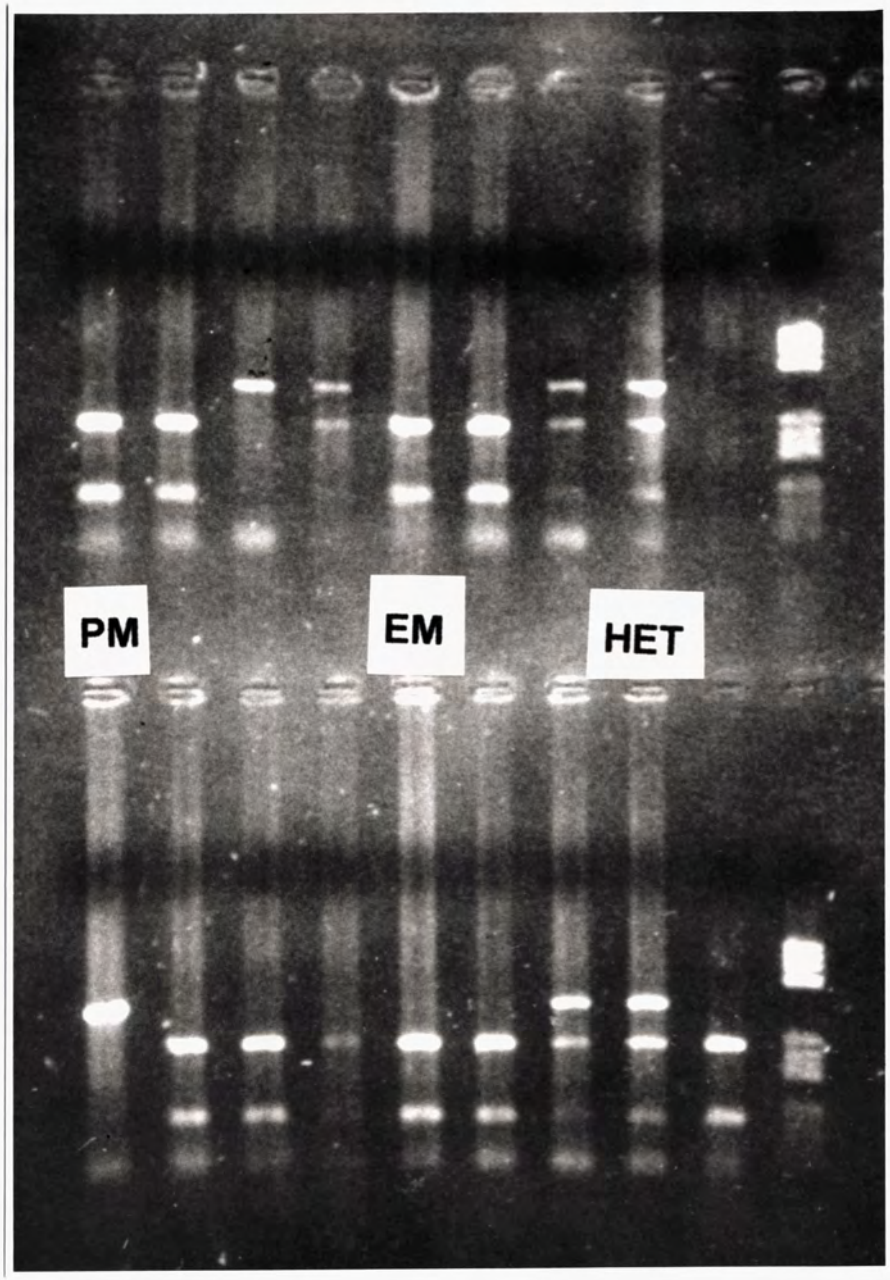
Table 43

	A	AA	B	A/B	BB	Null	EM	HET	PM	χ^2	*p*
GSTM1	4		6	0		9				4.47	0.216
(n = 19)	(21)		(31.6)			(47.4)				(DF = 3)	
GSTM3		17		2	1					1.84	0.40
(n = 20)		(85)		(10)	(5)					(DF = 2)	
GSTT1	16					4				0.37	0.545
(n = 20)	(80)					(20)				(DF = 1)	
GSTP1		5		9	5					4.79	0.092
(n = 19)		(26.3)		(47.4)	(26.3)					(DF = 2)	
CYP2D6							6	10	4	9.59	0.009
(n = 20)							(30)	(50)	(20)	(DF = 2)	
CYP1A1-1							16	3	0	0	0.956
(n = 19)							(84.2)	(15.8)		(DF = 1)	
CYP1A1-2							18	1	1	2.27	0.322
(n = 20)							(90)	(5)	(5)	(DF = 2)	

Detoxification enzyme phenotype frequencies (%) in patients with cervical lymph node metastases.

p = level of significance derived from χ^2 crosstabulation calculations comparing patients without nodal metastases with patients who developed nodal metastases. DF = degrees of freedom.

Figure 51

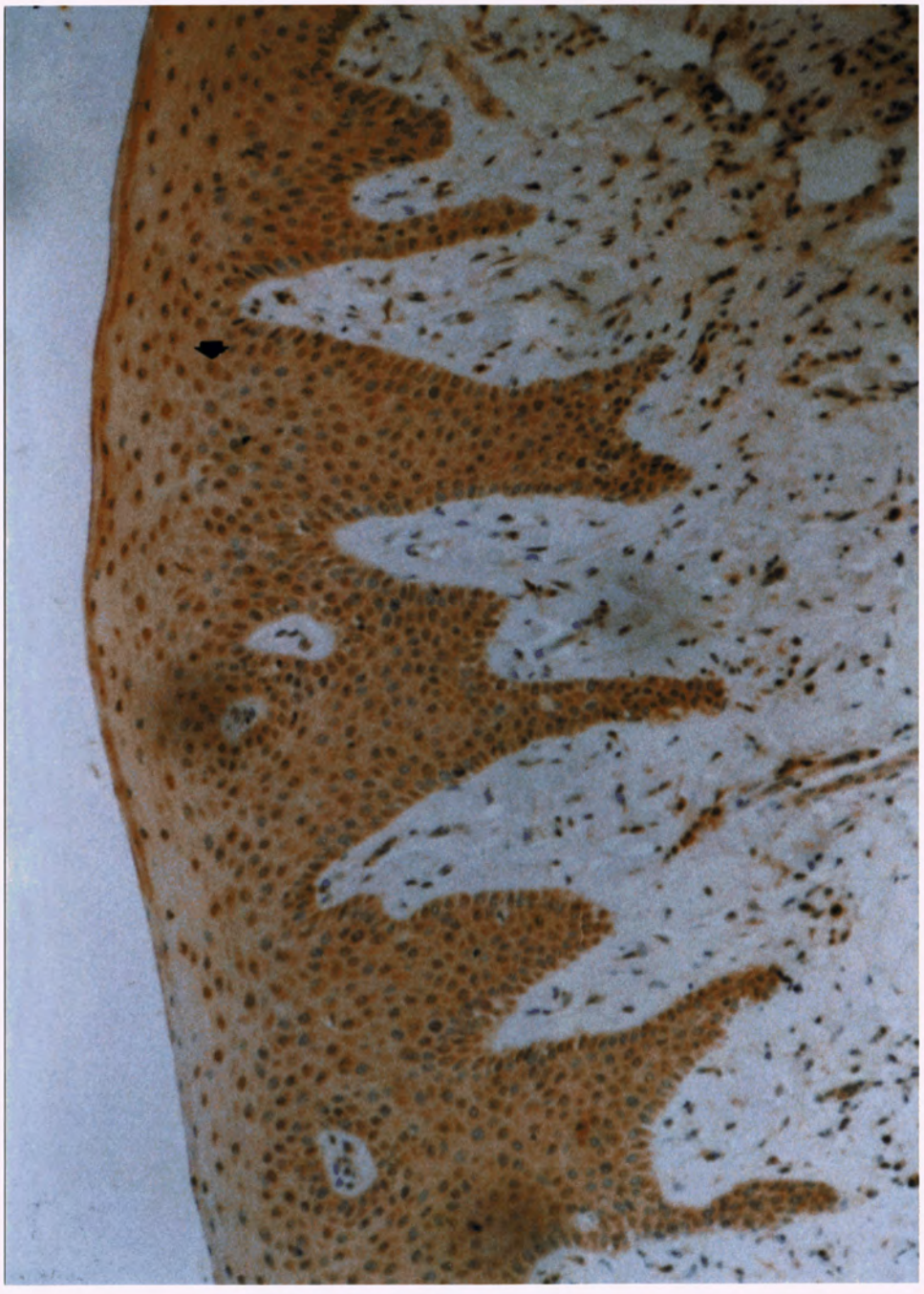


Photograph of a CYP2D6 B gel Polaroid detailing the typical *EM*, *HET* and *PM* banding pattern.

3.4 CYP2D6 Immunohistochemistry

Strong CYP2D6 expression was demonstrated in all sections **of** normal oral mucosa examined including gingiva, tongue, buccal mucosa, lip and hard palate. The majority of sections examined displayed strong cytoplasmic expression in the epidermis, particularly in the stratum spinosum and also in minor salivary glands and striated muscle. (Figure 52).

Figure 52



Photomicrograph of section of normal labial mucosa showing CYP2D6 immunostaining (arrow). x125 magnification.

Chapter 4

DISCUSSION

4.1 Review

All of the patients in the study group were extremely well characterised in terms of their demographics, risk factors, tumour, treatment and outcome affording an excellent opportunity to study the effects of the chosen polymorphisms on their susceptibility to oral cancer in comparison to a large group of oral cancer-free North Staffordshire patients. Although only 106 patients were recruited into the study group, oral cancer is an uncommon condition and very few studies of this type have reported on larger numbers **of** patients.

The ability to record and analyse a standardised clinical data set on all patients and correlate the findings with their detoxification enzyme genotype by using the computerised cancer database greatly facilitated the conduct of the study. Without it, it is likely that many data items would have been missed reducing the power of the subsequent data analysis.

The known risk factors for poor outcome such as poor tumour differentiation, high TNM stage, local recurrence and cervical lymph node metastasis were all identified as being statistically significant risk factors in the study population. This is good evidence that the clinical data recorded is reliable and that the North Staffordshire and Leeds oral cancer patients are typical of similar patients previously reported world-wide with oral cancer.

It was never a stated aim of this study to investigate the relationship between putative risk factors and patient outcomes because of the problems with short follow-up times. Rather, the main aim was to investigate oral cancer susceptibility in a large, homogenous and extremely well characterised patient sample. Nonetheless, the study has generated interesting pilot outcome data worthy of further analysis. While it is likely that some

factors that to date have not been shown to be significant in this group of patients will emerge in due course, it is unlikely that factors already shown to be significant will cease to be so over the same time period. This is especially likely to be the case for those factors that have also been shown to be significant by other workers in previous studies.

To a large extent I believe that the results of this study have enabled me to answer the **3** questions which generated its' original aims, namely:

- Is oral cancer susceptibility related to polymorphisms at loci coding for specific cytochrome P450 and glutathione S-transferase detoxification enzymes
- Is the *CYP2D6 PM* genotype a risk factor for oral cancer because of reduced detoxification of tobacco and/or alcohol-derived carcinogens
- Can any of these polymorphisms be used to improve prognosis prediction and hence improve outcome in an individual patient.

The study has clearly shown that individuals who have the *CYP2D6* PM genotype are at significantly increased risk of developing oral squamous cell carcinoma than those with the normal wild type EM genotype. None of the other CYP and GST genotypes investigated were shown to be significantly associated with oral cancer susceptibility.

However, the reasons for the increased risk in the *CYP2D6* PM patients are unclear and have not been determined by this study. The finding that CYP2D6 enzyme is strongly expressed in normal oral epithelium is fascinating and suggests that the enzyme may be important for the detoxification of xenobiotics that come into contact with the oral mucosa.

However, my study hypothesis that increased oral cancer risk in *CYP2D6* PM patients is mediated *via* reduced phase 1 metabolism of tobacco-smoke derived carcinogens such as

NNK is not supported by the study data. Therefore, it is likely that some other mechanism or an as yet unknown carcinogen is involved.

Allele frequencies were compared between the oral cancer patients and the cancer-free control patients in order to derive data on susceptibility by genotype. However, clinical data was not available for the control patients and so it was not possible to use more powerful statistical models such as **Cox** regression to control for other variables such as age, sex, tobacco and alcohol consumption. While I think it unlikely that such analysis would have reduced the significance of the association between *CYP2D6* genotype and oral cancer susceptibility, I do believe that it is important to achieve better clinical characterisation of the control patients in future studies of this type.

Although data analysis suggests that the *CYP2D6 PM* genotype is associated with poor outcome as a result of earlier and more frequent cervical lymph node metastases I do not believe that the data is not strong enough at present to enable its' presence or absence to be used as a prognostic factor to direct patient management strategies. For example, I do not believe that the data is sufficiently robust to suggest that all *CYP2D6 PM* patients with T1N0M0 oral tumours should undergo elective neck dissection. However, if this data is confirmed in 2 years time when actuarial survival data is available I think there may well be a case for such treatment, particularly with lingual carcinomas.

4.2 The Database

The computerised database proved invaluable for the collection, manipulation and analysis of both clinical and research data throughout this study. Its' usefulness extended beyond the strict confines of the study protocol and it has become an integral part of the infrastructure of our joint head and neck cancer clinics. Every speciality involved with the management of head and neck cancer in our hospital now enters clinical information on their patients onto a computerised database developed from the program used in this study.

The program has now been adopted by several other hospitals in the UK enabling them to audit their head and neck cancer practices and eventually to compare their results. This is an important and encouraging development in a frequently neglected area of patient care (Edwards et al., 1997).

Ideas firmly based on the dataset used in this database are now at the heart of a national data set for head and neck cancer sponsored by the British Association of Head and Neck Oncologists.

Accurate, comprehensive and standardised data collection covering not only the clinical findings at presentation but also management protocols, complications and outcomes are vital if we are to improve the standards of care, disease free survival times and the quality of life of our patients with oral cancer, (Tobias, 1987).

4.3 Oral Cancer Patients' Characteristics

The male:female ratio of the patient population in this investigation was 1.6:1 which is in good agreement with previous studies (Langdon *et al.* 1977; Henk and Langdon,1985; Worrall and Corrigan,1995). The mean population age at presentation was 63.3 years which is also in good agreement with previous studies (Langdon *et al.* 1977; Worrall and Corrigan, 1995). On average, males were 6 years younger at diagnosis than females (61 years and 67 years respectively). This is in contrast to Langdon *et al.* (1977) who found virtually no difference in mean age at diagnosis (63.6 years for males and 63.7 years for females) and Waterhouse (1974) who found that males were an average of 3 years older than females at diagnosis (63.5 years and 60.6 years respectively),

However, the present data are very similar to those previously reported by the author in a study of 116 patients (none included in the current investigation) with oral cancer presenting to the Leeds Dental Institute (Worrall and Corrigan,1995).

While Binnie *et al.* (1972) reported that 72% of all oral cancers occurred in patients aged between the ages of 55 years and 75 years, the present study found that this figure had fallen to 62% and that 19.8% of patients were under 55 years of age at diagnosis. This may well be further confirmation that oral cancer is affecting a younger population than in the past, particularly amongst males (Hindle *et al.*, 1996).

The TNM stage distribution at presentation of patients in this study was remarkably similar to the findings from an earlier project, particularly the distribution of stage 3 and 4 tumours (Worrall and Corrigan,1995). Almost a quarter of all patients presented with a stage 4 tumour while over 12% presented with a stage 3 tumour. This is a reversal of the findings reported by Henk and Langdon (1985) and although extremely worrying as it may reflect a tendency to later patient presentation, it is likely that it also reflects an increasing

willingness by clinicians to "upstage" some tumours, particularly lingual carcinomas. Indeed, there is some evidence that the current patient group actually presented with earlier stage disease than in other studies. Of the 118 patients with oral carcinoma reported by Rich and Radden (1984) 45% had early (stage 1 and 2) disease and 55% had late (stage 3 and 4 disease) at presentation. In the current study, 65% **of** patients presented with early disease.

Less than 16% **of** all patients in the study stated that they had never drunk alcohol or used tobacco products, while over 20% admitted to being heavy consumers of tobacco and alcohol products. Older patients were more likely to be never smokers and never drinkers (25%) than younger patients (5.5%) while younger patients were more likely to be heavy smokers and drinkers (33.3%) than older patients (7.7%). Over 77% of heavy drinkers and heavy smokers were young males, while over 68% of the never drinkers and never smokers were old females.

Similar findings were reported by Hodge ***et al.*** (1985) who found that of 945 patients with carcinoma of the aero-digestive tract only 3.4% had never used tobacco products and that the non-smokers were significantly older than the smokers.

The CYP2D6 enzyme was shown to be expressed in both normal oral mucosa and also in oral carcinomas. This is an important finding because previous work has suggested that CYP2D6 is primarily an hepatic enzyme which is rarely expressed outside the liver (Ingleman-Sundberg,1995). Zhou et al. (1996) using both polyclonal and monoclonal antibodies to various CYP and GST demonstrated that CYP1A1, CYP1A2, CYP2C9, CYP2E1 and CYP3A4 are present in normal human gingiva but failed to demonstrate the presence of both CYP2D6 and CYP2B6. The reasons for the disparity in findings with the

present study are unclear but may be due to the increased specificity **of** the anti-CYP2D6 antibody utilised by our laboratory.

4.4 Oral Cancer Susceptibility

Oral squamous cell carcinoma has a multifactorial aetiology and is the terminal result of many different mutational pathways (Cox *et al.,* 1991; Balaram *et al.,* 1995). Evidence is also emerging that in some patients head and neck cancer may be familial. Foulkes et al. (1996) investigated 1429 first degree relatives of 242 patients with head and neck cancer and found their relative risk for head and neck cancer compared to controls to be 3.79 (95% CI = 1.11 – 13.0). The relative risk rose to 7.89 (95% CI = 1.5 – 41.6) if the index case had multiple primary head and neck tumours. The greatest risks were seen with pharyngeal and oral cancer (relative risks 4.24 and 3.82 respectively) but there was no demonstrable increased risk associated with a family history of cancer outside of the head and neck.

It was hypothesised at the commencement of this study that certain individuals are at increased risk of developing oral cancer not only because of increased exposure to mutagenic environmental agents consequent on their lifestyle, but also because of a reduced ability to deal adequately with these agents due to inherited genetic defects in the loci coding for phase 1 and phase 2 detoxification enzymes.

As it is highly unlikely that oral cancer risk in an individual patient could be predicted from a single genetic marker, several detoxification enzyme polymorphisms were examined in the oral cancer patient group and their effects on susceptibility and outcome investigated both alone and in combination.

Detoxification enzyme polymorphisms have been shown to confer altered disease susceptibility in humans. Patients with the *CYP2D6* PM genotype possess a 2-3 fold increased risk of developing Parkinson's disease (Smith *et. al.,* 1992) and both astrocytoma and meningioma have been reported to be more frequent in patients who are *CYP2D6 PM*

or *GSTT1 null* (Elexpuru-Camiruaga *et. al.,* 1995). The *GSTM1 null* and *GSTT1 null* genotype frequencies have been reported to be significantly more common in patients with head and neck squamous cell carcinomas in general (Trizna *et. al.,* 1995) and oral squamous cell carcinomas in particular (Hung *et al.*, 1997).

However, of all the phase 1 and phase 2 detoxification enzyme polymorphisms investigated in this study, only *CYP2D6* showed a significant association with oral cancer susceptibility ($\chi^2_2 = 10.85, p = 0.005$).

In a study of 380 German patients with head and neck cancer (120 oral/pharyngeal, 260 laryngeal) Matthias et al. (1998a) found that the *GSTP1* AA genotype frequency was significantly lower in patients with oral/pharyngeal cancer than in 180 controls after correcting for the effects of age and gender ($p = 0.003$, OR = 0.47). It was not possible to perform the same statistical analysis in the present study because the control group data did not permit logistic regression analysis. However, it is interesting to note that the *GSTP1* AA, *GSTP1 AB* and *GSTP1 BB* allele frequencies observed in the oral cancer group in this study (37%, 51% and 12%) were virtually identical to those reported by Matthias et al. (1998a) in their oral/pharyngeal cancer group (37.5%, 50.8% and 11.7%)

Although the *GSTT1 null* allele frequency was higher in the oral cancer patients than the control group this difference was not statistically significant ($\chi^2_1 = 2.18, p = 0.14$). No association was found between the *GSTM1 null* genotype and oral cancer risk ($\chi^2_1 = 1.06, p = 0.30$).

13% of the oral cancer patients in the current study were both *GSTM1 null* and *GSTT1 null* whereas **9.6%** of the North Staffordshire control patients had this combined *null* genotype (Yates' $\chi^2_1 = 2.6, p = 0.105$). Hung *et al.* (1997) reported an increased oral cancer risk (OR = 3.1) in Taiwanese males who had the combined *GSTM1 null* / *GSTT1*

null genotype (7.3%) compared to disease free controls (20.3%) after correcting for age, smoking, betel chewing, alcohol consumption and ethnicity. By comparing the uncorrected *GSTM1* and *GSTT1* genotype frequencies using published data between cases and controls in their study group (Hung *et al.,* 1997, page 903 Table 2) I determined that they were similar to those found in the present study and that there was no statistical difference between the 2 groups (Yates' ($\chi^2_1 = 2.8$, $p = 0.094$). This finding emphasises the point that detoxification enzyme genotypes do not act as risk factors per *se* but in conjunction with exposure to xenobiotics.

The *GSTT1 null* genotype frequency in the Taiwanese control group (52.9%) was significantly higher ($p < 0.001$) than in the North Staffordshire control group (25.3%) highlighting the need for caution when comparing detoxification enzyme genotype frequencies and disease in different ethnic groups.

The current study data and previous work from our laboratory (Deakin *et al.*, 1996) do not support the observations by Trizna *et al.* (1995) that both the *GSTM1 null* and the *GSTT1 null* genotypes carry an increased risk for the development of oral cancer. There are a number of possible explanations for this.

Firstly, although the authors demonstrated an increased odds ratio (2.37) in the GSTM1 group, which was statistically significant ($\chi^2_1 = 6.38, p = 0.012$), the odds ratio for the GSTT1 group (1.47) was not statistically significant ($\chi^2_1 = 1.08, p = 0.30$) ($\chi^2_1$ and p values not originally reported but derived from the published data).

Secondly, the authors did not distinguish between patients with oral, pharyngeal and laryngeal cancer. **As** these three cancers are known to have different aetiological factors, with smoking playing a larger role in laryngeal cancer and alcohol being more

significant in the aetiology of pharyngeal cancer (Elwood ***et al.***, 1984), confounding factors may well have been present in the case group.

Thirdly, the racial composition of the case and the control groups (which also contained relatives of the cases) was not defined. Thus the known significant racial variation in allele frequencies may have lead to errors in interpretation of the study data.

The *CYP2D6* PM genotype was observed significantly more frequently in the oral cancer patients than it was in the control group. Together with the immunohistochemical demonstration of CYP2D6 enzyme activity in samples of normal oral mucosa, these data initially suggest that reduction or absence of normal CYP2D6 activity leads to a local perturbation of xenobiotic detoxification resulting in an increased risk of oral squamous cell carcinoma in some patients.

However, of the 22 oral cancer patients who were heavy drinkers and heavy smokers 18 (81.8%) had the *CYP2D6* EM genotype compared to only 1 (4.5%) with the *CYP2D6 PM* genotype. The *CYP2D6* EM genotype was also more common in the younger patient group. The 89% *CYP2D6* EM allele frequency in young heavy smokers and heavy drinkers was significantly higher than in the North Staffordshire controls ($\chi^2_1 = 5.05$, $p = 0.025$). While the *CYP2D6* EM allele frequency was higher in heavy drinkers and heavy smokers (81.8%) than in non-drinkers and non-smokers (57.1%) the difference was not statistically significant ($\chi^2_1 = 2.59$, $p = 0.11$), probably due to the small number of patients in each group (22 and 14 respectively).

These data are similar to the findings in lung cancer patients reported by Caporaso *et al.* (1990) who, in a case-control study of 188 patients (96 with lung cancer and 92 with non-malignant pulmonary diseases) found that patients with the CYP2D6 extensive metaboliser phenotype *(EM)* were at significantly greater risk of developing lung cancer

than those who were intermediate *(HET)* or poor metabolisers *(PM)* (OR = 6.1, 95% CI = 2.1 – 17.1). The authors speculated on the importance of this finding as a potential marker for lung cancer risk ". ..if a compound that undergoes an oxidation by the CYP2D6 enzyme could be identified in tobacco smoke, a completely new strategy for prevention could be devised" (Caporaso ***et al.,*** 1990).

The following year their colleagues at the National Cancer Institute in Bethesda, **USA** reported the discovery that the tobacco-smoke derived nitrosamine, **4-**(methylnitrosamino)-1-(3 pyridy1)-1-butanone **(NNK)**, the most potent of all tobacco-smoke derived carcinogens, is metabolically activated by CYP2D6 resulting in increased mutagenicity and reduced cell survival time (Crespi ***et al.,*** 1991).

One possible explanation for the preponderance of *CYP2D6 EM* individuals in the heavy smoking and heavy drinking group is that they represent a subgroup of oral cancer patients whose risk factor profile is different from the *CYP2D6 PM* oral cancer patients. Thus, it is not that *CYP2D6* PM is a risk factor because of reduced detoxification of carcinogens present in tobacco and alcohol, rather *CYP2D6 EM is* a risk factor in some individuals who consume large quantities of tobacco and alcohol because they are capable of activating procarcinogens in these products. It is interesting to speculate whether within this sub-group of patients there are individuals who are "ultra-rapid metabolisers" (Smith ***et al.,*** 1995) and as such capable of enhanced carcinogen activation.

The *CYP2D6* PM genotype was observed more frequently in the older oral cancer patients. **A** similar finding was reported by Caporaso ***et al.*** (1990) who found that the *PM* frequency was more prevalent in their ageing control patient group (non-malignant pulmonary disease) but not their lung cancer group. The authors speculated that this

phenomenon might be a result of the more susceptible *EM* individuals succumbing to lung cancer at an earlier age.

Such an explanation may also apply to the oral cancer patients but I believe it is unlikely in view of the longer survival experienced by these patients compared to those with lung cancer. **A** more plausible explanation is that *CYP2D6* EM individuals who smoke and drink heavily develop their oral cancer early because of phase 1 carcinogen activation. Whereas, in the absence of heavy tobacco and alcohol consumption, oral cancer presents later in life when the *CYP2D6* PM genotype becomes a distinct and separate risk factor. This explanation is consistent with the finding by this study that the *CYP2D6 PM* allele frequencies observed in both the heavy drinking and heavy smoking group (4.5%) and the young patient group (7.5%) were not significantly different to the *CYP2D6 PM* allele frequency observed in the control group (4.5%).

It is interesting to speculate that if some of the *CYP2D6* EM patients had not consumed the large quantities of tobacco and alcohol products that they did at an early age whether they would still have developed oral cancer. The provisional conclusions of this study suggest that many of them would not have.

4.5 Oral Cancer Outcome

It is extremely important to appreciate that although the median follow-up time for patients in this study was 1.7 years, less than 85% of patients had been followed up for more than 6 months and only 44% for more than 2 years at the time of data analysis. Henk and Langdon (1985) have demonstrated that for patients destined to develop a local recurrence, over 69% will do so within the first 6 months following diagnosis and treatment and a further 21% will do so within the following 18 months. **A** similar temporal relationship exists for lymph mode metastases and survival. For example, patients with carcinoma of the mandibular alveolus have been shown to continue to die of their disease up to 10 years following diagnosis and treatment (Langdon *et al.,* 1977). Accepted clinical practice **is** to follow-up patients with oral squamous cell carcinoma regularly until they have been disease-free for a minimum of 5 years or death.

Survival

Perhaps not surprisingly, survival was worse in the older patients. Previous reports have shown that patients younger than 60 years at presentation had a 5-year survival rate of 59.5% compared to 22% for those over 60 years old (Henk and Langdon, 1985). Comparable figures in this study were 8.7% and 24.8% respectively, underlining the need for caution when interpreting outcome data derived from this study.

No association was found between gender and length of survival. While Henk and Langdon (1985) reported that females had better survival times than males, Binnie *et al* (1972) were unconvinced that a real gender difference exists for survival in oral cancer patients.

The finding that patients with poorly differentiated tumours had significantly shorter survival times than those with well differentiated tumours is in agreement with previous reports (Langdon *et al.* 1977; Lighthelm *et al.* 1989). It does not support the assertion of Nason *et al.* (1990) that the association between the degree of tumour differentiation and prognosis is of little significance.

Also in keeping with previous reports (Langdon *et al.* 1977; Henk and Langdon, 1985; Rich and Radden, 1984; Worrall and Corrigan, 1995), prognosis worsened with increasing TNM stage. The reversal of stages 2 and **3** with respect to survival may well reflect the clinical uncertainty associated with the diagnosis of metastatic deposits in cervical lymph nodes (Shah *et al.*, 1990; Woolgar *et al.*, 1994).

Numerous authors have attested to the adverse prognostic significance of cervical lymph node metastases in patients with oral cancer (Hibbert *et al.*, 1983; Henk and Langdon, 1985; Phillips, 1989; Zatterstrom *et* al., 1991). In the present study the development of cervical node metastases following treatment of the primary oral tumour resulted in significantly shorter survival times, as did a recurrence at the primary site.

Even allowing for the short follow-up times, the results of this study with respect to patient survival are in very good agreement with previous reports in showing that patient age, degree of tumour differentiation, tumour stage, local recurrence and cervical lymph node metastases are all independently associated with poor prognosis. Of these 5 independent risk factors, a high TNM stage at presentation and the subsequent development of cervical lymph node metastases carried the worst prognosis.

No statistically significant associations were found between survival and any of the detoxification enzyme genotypes investigated.

Local recurrence

Poorly differentiated tumours were more likely to recur than well differentiated tumours and patients with stage 4 disease at presentation developed local recurrences earlier and more frequently than patients with earlier stage disease. The development of cervical lymph node metastases following initial therapy was significantly associated with recurrent disease at the primary site.

Alcohol was a strong independent predictor for local recurrence although no association was found between tobacco and recurrence. Similar findings were reported by Elwood *et al.* (1984) who, in a case control study of 374 patients with head and neck cancer found that while alcohol and tobacco were both significant independent risk factors for the development of oral cavity cancer, only alcohol remained a risk factor after controlling for other variables such as socio-economic status and dental care. Interestingly, Hindle *et al.* (1996) commented on the fact that oral cancer incidence in the UK has been increasing during a period of falling tobacco consumption. However, while the population consumption of tobacco products has been falling there has been a substantial increase in the consumption of alcohol, particularly amongst young males which many authors feel partly explains the recent changes in oral cancer incidence and age distribution (Hindle and Nally, 1991; Macfarlane *et al.*, 1992). The results from this study also suggest that alcohol is an important risk factor for local recurrence.

*GSTP1**AB*** was the only detoxification enzyme allele shown to have any statistically significant association with local recurrence ($\chi^2 = 4.33, p = 0.038$). The *GSTP1AA* allele frequency in patients who did not develop a local recurrence (39.8%) was over twice as high as in those that did (16.7%) although this difference was not statistically significant ($\chi^2 = 2.42, p = 0.121$). These findings are similar to those reported by Matthias

et al (1998a) who demonstrated significantly lower ($p = 0.003$) levels of the *GSTP1 AA* genotype in patients with orallpharyngeal cancer compared to controls. Their data suggests that individuals with the *GSTP1* ***AA*** genotype have a lower risk of developing oral/pharyngeal cancer, perhaps as a result of enhanced detoxification of environmental carcinogens.

A possible explanation for the protective effect of the *GSTP1* ***AA*** genotype in comparison to the *GSTP1 BB* genotype is that the substitution of Valine for Isoleucine at amino acid number 104 in the latter results in a reduced affinity for electrophilic substrates consequent on changes to the geometry of the hydrophobic substrate-bindingsite (Zimniak. et al., 1994).

The low numbers of patients with local recurrence in the present study may account for the lack of a statistically significant difference between the case and control groups being found.

Cervical lymph node metastasis

Unlike the findings for local recurrence and survival, overall TNM stage at presentation was not significantly associated with nodal metastasis. However, as shown graphically in Figure 57 the time to first nodal metastasis was a function of TNM stage at presentation, although the relationships were different to those seen for local recurrence and survival. Patients with stage 2 and 3 tumours developed new nodal metastases earlier and more frequently than those with stage 1 and 4 tumours. This is, of course, exactly what one would predict from knowledge of tumour behaviour and current clinical management.

The majority of patients with stage 4 disease will have had clinically palpable cervical lymph nodes at presentation. These will have been treated by either a neck

dissection or in a minority of cases by radiotherapy. All patients with stage 2 disease will have had no clinically detectable cervical nodes at presentation but subsequently developed nodal metastases following treatment for their primary tumour.

The likeliest explanations for nodal metastases in the stage 3 and 4 patients are the development of nodal metastases in patients who were clinically N0 at presentation but staged 3 or 4 because of their large tumour size and failure of primary modality treatment in the neck with subsequent nodal recurrence in patients who had nodal disease at presentation.

The strongest predictors for the development of cervical node metastasis were local recurrence, poor tumour differentiation and the *CYP2D6* genotype. The *CYP2D6 HET* and PM genotypes were both associated with an increased risk of nodal metastasis (Figure 58) indicating that the loss of a single *CYP2D6* allele is associated with earlier and more frequent nodal metastases in oral cancer. However, the *CYP2D6* EM genotype was associated with a much lower risk of nodal spread as shown by both the **Cox** hazards model and the significantly lower EM allele frequency in patients with nodal disease (30%) in comparison to those without nodal disease (67.5%) ($\chi^2 = 9.37, p = 0.002$).

Individuals with the *GSTP1 BB* genotype were at increased risk for the development of cervical lymph node metastases ($p = 0.057, HR = 2.69, 95\%\ CI = 0.97 - 7.49$). Taken together with the findings for local recurrence, these data suggest that the *GSTP1 BB* genotype may be a risk factor for disease progression and poor outcome in oral squamous cell carcinoma.

4.6 The *CYP2D6 PM* and *HET* Genotypes and Oral Cancer

In sections 4.3 and 4.4 I have summarised the data available from this study, which support the hypothesis that *CYP2D6* polymorphisms are associated with both an increased risk for, as well as a poorer prognosis of, oral squamous cell carcinoma.

The *CYP2D6* PM genotype was found at a significantly higher frequency in patients with oral cancer compared to a large group of oral cancer-free control patients. Furthermore, both the *CYP2D6 PM* and HET genotypes were associated with the development of earlier and more frequent cervical lymph node metastases.

However, oral cancer patients with the *CYP2D6* EM genotype were the heaviest consumers of tobacco and alcohol products rather than those with the PM or HET genotypes. There was also a definite predilection for the *PM* and HET genotypes to occur in patients who were older at initial presentation than those with the EM genotype. It is thus difficult to reconcile these data with the study hypothesis that reduced detoxification of tobacco smoke-derived carcinogens in individuals with the PM genotype results in an increased oral cancer risk.

In his extensive review of the literature and current knowledge on the cytochrome P450's, Ingleman-Sundberg (1995) concludes that *CYP2D* is essentially a functionless gene and that data purporting to show an association between CYP2D6 pheno- and/or genotypes are controversial. It has also been suggested that the CYP2D6 protein serves no useful function (Rannug *et* al., 1995). Nonetheless, data from this study and others previously cited, clearly shows that patients with the *CYP2D6* PM genotype are at Significantly increased risk from certain diseases including oral squamous cell carcinoma.

Perhaps the comment that these observations represent "some kind of functional association" Ingleman-Sundberg (1995) is the key to this puzzle and that any association between the *CYP2D6* genotype is casual not causal.

There is extensive evidence that the *CYP2D* locus at 22q 13.1 is intimately associated with several putative tumour suppressor genes. Bryan *et al.* (1996) have identified 2 candidate tumour suppressor genes for ovarian carcinoma that flank the *CYP2D* locus. LOH at these loci was observed in 53% of 110 ovarian carcinomas. The authors found that the frequency of LOH at 22q 12 in ovarian cancers increased with increasing tumour stage and concluded that 22q 12 LOH is more relevant to tumour progression than initiation.

Ewing's sarcoma is associated with the specific chromosomal translocation t(11; 22) (q24; q12) resulting in the substitution of a putative RNA-binding domain (EWSR1) for *Hum-FLI-1,* the DNA-binding domain of the human homologue of the mouse *FLI-1* retroviral integration site associated with murine erythroleukaemia (Ben-David ***et al.,*** 1991; Delattre *et al.*,1992). The translocation results in a novel gene sequence that codes for a 656 amino acid protein termed EWS, which appears to be essential for tumorigenesis. Also, because *Hum-FLI-1* is now subject to control by the *EWS* promoter region and not its normal promoter, the translocation results in both deregulation and aberrant activation of the *Hum-FLI-1* target genes (Delattre *et al.,* 1992).

EWS is located in the same 22q 12 region as the gene defect causing neurofibromatosis type 2 (NF2) (Delattre *et al.,* 1992). NF2 and a subset of sporadic meninigiomas are associated with inactivation of the tumour suppressor gene *NF2* located at 22q 12 (Trofatter *et al.*, 1993; De Vitis ***et al.,*** 1996). The *NF2* gene codes for a 587 amino acid moesin (membrane-organising extension spike protein)-ezrin-radixin protein

named merlin (Trofatter *et al.,* 1993). The moesin-ezrin-radixin proteins are involved with linking certain cell membrane proteins to the cytoskeleton (De Vitis *et al.,* 1996).

The cytoskeleton plays a major role in many normal cell functions including changes in cell shape, cell motility, mitosis and anchorage-dependent growth. These functions are also critically involved in neoplastic cell invasion and metastasis (Bernal and Stahel, 1985). Inactivation of *NF2* and aberrant expression or merlin could thus be expected to interfere with many of these functions with a consequent effect on cell growth control and migration (Bernal and Stahel, 1985; Trofatter *et al.,* 1993).

These data are compatible with the findings of the present study in which the *CYP2D6* PM allele frequency was higher in cases than controls, the *CYP2D6* HET allele frequency was higher in non-survivors than survivors, and both the *PM* and HET genotypes were associated with more frequent and earlier cervical lymph node metastases than the EM genotype.

It is thus possible that the *CYP2D6* PM and HET genotypes are linkage markers for mutations at 22q 12, and that it is actually the loss of a tumour suppressor gene at or close to the *NF2* locus that mediates the increased oral cancer susceptibility and early nodal metastases seen with these genotypes.

An extensive literature search (Medline and the Internet) has failed to identify any published work that demonstrates LOH at 22q 12 in oral squamous cell carcinoma. Indeed, in their comprehensive study of LOH on 39 chromosome arms in 52 head and neck squamous cell carcinomas, Field *et al.* (1995) failed to identify LOH on 22q perhaps because the micro-satellite markers used were directed against the wrong region.

An alternative explanation for these findings is that individuals with the *CYP2D6* PM genotype fail to effectively detoxify an as yet unknown substrate involved in oral cavity carcinogenesis. The data on tobacco and alcohol consumption in the *CYP2D6 PM* group in this study would tend to suggest that this putative carcinogen is unlikely **to** be contained in these products.

4.7 Conclusions

The study has shown that not only is the *CYP2D6 PM* genotype associated with an increased risk for the development of oral squamous cell carcinoma but also, together with the *CYP2D6 HET* genotype, with an increased risk for the development of cervical lymph node metastases. Although it was not possible to show any association between the *CYP2D6* PM genotype and survival, it is interesting to speculate that as this group of patients gets older whether an association with poor survival will become apparent. In my opinion, the extremely strong association between cervical lymph node metastasis and poor survival shown by this and other studies makes such an association likely.

Contrary to expectations implicit in my initial working hypothesis, the study demonstrated that it was the oral cancer patients with the *CYP2D6* EM and not the *PM* genotype who were the heaviest consumers of tobacco and alcohol products. This finding casts severe doubt on the idea that oral cancer risk is increased in patients with the poor metaboliser phenotype because of their reduced ability to detoxify carcinogens present in tobacco smoke.

Data analysis suggests that this study has identified 2 distinct sub-groups of oral cancer patients, each with different risk factors for susceptibility and outcome. It appears likely that in one group of patients, inactivation of a tumour suppressor gene at or close to the *NF2* gene on 22q 12 leads to increased susceptibility to, and poor outcome from, oral squamous cell carcinoma. Data analysis suggests that the *CYP2D6* PM and *HET* genotypes are linkage markers for this putative tumour suppressor gene.

The *CYP2D6* EM genotype appears to be a risk factor for oral cancer susceptibility in patients who smoke and consume alcohol heavily, possibly ***via*** phase 1 activation of tobacco smoke- and/or alcohol-derived carcinogens. These patients appear to develop their

tumours at an earlier age than individuals with the *PM* genotype. *CYP2D6 EM* activation of nitrosamines such as 4-(methylnitrosamino)-1-(3 pyridyl)-1-butanone present in tobacco smoke with the subsequent production of mutagenic compounds is an attractive oral tumorigenesis model in these patients and is compatible with the association between smoking, *CYP2D6* EM genotype and lung cancer risk previously reported (Caporaso ***et al.***, 1990; Crespi ***et al.***, 1991). The demonstration by this study that CYP2D6 is expressed locally in the oral mucosa is further support for this hypothesis.

Of all the detoxification enzyme genotypes investigated in this study, CYP2D6 was the only one to have been shown to be significantly associated with both oral cancer susceptibility and outcome. Furthermore, no significant interactions between the detoxification enzymes studied and either oral cancer susceptibility or outcome were observed.

However, the study has also shown that the *GSTP1* genotype appears to play a role in oral cancer tumorigenesis and progression confirming the findings of Matthias et al. (1998a). *GSTP1* ***AA*** appears to be protective while *GSTP1 BB* appears to enhance the risk for the development of local recurrence and cervical node metastasis in oral squamous cell carcinoma. Although the study was unable to show a statistically significant association between the *GSTP1* ***AA*** genotype and oral cancer susceptibility, the very strong correlation of *GSTP1* allele frequencies observed in oral cancer patients in this study with those reported by Matthias et al. (1998a) suggests that an association is likely.

4.8 Study Critique and Suggestions for Future Research

Although I am happy with the study results and I have learned an enormous amount about research methodologies in general and detoxification enzyme polymorphisms in particular, there are several areas where the study could have been improved.

Firstly, the number of patients recruited is less than ideal. While this is a function of the rarity of the disease under investigation this fact should not be allowed to dictate future study protocols. The logical approach is to ensure that all future studies of this type are truly multicentre so that as far as possible, all newly diagnosed patients in the UK are recruited and their data made available for study and audit. The availability of a standardised database and a nationally agreed head and neck cancer minimum dataset greatly facilitates such multicentre collaboration. If study populations become multinational due regard will need to be given concerning the variation in allele frequencies with the racial mix of the population.

Mention has already been made of the need to improve the characterisation of the control patients used in studies of this type. In future I would wish to collect the same demographic and risk factor data on control and study patients. This would allow me to repeat previous studies in which the recruitment of age, sex, ethnicity and risk factor matched control subjects enabled the demonstration of a significant association between the *GSTM1 null* and *GSTT1 null* genotypes both alone (Trizna ***et*** *al.,* 1995) and in combination (Hung *et al.,* 1997) and oral cancer risk after allowing for the effect of known risk factors.

Smoking and alcohol data were not available on the control patients in this study and the numbers **of** oral cancer patients in the non-drinking non-smoking group and heavy drinking heavy smoking group were too small to show any significant differences in allele

frequencies between them. In order to substantiate the hypothesised link between the *CYP2D6 EM* genotype, phase 1 nitrosamine activation, smoking and earlier tumorigenesis a prospective case-control study is required in which the tobacco and alcohol consumption in controls as well as cases is recorded.

Further research is needed to unravel the mechanism by which the *CYP2D6 PM* genotype exerts its' influence on oral cancer susceptibility and perhaps, outcome.

To this end, investigation of LOH around 22q 12 is urgently required in this patient group to ascertain whether the PM and *HET* polymorphisms are acting as genetic markers for tumour suppressor genes or other detoxification enzyme polymorphisms which are present close to this locus. One arm of such an investigation is currently underway in our laboratory using matched tumour and blood sample pairs taken from patients reported on in this study.

The newly described *GSTZ* (Board ***et al.,*** **1997)** and *GSTT2* (Strange RC, personal communication) polymorphisms are candidate susceptibility genes for oral cancer. The fact that their coding genes are located close to the *CYP2D6* locus makes them of special interest as they may display linkage disequilibrium with *CYP2D6* polymorphisms and as such may offer an explanation for the increased incidence of oral cancer seen in patients with the *CYP2D6 PM* genotype.

After establishing reliable and repeatable PCR methodology for detecting *GSTZ* and *GSTT2* polymorphisms they will be looked for in the DNA of the current study population and any association with oral cancer susceptibilityand outcome both alone and in combination with *CYP2D6 PM* polymorphisms will be investigated.

Of critical importance for outcome data verification **is** the absolute requirement to follow-up the oral cancer patient group until death or for a minimum of 2 years (preferably

5 years) from diagnosis. Only then can the effects of any detoxification polymorphisms on actuarial outcome be fully assessed and verified. With this in mind, I intend to re-analyse the current 106 patients in 2 years and 5 years time. Recruitment into this study is still on-going and so as well as being able to produce actuarial **2** and 5-year local recurrence, nodal metastasis and survival data for the current group of patients, susceptibility data will be available on approximately a further 200.

This study has provided interesting data suggesting that the *GSTP1 BB* genotype is associated with a poor outcome in patients with oral cancer and possibly also with an increased susceptibility to the disease. With better characterisation of the oral cancer control group, and increased recruitment into the oral cancer case group in the future it will be possible to investigate the association between *GSTP1* polymorphisms and oral cancer susceptibility and outcome more completely than was possible in the current study.

Finally, it would be extremely interesting to ascertain the *CYP* and GST polymorphisms in first-degree relatives of patients with oral cancer, particularly those relatives who have a history of malignant disease themselves. Our laboratory has had success in extracting **DNA** from buccal epithelial squames collected using a simple 5ml 0.9% saline mouthwash. Living first-degree relatives will be asked to supply a mouthwash sample from which their **DNA** will be extracted and their *CYP* and GST genotype ascertained. The results will be analysed particularly looking for genotypes that display a high frequency of correlation between generations of patients with oral cancer. If pilot data is encouraging this study will need to be organised on a multi-centre collaborative basis in order to recruit sufficient numbers of affected first-degree relatives to enable reliable statistical analysis of the study data to be performed.

APPENDIX

Publications in scientific journals on areas of study in this thesis written by the author

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