

# Prognostic Markers in Head and Neck Cancer

# Prognostic Markers in Head and Neck Cancer

A thesis submitted to The University of Manchester for the degree of Doctor of Medicine in the Faculty of Medical and Human Sciences and School of Cancer and Enabling Sciences


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Catriona Mairi Douglas

School of Medicine

This project was undertaken in the Translational Radiobiology Group of the School of Cancer & Enabling Sciences and the Department of Head and Neck Surgery, Christie Hospital NHS Trust, Christie Hospital, Wilmslow Road, Withington, Manchester, M20 4BX.

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# List of Abbreviations

| | |
|---|---|
| ARCON | Accelerated radiotherapy with carbogen and nicotinamide |
| Bcl-2 | B cell lymphoma-2 |
| CA9 | Carbonic anhydrase 9 |
| CI | Confidence interval |
| CSS | Cancer-specific survival |
| CT | Computer tomogram |
| DAHANCA | Danish Head and Neck Cancer Group |
| DFS | Disease free survival |
| DSS | Disease specific survival |
| EBV | Epstein Barr virus |
| EGFR | Epidermal growth factor receptor |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EORTC | European Organisation for Research and Treatment of Cancer |
| EPO | Erythropoietin |
| EU | European Union |
| FF | Fresh frozen |
| FFPE | Formalin fixed paraffin embedded |
| Gy | Gray |
| Hb | Haemoglobin |
| HIF-1α | Hypoxia inducible factor – 1α |
| HNSCC | Head and neck squamous cell carcinoma |
| HPV | Human papillomavirus |
| HR | Hazard ratio |
| IHC | Immunohistochemistry |
| INHANCE | International Head and Neck Cancer Epidemiology Consortium |
| ISD | Information and statistics department, Scotland |
| ISH | In situ hybridization |
| LC | Local control |
| LRF | Locoregional failure |
| MRI | Magnetic resonance imaging |
| MV | Megavolt |
| MVA | Multivariate analysis |
| MVD | Microvessel density |
| NICR | Northern Ireland cancer registry |
| ONS | Office for national statistics, England |
| OR | Odds ratio |
| OS | Overall survival |
| OPN | Osteopontin |
| OTT | Overall treatment time |
| PCR | Polymerase chain reaction |
| PFC | Perflurocarbons |
| *CDKN2A* | Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) protein product |
| RT | Room temperature |
| RTOG | Radiation Therapy Oncology Group |
| TBS | Tris buffered saline |
| TCP | Tumour control probability |
| TPZ | Tirapazamine |
| TNM | Tumour node metastasis |
| UVA | Univariate analysis |
| VEGF | Vascular endothelial growth factor |
| WCISU | Welsh cancer intelligence and Surveillance unit |

# Abstract

**The University of Manchester**

Abstract of Thesis submitted by Catriona Mairi Douglas for the Degree of Doctor of Medicine. Prognostic markers in head and neck cancer. Submitted December 2010.

Purpose: The management of head and neck squamous cell carcinoma (HNSCC) is complex and often involves multimodality treatment. Currently, most management decisions are based on clinical parameters with little appreciation of patient differences in underlying tumour biology. The identification of biomarkers that predict response to radiotherapy would be clinically useful in determining optimal management. The purpose of the thesis was to investigate potential biomarkers that might predict radiotherapy outcome in patients with HNSCC.

Aims: 1) To investigate the hypoxia-associated biomarkers carbonic anhydrase 9 (CA9) and hypoxia-inducible factor -1α (HIF-1α) in patients with early glottis cancer who underwent radiotherapy as their primary mode of treatment, furthermore to investigate the role of accelerated hypofractionated radiotherapy in the management of T2 glottic cancer. 2) To investigate markers of hypoxia (CA9 and HIF-1α) and viral infection in oropharyngeal cancer, and in particular to test for an association between hypoxia markers and viral infection. 3) To investigate HIF-1$\alpha$ and CA9 in a series of patients undergoing surgery as their primary mode of treatment to explore whether they are associated specifically with radioresponsiveness or a general poor prognosis.

Results: 1) Adverse prognostic factors for locoregional control were low pre-treatment haemoglobin (Hb; p = 0.010), advancing T stage (p = 0.001) and high CA9 expression (p = 0.032). Low Hb and high CA9 expression were independent factors on multivariate analysis; and combined predicted locoregional recurrence with an odds ratio of 8.0 (95% CI: 2.7-23.9), or either/or with an odds ratio of 3.3 (95% CI 1.5-7.1). In the subset of T2 patients, five-year locoregional control following radiotherapy was 82% and cancer specific survival was 90%. Serious morbidity occurred in 1.8% of patients. T stage subdivided by vocal cord movement was significant for local control. 2) Features associated with a poor locoregional control were older age (p=0.002), tongue base subsite (p=0.002), heavy alcohol use (p=0.004), heavy smoker (p=0.0002), low Hb level (p=0.001), advancing T (p=<0.0001), N (p=0.001) and AJC (p=0.001) stage, high CA9 expression (p=0.020) and high HIF-1α expression (p<0.0001). In multivariate analysis T stage (p=0.003) and high HIF-1α expression (p=0.001) remained significant. 3) Extracapsular spread was significantly associated with poor cancer specific survival (p=0.022). No other patient variables were associated with outcome. HIF-1α expression was significantly associated with poorly differentiated tumour (p=0.019) and the tumour having a cohesive front (p=0.026).

Conclusion: 1) Hb and CA9 have potential to be used together as a biomarker to identify glottis cancer patients with a high probability of a poor outcome following radiotherapy, furthermore, vocal cord movement should be taken into consideration when managing glottis cancer. 2) As it does not appear to be influenced by HPV status, HIF-1α warrants further investigation as a biomarker in oropharyngeal patients treated with primary radiotherapy. 3) As HIF-1α and CA9 had no prognostic significance in patients undergoing surgery, they should be explored further as markers to help guide management decisions in patients with HNSCC.

# Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

# Copyright Statement

# Dedication

To my father, my husband and my son.

# Acknowledgements

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I would like to thank the virology department at Manchester Royal Infirmary for performing the PCR analysis on the oropharyngeal specimens.

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# The Author

Catriona M Douglas graduated BSc, MBChB from the University of Glasgow in 2002. She completed the West of Scotland basic surgical training and passed the Membership of the Royal College of Surgeons (Glasgow) examination in 2005. The work carried out in this thesis was completed whilst she was employed as a Clinical Research Fellow in the Department of Head and Neck Surgery, and the Academic Department of Radiation Oncology, Christie Hospital NHS trust. In 2008 she was appointed to the West of Scotland ENT surgery specialist registrar rotation and she plans to sub-specialise in head and neck surgery.

# 1 Introduction

## 1.1 Head and neck cancer

Head and neck cancer does not refer to a single site, single histological cancer. Rather it refers to a heterogeneous group of malignancies that arise from the upper aerodigestive tract, the salivary glands, thyroid and parathyroid glands, paranasal sinuses and the skin of the head and neck. However, most often the term “head and neck cancer” refers to squamous cell carcinoma arising from the upper aerodigestive tract. Around 90–95% of head and neck cancers are squamous cell carcinoma (HNSCC) (Batsakis, 1974, Argiris et al., 2008). The ICD-10 classification for head and neck tumours includes tumours of the larynx, hypopharynx, oropharynx and oral cavity (Table 1.1).

Table 1-1 Head and neck squamous cell carcinoma subsites

| ***Site*** | **Subsite** |
|---|---|
| Larynx | Supraglottis<br>Glottis<br>Subglottis |
| Hypopharynx | Post cricoid<br>Piriform sinus<br>Posterior pharyngeal wall |
| Oropharyngeal | Tongue base<br>Tonsil<br>Soft Palate |
| Oral Cavity | Buccal mucosa<br>Retromolar triangle<br>Alveolus<br>Hard palate<br>Anterior two-thirds tongued<br>Floor of mouth<br>Mucosal surface of lip |

Each year about 650,000 people are diagnosed with cancer of the head and neck worldwide, and 350,000 people will die from the disease (Boyle and Ferlay, 2005). Over the past 30 years, survival rates for HNSCC have remained relatively static. The 5-year survival rate for all stages combined, on the basis of Surveillance Epidemiology and End Results (SEER) data is about 60% (Ries, 2006). Two-thirds of HNSCC patients still present with locally advanced disease (Horner, 2009) and these patients have a 5 year survival rate of <50%, with a poor post treatment quality of life

(Carvalho et al., 2005). These figures are despite advances in surgical and oncological practice. Treatment failure still occurs in the form of locoregional recurrence or distant metastasis. Head and neck cancer patients are also at risk of developing a second primary malignancy, the incidence being between 10-25% (McGarry et al., 1992, Cooper et al., 1989, Argiris et al., 2004). Do et al looked at the development of second primary cancers in 1181 head and neck patients enrolled in a placebo-controlled chemoprevention trial of 13-cis-retinoic acid. He found that 17% of patients developed a second primary cancer. An increased risk of second primary was associated with: smoking at time of diagnosis (RR = 2.1; 95% CI 1.3-3.6); continued alcohol consumption post-diagnosis (RR = 1.3; 95% CI 1.0-1.7); increased age (RR = 2.1; 95% CI 1.5-2.8); stage II diagnosis (RR = 1.5; 95% CI 1.1-2.1) and the diagnosis of a pharyngeal cancer (RR = 1.6; 95% CI 1.1-2.5) (Do et al., 2003). The diagnosis of a second primary lesion is important with regards to survival, as it has a better prognosis than a metastasis probably because the patients are under regular follow up and the second cancers are diagnosed early (Jones et al., 1995).

## 1.2 Epidemiology

Head and neck cancer is the sixth most common cancer worldwide, representing about 6% of all new cancers. It accounts for around 650,000 new cases of cancer and 350,000 cancer deaths, worldwide each year (Parkin et al., 2005). When broken down, this is composed of 389,000 cases of oral cavity cancer, 160,000 cases of laryngeal cancer and 65,000 cases of pharyngeal cancer diagnosed worldwide each year (Boyle, 2008). The age standardised incidence rate of head and neck cancer in males, worldwide is about 30/100,000. However, there is significant variation in incidence by subsite of head and neck cancer, likely related to differences in the incidence of risk factors (Sankaranarayanan et al., 1998, Parkin et al., 2005). In the UK, head and neck cancers comprise 3% of all cancers diagnosed each year. Government statistics group head and neck cancer into: 1) cancer of the lip, mouth and pharynx (oral cancer); and 2) cancer of the larynx (Westlake, 2009). Table 1.2 shows the latest head and neck cancer statistics from the office for National Statistics. Table 1.3 shows the latest HNSCC deaths with age-standardised mortality rates per 100,000 of the population.

### 1.2.1 Oral/oropharyngeal cancer

Oral cancer constitutes a group of cancers, namely cancer of the lip, tongue, mouth, oropharynx, piriform sinus, and hypopharynx. Table 1.2 shows the number of new cases of oral cancer in the UK diagnosed in 2007, broken down by subsite. Oropharyngeal cancer is more common in men than women. However, over the past

50 years there has been a dramatic change in the gender ratio, changing from around 5:1 fifty years ago to less than 2:1 today. The risk of developing oropharyngeal cancer increases with age, and in the UK the majority of cases (87%) occur in people aged 50 years or over (CRUK, 2010b, OfficeforNationalStatistics., 2007, ISD, 2010, NICR, 2010, WCISU, 2010). There are differences in cancer rates throughout the UK. Table 1.3 and table 1.4 shows that Scotland has significantly higher rates of cancer than other parts of the UK, which correlate with the higher rates of alcohol and tobacco consumption in Scotland (Conway et al., 2010). In Scotland, for patients diagnosed in 1991-95, the incidence rates for cancer of the head and neck were twice as high for those in the most disadvantaged category compared with the least disadvantaged, reflecting the higher consumption of alcohol and tobacco in more disadvantaged groups (ISD, 2010) (Conway et al., 2008a, Conway et al., 2008b).

The incidence of oral cancer remained relatively static between 1975 and 1989, with the age standardised incidence for UK males around 7 per 100,000 males. However, since 1989 there has been an increase, with the age standardised incidence of 11 per 100,000 in 2007, an increase of more than 50% since 1989 (Figs 1.1 and 1.2 (CRUK, 2010b)). The change in incidence of oral cancer in males has been greatest for men in their forties and fifties; with rates doubling from 3.6 to 9.3 per 100,000 for men aged 40-49 and from 11.5 to 29.7 for men aged 50-59 years. For men over 80 years old, the incidence of oral cancer has halved since 1975, while rates for men in their 70s has remained stable (Fig 1.2). These trends have been mirrored worldwide, with a rising incidence of oral cancer in both Europe and America (Conway et al., 2009, Shiboski et al., 2005, Annertz et al., 2002, CRUK, 2010b). Although female rates of oral cancer are still lower than males, they have also seen a steady increase in incidence by around 3% each year since 1989 (Fig 1.1) (Shiboski et al., 2005, Annertz et al., 2002, CRUK, 2010b).

Table 1-2 Number of new cases of oral cancer, by type, UK 2007

| *Site* | *Males* | *Females* | *Persons* | *M:F ratio* |
|---|---|---|---|---|
| **Lip (ICD10 C00)** | 220 | 113 | 333 | 1.9:1 |
| **Tongue (ICD10 C01-02)** | 1008 | 590 | 1598 | 1.7:1 |
| **Mouth (ICD10 C03-06)** | 954 | 620 | 1574 | 1.5:1 |
| **Oropharynx (ICD10 C09-10)** | 799 | 264 | 1063 | 3:1 |
| **Piriform sinus (ICD10 C12)** | 267 | 59 | 326 | 4.5:1 |
| **Hypopharynx (ICD10 C13)** | 109 | 62 | 171 | 1.8:1 |
| **Other & ill-defined (ICD10 C14)** | 183 | 77 | 260 | 2.4:1 |
| **Oral cancer** | 3,540 | 1,785 | 5,325 | 2:1 |

Adapted from (CRUK, 2010b)

Table 1-3 Newly diagnosed cases of head and neck cancer and directly age-standardised incidence rates per 100,000 population, 2004–6[1]

| ICD–10 | Site description | Sex | United Kingdom | | England | | Wales | | Scotland | | N Ireland | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Number | Rate | Number | Rate | Number | Rate | Number | Rate | Number | Rate |
| **C00-C14** | Lip, mouth & pharynx | M | 3,821 | 11.7 | 3,011 | 11.1 | 228 | 13.5 | 476 | 16.9 | 107 | 12.7 |
| | | F | 2,031 | 5.2 | 1,642 | 5.0 | 110 | 5.5 | 222 | 6.5 | 57 | 5.5 |
| **C32** | Larynx | M | 1,809 | 5.3 | 1,429 | 5.0 | 91 | 5.0 | 232 | 8.1 | 58 | 6.8 |
| | | F | 394 | 1.0 | 289 | 0.9 | 22 | 1.0 | 68 | 2.0 | 14 | 1.4 |

[1] Using the European standard population and all numbers and rates calculated as three-year averages. Adapted from (Westlake, 2009).

Table 1-4 Deaths from head and neck cancer and directly age-standardised mortality rates per 100,000 population, 2004-6[1]

| ICD–10 | Site description | Sex | United Kingdom | | England | | Wales | | Scotland | | N Ireland | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Number | Rate | Number | Rate | Number | Rate | Number | Rate | Number | Rate |
| **C00-C14** | Lip, mouth & pharynx | M | 1,323 | 3.9 | 1,042 | 3.7 | 75 | 4.3 | 171 | 6.0 | 35 | 4.1 |
| | | F | 690 | 1.5 | 559 | 1.5 | 35 | 1.5 | 76 | 1.9 | 21 | 1.8 |
| **C32** | Larynx | M | 628 | 1.8 | 492 | 1.7 | 41 | 2.2 | 76 | 2.6 | 19 | 2.1 |
| | | F | 166 | 0.4 | 129 | 0.3 | 8 | 0.3 | 25 | 0.6 | 5 | 3 |

[1] Using the European standard population and all numbers and rates calculated as three-year averages. Adapted from (Westlake, 2009).

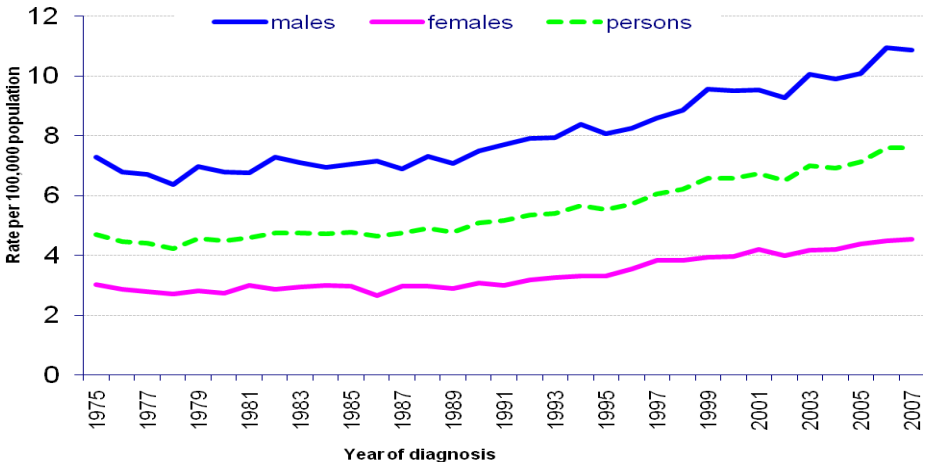



Figure 1-1 Age standardised (European) incidence rates of oral cancer in Great Britain from 1975 to 2007 by gender. Taken from (CRUK, 2010b).

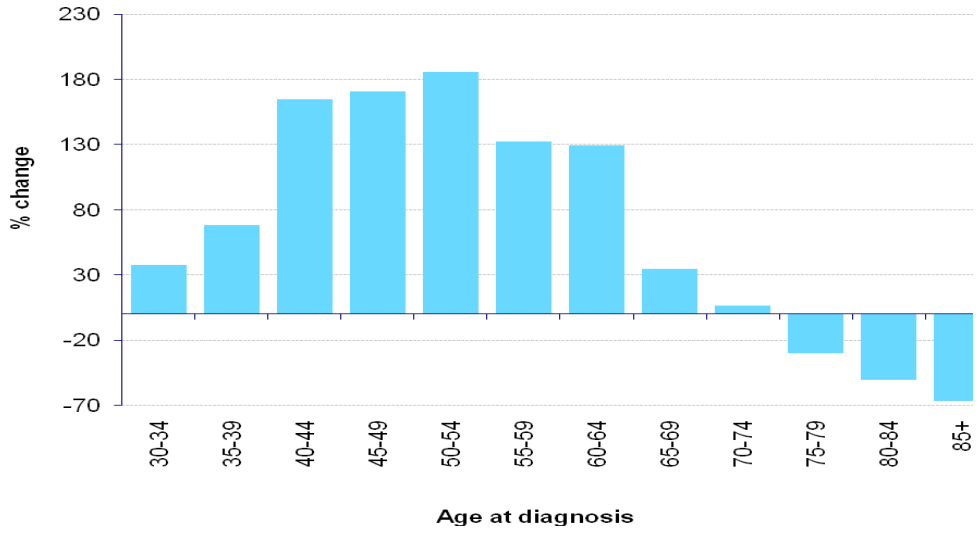



Figure 1-2 Percentage change in incidence rates for oral cancer in British men between 1975 and 2007. Taken from (CRUK, 2010b).

### 1.2.2 Laryngeal cancer

In 2007, 2,205 people in the UK were diagnosed with laryngeal cancer and in 2008, 850 people died from the disease. Laryngeal cancer is much more commonly diagnosed in males compared to females. It is in the top twenty most common cancers

in UK males (number 18), with 1,844 new cases diagnosed in 2007, compared to 361 cases in females - giving a male: female ratio of approximately 5:1. Cancer Research UK estimated the lifetime risk of developing laryngeal cancer in the UK as 1 in 181 for men and 1 in 849 for women. The risks were calculated on February 2009 using incidence and mortality data for 2001-2005 (CRUK, 2010a). Like oral cancer, larynx cancer is rarely diagnosed in people under the age of 40 years, but incidence rises steeply thereafter peaking in people aged 70-74 years. Most cases (73%) occur in people over the age of 60 years (CRUK, 2010a).

There is significant geographical variation within the UK with Scotland having the highest incidence (Table 1.2) (ISD, 2010, NICR, 2010, WCISU, 2010). Within England there is a very clear north south divide with incidence rates being higher than average in the North West, Northern and Yorkshire regions and lower than average in the Eastern, South East and South West regions (Coleman et al., 2004). There is also a similar geographical pattern for mortality of laryngeal cancer (Table 1.3) (ISD, 2010, NICR, 2010, WCISU, 2010). The highest mortality rate is in Scotland (Table 1.3). Mortality is also higher than average in the North West, and Northern and Yorkshire regions of England and lower than average in the Eastern, South West, and South East regions of England. The highest mortality rate for laryngeal cancer is seen in Glasgow then Manchester. The areas of highest incidence and mortality are also the areas with high socio-economic deprivation (Coleman et al., 2004). Using the Carstairs' index ( a marker of socio-economic status), there appears to be a strong relationship between the incidence of laryngeal cancer and socio-economic deprivation (Carstairs and Morris, 1989, Carstairs, 1981). In 1991 the incidence of laryngeal cancer in males living in the most deprived areas was almost three times greater than males living in affluent areas (Harris 1998, Quinn et al., 2001). In Scotland there is a significant association between incidence and mortality of laryngeal cancer and deprivation (Table 1.5) (ISD, 2010). The age-standardised incidence rates for laryngeal cancer in Great Britain are very different for males and females. For males, the rate has been falling over the last 8 years and is currently less than 5.3 per 100,000 of the population; however, the rate for females has remained close to 1 per 100,000 of the population (Fig 1.4).

Table 1-5 Scotland: age-standardised incidence and mortality rates (EASRs)[1] by SIMD 2006 deprivation quintile.

| ***SIMD 2006 deprivation quintile*** | ***Incidence 2004-2008*** | | | ***Mortality 2005-2009*** | | |
|---|---|---|---|---|---|---|
| | Incidence registrations | EASR | 95% CI | Death registrations | EASR | 95% CI |
| 1 (Least deprived) | 150 | 2.5 | 2.1-2.9 | 31 | 0.5 | na |
| 2 | 188 | 2.9 | 2.5-3.3 | 58 | 0.9 | 0.7-1.1 |
| 3 | 271 | 4.2 | 3.7-4.7 | 93 | 1.4 | 1.1-1.7 |
| 4 | 375 | 6.3 | 5.7-7.0 | 126 | 2.0 | 1.6-2.3 |
| 5 (Most deprived) | 501 | 9.4 | 8.6-10.2 | 179 | 3.2 | 2.8-3.7 |
| Test for trend[2] | | <0.0001 | | | <0.0001 | |

[1]Rates are calculated using the populations in 2006. EASR: age-standardised incidence rate per 100,000 person-years at risk (European standard population). Note: confidence intervals for age-standardised rates (EASR) have been calculated using a formula which works only when numbers are sufficiently large. They are therefore set to 'not applicable (na)' in the event of there being 50 cases or less. Sources: Scottish Cancer Registry, ISD (incidence); General Register Office for Scotland (GROS) (mortality and populations). Data extracted September 2010. Taken from (ISD, 2010).
[2]Poisson regression.

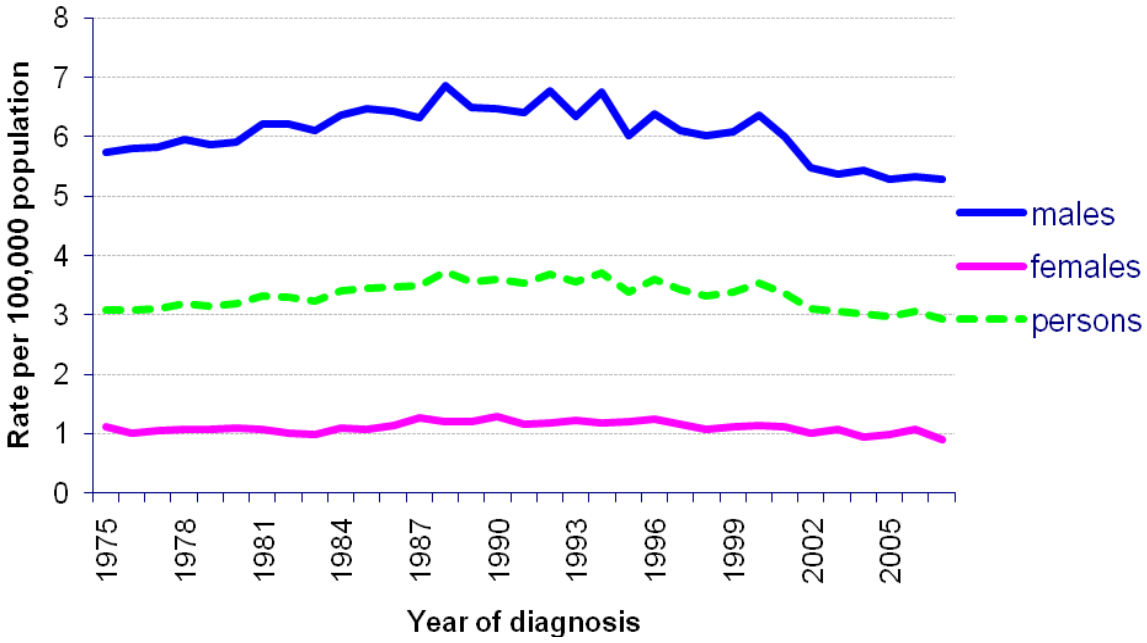



Figure 1-3 Age standardised (European) incidence rates for laryngeal cancer in Great Britain between 1975 and 2007 by gender. Taken from (CRUK, 2010a).

## 1.3 Aetiology

### 1.3.1 Smoking

Tobacco and alcohol are the two main established risk factors for development of HNSCC and at least 75% of head and neck cancers diagnosed in Europe and the United States are attributable to tobacco and alcohol (Blot et al., 1988, Ferlay et al., 2004, Hashibe et al., 2007, Marron et al., 2010, Johnson, 2001). In the 1950s, an historic case control series by Wynder et al established the link between cancer of the oral cavity and tobacco use (Wynder et al., 1957). Since then numerous studies have confirmed this link (Blot et al., 1988, Cattaruzza et al., 1996, La Vecchia et al., 1997, Johnson, 2001, Basu et al., 2008). Importantly the strength and consistency of the association has been demonstrated in many studies. Several studies have demonstrated up to a 20 fold increased risk of laryngeal cancers in smokers compared with non-smokers. These studies have also shown a dose-response effect between duration of smoking and increased risk of cancer and risk reduction after cessation of smoking (Talamini et al., 2002, De Stefani et al., 1987, Wynder et al., 1976, Franceschi et al., 1990). Several large series have suggested a reduction of head and neck

cancer risk of 16-85% after stopping smoking (Kenfield et al., 2008, Bosetti et al., 2008, Bosetti et al., 2006). Marron et al pooled individual-level data from case–control studies in the International Head and Neck Cancer Epidemiology Consortium (INHANCE). Data were available from 17 studies looking at smoking cessation. Stopping smoking for 1-4 years resulted in a cancer risk reduction [OR 0.70, confidence interval (CI) 0.61–0.81 compared with current smoking]. After 20 years, the risk had reached that of never-smokers (OR 0.23, CI 0.18–0.31) (Marron et al., 2010).

The increased risk in smokers is due to the genotoxic effects of the carcinogens in the tobacco, causing structural changes in DNA. Benzo[α]pyrene diol epoxide (BPDE) is a known tobacco carcinogen. It forms covalent bonds to create DNA adducts, throughout the genome inducing genetic damage (Denissenko et al., 1996). Several studies have shown that sequence variations in NER/BER genes contribute to HNSCC susceptibility (Hung et al., 2005, Li et al., 2007, Cheng et al., 2002, Handra-Luca et al., 2007).TP53 mutations in HNSCC occur more frequently in patients who smoke, compared to those who do not (Brennan et al., 1995). However, not everyone that smokes acquires a TP53 mutation and develops HNSCC, suggesting, as discussed above, that the risk of developing HNSCC is a complex interaction of exposure, activation, detoxification and repair of DNA damage (Ho et al., 2007).

Regional variations in the development of HNSCC around the world are strongly related to habitual and cultural risk factors which are prevalent in these areas (Goldenberg et al., 2004). The use of smokeless tobacco is prevalent in Northern Europe and America, and has been suggested as an aetiological factor in the development of oral cancer, increasing the risk by a factor of 2.8 (Rodu and Cole, 2002). Betel nut is the main ingredient of widely chewed products in Southeast Asia and parts of the Pacific Rim. It is a strong risk factor, independent of tobacco for development of oral cavity squamous cell carcinoma (Merchant et al., 2000).

Passive smoking, i.e. involuntary exposure to tobacco or second hand tobacco smoke, exposes individuals to the carcinogens present in tobacco smoke. There is evidence that tobacco carcinogens are metabolised by passive smokers, therefore having the potential to increase their risk of cancer (IARC, 2004). A pooled analysis by INHANCE showed a significant increased risk of HNSCC associated with a long exposure to involuntary smoke either at home or work (Lee et al., 2008). This finding suggests that the elimination of involuntary smoking may reduce the risk of HNSCC among those that have never smoked.

Non-smokers account for 5-30% of HNSCC (Hashibe et al., 2007) and appear to be a distinct group, compared to the smokers. Loss of heterozygosity occurs when there is loss of function of one allele of a gene, where the other allele was already inactivated. In non-smokers, they have fewer *TP53* gene mutations, a loss of

heterozygosity on chromosome arms 3p and 4q and at chromosome 11q13 (site at which loss of heterozygosity occurs in oral cancer,) (Koch and McQuone, 1997). The study of patients with HNSCC who have never smoked is important in order to establish the aetiology of other risk factors other than tobacco (IARC, 2004).

As mentioned above, smoking and alcohol account for the majority of HNSCC. However, the individual contributions of these two risk factors are difficult to determine, as most people who smoke drink. (Hashibe et al., 2007). An understanding of the association between these factors and the risk of HNSCC has significant implications for the understanding of the mechanism of carcinogenesis, and for calculating the results of interventions to modify risk factors. One of the difficulties of assessing the two risks individually has been that previous studies have only had small numbers as few patients were never drinkers or never smokers (Bosetti et al., 2002a, Fioretti et al., 1999, Talamini et al., 1998). Hashibe et al looked at HNSCC associated with smokers who never drank alcohol and drinkers who never smoked. They used individual-level data from 15 case control studies, finding that in those that never drank alcohol, cigarette smoking was associated with an increased risk of head and neck cancer (OR for ever versus never smoking = 2.13, 95% CI = 1.52 - 2.98). There was also a clear dose–response relationship for the frequency, duration, and number of pack-years of cigarette smoking. In their study 24% (95% CI = 16 - 31%) of head and neck cancer cases among non-drinkers would have been prevented if those patients had not smoked (Hashibe et al., 2007).

### 1.3.2 Alcohol

Alcohol is second only to smoking as an aetiological factor in the development of HNSCC. As discussed above, it is difficult to distinguish the separate effects of alcohol and smoking due to smokers tending to drink, and drinkers tending to smoke. However, alcohol is said to contribute to at least 75% of cases (Blot et al., 1988, Blot, 1999). The risk of developing cancer in patients who both smoke and consume alcohol has a multiplicative effect rather than an addative risk (Talamini et al., 2002) The causal mechanism of alcohol causing cancer is not fully understood. Alcohol is not a carcinogen, but its metabolite, acetaldehyde, forms DNA adducts that interfere with DNA synthesis and repair (Brooks and Theruvathu, 2005). The ability of alcohol to enhance the effects of smoking is thought, in part, to be due to its solvent nature, enhancing and prolonging mucosal exposure of the carcinogens in smoke (Pai and Westra, 2009). It is also thought that other ingredients in alcohol may influence cancer risk: namely tannins, N-nitroso compounds, urethane and asbestos filtration products that are found in some alcoholic drinks (Blot, 1999, Ogden and Wight, 1998). INHANCE carried out a pooled analysis at 15 case control studies looking at the risk of

head and neck cancer associated with drinking beer, wine and spirits. A similar risk of head and neck cancer was seen for beer-only and spirit only drinkers. There was a weaker association with wine only drinking, thought possibly to be due to differences in lifestyle and diet that may confound the finding (Purdue et al., 2009).

In contrast to the large number of studies looking at the effects of stopping smoking (see Section 1.3.1), few studies have been conducted looking at the effects of quitting alcohol drinking, with the results inconsistent. In two of the studies there appeared to be a risk reduction of oral and pharyngeal cancer after cessation of drinking (Garrote et al., 2001, Castellsague et al., 2004), however two other studies did not find any association (Balaram et al., 2002, Hayes et al., 1999). It has even been reported that there is a higher risk of oral and pharyngeal cancer 7-10 years after stopping drinking, compared to current drinkers (Franceschi et al., 2000). However, these studies are small and it is difficult to draw any firm conclusions. Marron et al pooled individual-level data from 13 case–control studies (9167 cases and 12 593 controls) on drinking cessation. They found that a beneficial effect on the risk of head and neck cancer was only observed after ≥20 years of quitting drinking alcohol (OR 0.60, 95% CI 0.40–0.89 compared with current drinking), reaching the level of never drinkers (Marron et al., 2010). Hashibe et al looked at the risk of HNSCC in alcohol drinkers that had never smoked. They pooled 15 case control studies (1598 case subjects and 4051 control subjects), finding that among those that had never smoked, alcohol consumption was associated with an increased risk of head and neck cancer only when alcohol was consumed at high frequency (OR for three or more drinks per day versus never drinking = 2.04, 95% CI 1.29–3.21). The association with high-frequency alcohol intake was limited to cancers of the oropharynx/hypopharynx and larynx (Hashibe et al., 2007).

### 1.3.3 Diet

Other risk factors have also been implicated in the aetiology of head and neck cancer. Epidemiological studies suggest that diets low in fruit and vegetables and high in animal fats may be a risk factor for the disease (McLaughlin et al., 1988, Winn et al., 1984). The key components of a Mediterranean diet (citrus fruit, tomatoes, olive oil and fish) appear to confer some protection from HNSCC (Franceschi et al., 1999, Uzcudun et al., 2002). High fat and high red meat intake have also been linked with an increase risk of laryngeal cancer (Bosetti et al., 2002b, Oreggia et al., 2001). A large retrospective case control study also suggested a link for diagnosis of gastro-oesophageal reflux with laryngeal and pharyngeal cancer, independent of smoking and alcohol intake (El-Serag et al., 2001). Tea and coffee are the most widely consumed hot drinks in the world. INHANCE looked at nine case-control studies to explore the

relationship between coffee and tea intake and development of HNSCC. Caffeinated coffee intake was inversely related with the risk of oral cavity and pharynx cancer with an OR of 0.96 (95% CI, 0.94-0.98) for an increment of 1 cup per day. There was no association with laryngeal cancer and caffeinated coffee drinking. The authors were unable to draw any firm conclusions about decaffeinated coffee as the data were insufficient. Tea intake was not associated with head and neck cancer risk (Galeone et al., 2010).

## 1.4 Treatment of HNSCC

The management of patients with HNSCC poses a significant challenge for doctors. The overall aim is to achieve the highest cure rate with the lowest morbidity to the patient, ideally preserving the key functions of swallowing, speaking and breathing. Patients should be managed under the care of a multidisciplinary team of health care professionals and the team should include: a head and neck surgeon, a clinical oncologist, a radiologist, a pathologist, a clinical nurse specialist, a speech and language therapist and a dietician. After a histological diagnosis has been made and the clinical staging established, the treatment decisions are based on a number of factors including patient parameters (performance status, age, other co-existing diseases, patient's wishes) and clinicopathological features (site, stage). Another key consideration that should be taken into account is the potential for recurrences after treatment, and its subsequent management.

Currently the evidence base for treatment of HNSCC is scant. There is variation in the management of patients across the country (Edwards and Johnson, 1999). However, two national documents have been published to guide the clinicians in the management of these patients (SIGN, 2006). The mainstays of treatment are surgery, radiotherapy, chemotherapy and to a lesser extent novel targeted therapies. The purpose of treatment is to remove the cancer load, prevent subsequent recurrence or metastasis, and maintain a quality of life (Shah and Lydiatt, 1995). In general, for small primaries without regional metastases, one treatment modality is used, usually wide surgical excision or curative radiotherapy. For more advanced primary tumours, with or without regional metastases, the treatment is usually a combination of surgery and post-operative radiotherapy or chemoradiotherapy. However, when looking at the evidence to find the optimal therapeutic approach, it is very clear that no single therapeutic regimen offers a clear benefit over other regimens (Corvo, 2007). Ten percent of patients present with metastatic disease (Horner, 2009) and 50% of patients treated for advanced disease will recur (Boyle, 2008, Clark et al., 2005). With these patients the goals of management are palliation of symptoms and improved survival. Management of these patients can be diverse, ranging from salvage surgery if

possible, re-irradiation or the use of single or multi-agent chemotherapy (Argiris et al., 2008). Generally, for most patients with recurrent or metastatic disease the standard of care would be multi-agent chemotherapy; a combination of a platinum compound and 5-fluorouracil (5-FU) being the most widely used regimen (Ferrari et al., 2009).

### 1.4.1 Surgery

With regards to surgery, some studies have compared surgical techniques, but these are generally non-randomised, retrospective observational studies (Mitchell and Crighton, 1993, Davidson et al., 1991). There is some evidence to support the use of combined surgery and radiotherapy for advanced tumours, in particular tongue, pharynx and larynx (Weber et al., 1990, Nisi et al., 1998, Fein et al., 1994). When comparing pre- and post-operative radiotherapy, the North American Radiation Therapy Oncology Group (RTOG) study found the use of post operative radiotherapy significantly improved local control of the disease, but did not improve overall survival (Tupchong et al., 1991). There is only one published randomised controlled trial directly comparing surgery with post-operative radiotherapy versus chemoradiotherapy for treatment of stage III/IV head and neck patients. Unfortunately the study was underpowered and did not enable any meaningful conclusions to be drawn about the treatments. The study also highlighted the difficulty of doing a trial of such magnitude; the patient group was very heterogeneous, recruitment to the study was difficult and patients were not keen to have decisions about their treatment subject to randomisation (Soo et al., 2005).

### 1.4.2 Radiotherapy

The use of radiotherapy to treat malignancy began soon after the discovery of X-rays by Wilhelm Roentgen in 1895. The first “cure” of a malignancy was reported in 1899 when Stenbeck used ionising radiation to treat a basal cell carcinoma on a patient’s nose. Another early descriptions of radiotherapy for a head and neck cancer was by Coutard in 1937 (Coutard, 1937). Radiotherapy uses high energy photons of electromagnetic radiation. The high energy photons will initially interact with tissue and produce high energy electrons, which create secondary ionising events as they travel through tissue. Within the cell, nuclear DNA is a critical target, but damage to other structures, such as cellular and nuclear membranes is also important. Radiation results in DNA damage in the form of, intra and inter strand cross-links and single and double strand breaks. Unrepaired double strand breaks are thought to be the main cytotoxic lesions. The interaction of radiation with tissues results in the production of free radicals. Free radicals are short-lived but their half-life is increased in the presence of oxygen which is said to ‘fix’ the radicals and increase their likelihood of interacting

with DNA. Rapid reduction of radiation-induced free radicals in hypoxia reduces their interaction with DNA. Therefore in hypoxic conditions DNA damage is less than in oxygen. External beam radiotherapy is delivered in fractions, which means that the total dose is delivered over time in smaller doses per fraction. The ability of tissues and tumours to respond to ionising radiation and repair the damage caused varies between patients. The outcome will depend not only on the dose of ionising radiation given, but also on the cells ability to detect the damage and repair it. It has always been accepted that some tumours respond better to radiotherapy than others. A number of biological factors underlie differences in tumour response to radiotherapy and these include hypoxia (see below), proliferation and intrinsic tumour cell radiosensitivity (Deacon et al., 1984).

As mentioned previously, patients with early stage disease are generally managed with a single modality treatment. There is currently no level 1 evidence directly comparing radiotherapy to conservative surgery for the evaluation of local control and survival in HNSCC (SIGN, 2006). The current evidence is limited to prospective and retrospective cohort studies. Conventional radiotherapy has been the mainstay of management in patients with early stage disease for decades. Historically, the doses employed (60-70 Gy in 30-35 fractions over 6 – 7 weeks) can have excellent results on small T1/T2 tumours limited to the mucosa, with definitive cure in 70 – 90% of patients (Skladowski et al., 1999). However, when bone and muscle become involved in these tumours, the probability of cure drops to 50-70% after definitive conventional radiotherapy (Laskar et al., 2006). Conventional radiotherapy for early disease offers the best chance of cure if the treatment is delivered in the planned overall treatment time, local failure is reported more frequently when there is an interruption to treatment (Peters and Withers, 1997). Withers et al analysed 500 oropharygneal cancer patients that showed evidence of rapid tumour growth when their treatment was extended from 5 to 8 weeks. They estimated doses required to achieve local control in 50% of cases from published local control rates, and the dependence of these doses on overall treatment time was evaluated. In parallel, published scattergrams were analysed to estimate the rate of tumour regrowth over the period of 4-10 weeks from initiation of therapy. Analysis showed that clonogen repopulation in HNSCC accelerates only after a lag period of the order of 4±1 weeks after initiation of radiotherapy and that a dose increment of about 0.6 Gy per day is required to compensate for this repopulation (Withers et al., 1988).

Advanced stage tumours or small tumours with positive neck nodes are not commonly treated with conventional radiotherapy alone. A dose of 70 Gy will only control 50% of T3 and T4 tonsil tumours and 50% of N2 neck nodes i.e treat the cancer with no recurrence (Laskar et al., 2006). As disease advances in stage, it will

include different tumour subsites, with complex biological restraints that lead to radioresistance. Many experimental and preclinical studies have demonstrated many factors involved in the failure of conventional radiotherapy including; hypoxic cells with surrounding neo-angiogenesis, accelerated repopulation during radiotherapy course and overexpression of growth factor receptors (Antognoni et al., 2005) (Baumann and Krause, 2004). All the above factors lead to poor locoregional control with ultimate 5-year survival only 30–50% (Laskar et al., 2006). Several steps have been taken to try and improve the outcome of patients with locally advanced disease including altered fractionation of radiotherapy. A meta-analysis by Bourhis et al looked at 15 trials with 6515 patients. They concluded that altered fractionated radiotherapy improves survival in HNSCC. When comparing the different types of altered radiotherapy, hyperfractionation had the greatest benefit (Bourhis et al., 2006). The addition of chemotherapy to radiotherapy improves survival by 4.5%, discussed further in Section 1.4.3 (Pignon et al., 2009).

### 1.4.3 Chemotherapy

The mainstay of treatment in HNSCC is surgery and/or radiotherapy. There is no evidence to support the use of chemotherapy alone in the management of head and neck cancer, other than in the palliative setting for recurrent and metastatic disease. Although radiotherapy is effective in treating patients with HNSCC, in an attempt to improve outcomes further trials combing radiotherapy and chemotherapy were developed. The motivation was twofold: to hopefully increase locoregional control by intensifying the radiotherapy efficacy by using two tumourocidal agents and to reduce the incidence of distant metastasis (Brizel and Esclamado, 2006). Originally chemotherapy was given to patients with unresectable tumours where conventional radiotherapy was unlikely to be curative. Over time, chemotherapy and radiotherapy were investigated in resectable tumours in an effort to preserve organ function. (Adelstein et al., 2003, Bensadoun et al., 2006, Budach et al., 2005). The investigating trials differed in many respects: timing of chemotherapy (sequential or concomitant schedules); drugs used (5-FU, mitomycin, cisplatin, carboplatin, methotrexate, taxanes); single agent chemotherapy or polychemotherapy; type of drug administration (bolus or infusion); conventional or altered fractionation radiotherapy; radiotherapy technique; endpoint (locoregional control, progression free survival, overall survival, organ preservation) (Browman et al., 2001) (Calais et al., 1999).

There are many phase III trials of chemoradiotherapy in HNSCC. Appendix 1 shows some of the phase III trials of chemoradiotherapy, demonstrating the wide range of difference between the trials, highlighting the difficulty in interpreting the results and extrapolating the best chemoradiotherapy regimen. However, despite these difficulties,

the trials do show interesting results; several large meta-analyses have been carried to demonstrate whether chemoradiotherapy or radiotherapy alone is better for locoregional control and survival. The first major meta-analysis was published by Pignon et al in 2000, with an update in 2004 and again in 2009. (Pignon et al., 2000, Pignon et al., 2007, Pignon et al., 2009). The initial meta-analysis demonstrated that the addition of chemotherapy to radiotherapy in locally advanced disease: (1) improved overall survival by 5% at 5 years, with the use of any chemotherapy association or timing of association; (2) increased overall survival by 8% at 5 years when concomitant chemotherapy was used; (3) improved outcome when concomitant rather than neoadjuvant chemotherapy was used; (4) was associated with a similar benefit for polychemotherapy and monochemotherapy; (5) improved outcome if cisplatin was included in the combined approach; (7) did not benefit patients over the age of 70 years. In the updated 2004 publication the overall survival gain was higher in the chemotherapy/altered fractionation group (HR 0.73) compared to the chemotherapy/conventional fractionation radiotherapy group (HR 0.83), suggesting that the alteration of fractionation may boost the effect of chemoradiotherapy (Bourhis et al., 2004, Pignon et al., 2005).

The 2009 meta-analysis included 93 randomised trials and 17,346 patients (Pignon et al., 2009). The increased statistical power allowed a more complete analysis, enabling a more detailed evaluation of the effect of chemotherapy. Adding more studies did not change the previously observed survival benefit of chemotherapy, it merely verified the improved survival of 4.5% at 5 years. It did however show that there is a significant difference ($p < 0.0001$) between chemotherapy timing ( induction or concomitant) and benefit. The benefit was seen for concomitant chemotherapy, whereas there was no clear evidence of a benefit for induction and adjuvant chemotherapy. Interestingly, concomitant chemotherapy had a pronounced effect on loco-regional failure, which was not observed for induction chemotherapy, but induction chemotherapy provided a relatively more pronounced effect on distant metastases, compared to concomitant chemotherapy. This finding suggests that high doses of chemotherapy may be required to affect the occurrence of distant metastasis. The observation also suggests that the two methods of delivery may be complementary for HNSCC, giving weight to the ongoing trials looking at the benefit of adding induction chemotherapy before concomitant radiochemotherapy. Cisplatin alone, cisplatin or carboplatin associated with 5-FU or other poly-chemotherapy including either platin or 5-FU gave a benefit of the same order of magnitude. However, when only one drug was used, and not cisplatin, this gave inferior results, to the extent that this practice is no longer recommended. The method of radiotherapy delivery, either conventionally or using altered fractionation did not appear to be significant when concomitant

chemotherapy was used. Further, this large meta-analysis confirmed that the benefit of concomitant chemotherapy is less in older patients (Pignon et al., 2009). This has also been observed with differences in radiotherapy fractionation (Bourhis et al., 2006). It is thought that as older patients frequently die from other causes, it is more difficult to observe any benefit in these patients.

Recently, three taxane-based induction chemotherapy regimens have proved to be superior to the reference 5-FU-platin-based induction chemotherapy regimens (Hitt et al., 2005, Posner et al., 2007, Vermorken et al., 2007). While these results are promising, they also emphasise the importance of continuing to carry out randomised trials. Lawrence et al (Lawrence et al., 2003) stated that the enhanced activity of radiotherapy when combined concurrently with platinum derivatives and 5-FU is due to a number of factors: (1) radiosensitisation of hypoxic cells; (2) reduction of tumour load leading to an enhanced blood supply; (3) inhibition of repair of the lethal and sublethal damage caused by radiotherapy; (4) organisation and redistribution of tumour cells into the more radiosensitive G2-M cell-cycle phases; (5) induction of apoptosis.

### 1.4.4 Emerging drugs to treat HNSCC

Therapies that specifically target cellular pathways associated with carcinogenesis are now routinely used in many areas of oncology and haematology. The primary goals of any new drug therapy to treat HNSCC are to increase survival and reduce treatment toxicity. Over the past few decades there have been significant improvements in optimising radiotherapy and chemotherapy. However, both approaches have almost reached their limit of effectiveness when balancing toxicity and efficacy Consequently, the focus has now turned to the development of molecular targeted therapies specific to the biology of HNSCC. These research efforts can be split into three groups: the continuing optimisation of epidermal growth factor receptor (EGFR) targeted therapies, the clinical development of established therapeutic targets and the preclinical investigation of new mechanisms of action (Fig 1.4 and Table 1.6) (Fung and Grandis, 2010).

EGFR is a member of the human epidermal receptor (HER)/Erb-B family. It is a transmembrane glycoprotein that plays a pivotal role in mediating the relationship between cellular homeostasis and extracellular signals. Structurally it is made up of an extracellular ligand binding domain, transmembrane domain and an intracellular tyrosine kinase domain (Lurje and Lenz, 2009). It can bind to protein ligands, including EGF, amphiregulin and TGF-α. Signalling via EGFR begins with ligand binding to the extracellular domain followed by dimerisation with other EGFR and other receptors in the HER family. Dimerisation allows for auto- and *trans*-phosphorylation of the tyrosine kinase domain which leads to the activation of several well-characterised cell survival and proliferation pathways including the MAPK, Jak/STAT and AKT pathways (Lurje and Lenz, 2009).

Abnormal EGFR activity has been documented in squamous carcinoma. In HNSCC, EGFR over expression is present in the majority of tumours (Ongkeko et al., 2005, Bei et al., 2004) with EGFR gene amplification being present in up to 30% of HNSCC (Sheu et al., 2009, Braut et al., 2009). EGFR over expression has also been linked to radiotherapy and drug resistance (Liang et al., 2003, Dai et al., 2005). Various studies have suggested that increased EGFR expression and gene copy number are linked to poorer patient outcomes in HNSCC (Chung et al., 2006, Ang et al., 2002, Temam et al., 2007).

Cetuximab is a monoclonal antibody that binds to the EGFR with a higher affinity than its endogenous ligands. Cetuximab enhances tumour cell apoptosis and inhibits invasiveness and proliferation (Huang et al., 1999). Cetuximab was approved by the United States FDA in 2006 as the first molecular targeted therapy for HNSCC (Fung and Grandis, 2010). This approval was based on the Phase III trial carried out by Bonner et al which demonstrated a significant improvement in locoregional control and

survival, when cetuximab was added to radiotherapy (Bonner et al., 2006). An updated report showed that cetuximab plus radiotherapy provided a sustained survival benefit of ~9% (5-year overall survival of 45.6 vs 36.4%) (Bonner et al., 2010). This result compares favourably with the 6% survival benefit seen with the addition of platinum based chemotherapy in Pignon's substantial meta-analysis (Pignon et al., 2009).

Based on the phase III EXTREME study in patients with recurrent or metastatic disease, the FDA has recently approved the addition of cetuximab to cisplatin and 5-FU chemotherapy regimens. EXTREME showed that the addition of cetuximab improved overall survival by 2.7 months (10.1 vs 7.4 months for cisplatin and 5-FU alone) (Vermorken et al., 2008). Furthermore, cetuximab has also been shown to be effective as a second line treatment for patients with recurrent or metastatic HNSCC (Burtness et al., 2005, Herbst et al., 2005).

Another important factor in the use of cetuximab is that the toxic effects (primarily an acneiform skin rash) appear to be less severe than the side effects from traditional chemotherapy and do not affect compliance (Bonner et al., 2006, Bonner et al., 2010). Quality-of-life is an important issue in patients with advanced or recurrent HNSCC. The EXTREME trial showed the addition of cetuximab to traditional chemotherapy improved patients' overall quality-of-life and did not adversely affect social functioning. Furthermore, the addition of cetuximab significantly improved the symptoms of pain, dysphagia, speech and social eating (Mesia et al., 2010).

While EGFR is the only approved biological treatment for HNSCC, there are several other pathways currently being investigated (Fig 1.4 and Table 1.6). It has long been recognised that for a tumour to grow it needs an adequate blood supply (Kerbel, 2008). The progress of antiangiogenic treatments has been limited by the incomplete understanding of the exact mechanisms involved in the development of tumour vasculature (Ribatti, 2009). Currently is it well known that hypoxia can trigger a chain of cytokine mediated signalling events between endothelial and tumour cells (Gerber et al., 2009, Montag et al., 2009). One of the most important groups of cytokines are members of the VEGF family (Cortes-Funes, 2009). VEGF-A (VEGF) is a proangiogenic factor secreted by tumour cells that mediates angiogenesis by binding to receptors expressed on endothelial cells (Kerbel, 2008). In animal and in vitro models, VEGF signalling has been shown to promote radioresistance along with tumour growth, vasculagenesis and invasiveness (Kim et al., 1993, Riedel et al., 2003, Bock et al., 2008, Tong et al., 2008). In a number of cancer and preclinical models, interruption of VEGF signalling was shown to limit tumour growth and metastatic potential (Kerbel, 2008). Several studies have shown VEGF receptor expression in HNSCC, and this has been associated with a worse prognosis (Kyzas et al., 2005c, Kyzas et al., 2005a, Neuchrist et al., 2003).

There are two main approaches for targeting angiogenesis: inhibition of the VEGF ligand itself and small molecule inhibition of VEGF receptor tyrosine kinases. Bevacizumab is a humanised mAb that binds and sequesters all five isoforms of VEGF therefore decreasing the levels of circulating VEGF (Ferrara et al., 2004). VEGF targeted therapies (bevacizumab) have been approved, in combination with other chemotherapy, for first or second line treatment of non small cell lung cancer (Horn and Sandler, 2009). Cohen et al carried out a phase I/II trial using bevacizumab. Patients tolerate the treatment well and some patients seemed to derive a sustained response (Cohen et al., 2009). A phase III trial using bevacizumab and chemotherapy is currently underway for recurrent or metastatic HNSCC (NCT00588770) (Goerner et al., 2010).

Another group of promising targeted therapies in late stage clinical development are agents that target molecules involved in intracellular signalling networks downstream of EGFR, VEGFR and other receptors. STAT3 is a member of a family of transcription factors that control important genes involved in tumour growth and survival regulating the expression of many critical genes in tumour growth and survival (Bowman et al., 2000). STAT3 is an oncogene that acts as an intermediary between EGFR and target gene expression in HNSCC (Shao et al., 2003). In HNSCC models, activated STAT3 has also been shown to play a role in migration, inhibition of apoptosis and cell growth (Neiva et al., 2009, Kijima et al., 2002), which is thought to contribute to treatment resistance (Kim et al., 2009). There is currently a Phase 0 proof-of-concept trial (NCT00696176) underway to evaluate the effect of intratumourally injected STAT3 decoy on target gene expression in HNSCC (Fung and Grandis, 2010).

The human papilloma virus (HPV) is discussed in detail in Section 1.8.2. The appearance of a different risk factor profile and significantly better outcomes for HPV positive HNSCC suggest that a treatment approach tailored specifically to these patients may be appropriate. At present, there are two preventative HPV vaccines, Gardasil and Cervarix, for use in preventing cervical cancer (Lin et al., 2010). The role of these two vaccines, in both males and females, has not yet been studied in HNSCC. In addition to prevention, vaccines that are targeted against HPV may also be used therapeutically in HNSCC. While the majority of vaccines are being studied in cervical cancer, a small number are being trialled in HNSCC. Two Phase I studies of ~140 total patients are examining the use of HPV-16 peptide epitopes in recurrent HPV-16 positive HNSCC (NCT00257738, NCT00704041) (Fung and Grandis, 2010).

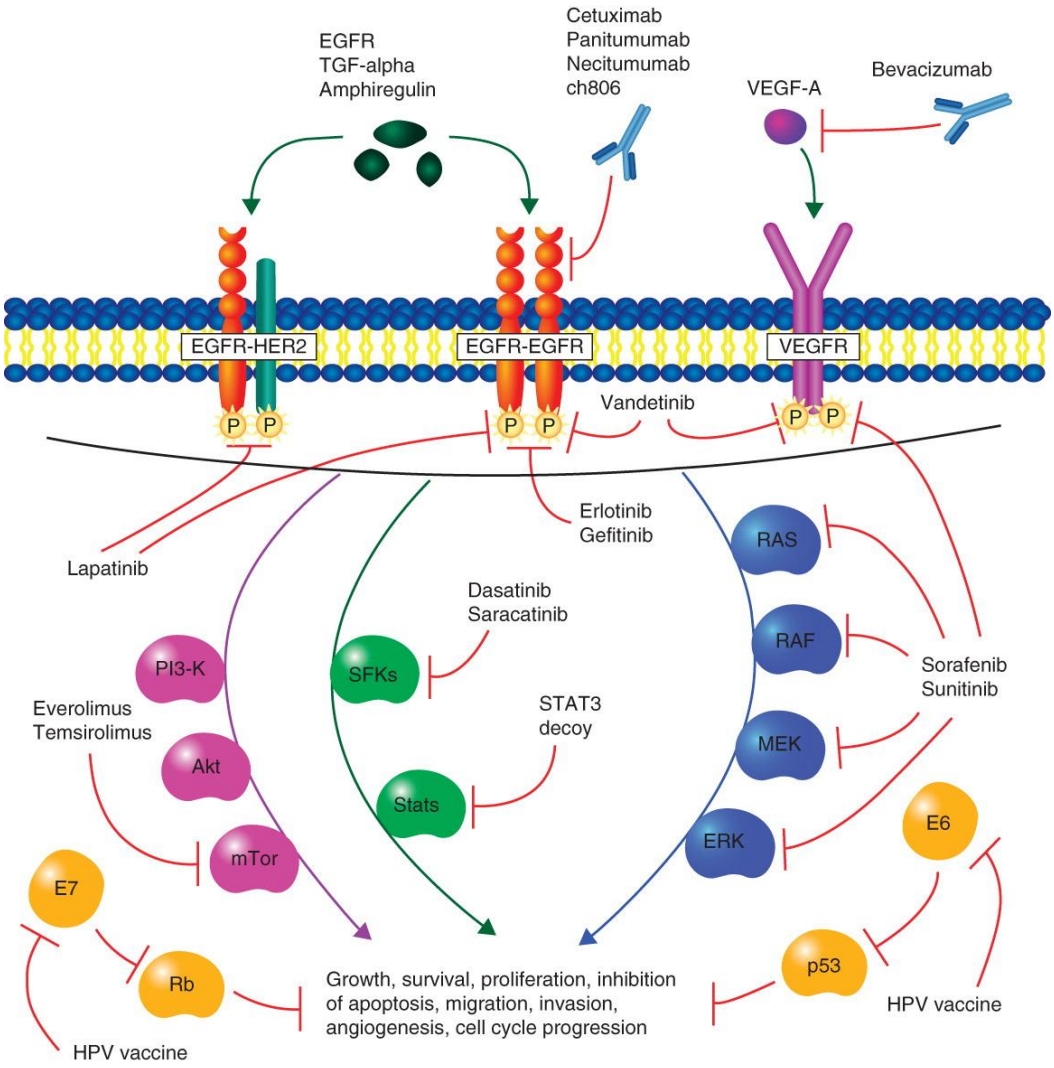



Figure 1-4 Targeted molecular pathways in head and neck cancer.

Selected agents that are currently in clinical trials are represented here. Briefly, EGFR and VEGFR signals use a variety of downstream molecular pathways including the PI3K-Akt-mTOR, STAT and Ras-MAPK (ERK) pathways. Agents targeting these pathways include mAbs (cetuximab, panitumumab, necitumumab, ch806, bevacizumab), tyrosine kinase inhibitors (erlotinib, gefitinib, vandetanib, lapatinib), multikinase inhibitors (sorafenib, sunitinib), Src family kinase inhibitors (dasatinib, saracatinib), mTOR inhibitors (everolimus, temsirolimus) and nucleic acid decoy (STAT3 decoy). p53 and Rb are tumour suppressors that are inhibited by HPV proteins E6 and E7, respectively. E6 and E7 are constitutively expressed in HPV-16 and -18 infected tumor cells and are targeted by HPV peptide vaccines. Vaccination against E6 and E7 allows cytotoxic T-cell recognition of infected cells (Table 1.7). Taken from (Fung and Grandis, 2010).

Table 1-6 Selected targeted therapies in clinical development for HNSCC in 2010

| Compound | Targeted pathway | Mechanism of action | Manufacturer | Developmental stage for HNSCC |
|---|---|---|---|---|
| **Bevacizumab** | Angiogenesis, VEGF | mAb against VEGF ligand | Genentech, Roche | Phase III |
| **Vandetanib** | Angiogenesis, VEGFR, EGFR | TKI specific for VEGFR and EGFR | AstraZeneca | Phase II |
| **Sorafenib** | Angiogenesis, kinases | Multi-TKI | Bayer, Onyx | Phase II |
| **Sunitinib** | Angiogenesis, kinases | Multi-TKI | Pfizer | Phase II |
| **STAT3 Decoy** | Intracellular signaling | STAT3 transcription factor decoy | University of Pittsburgh | Phase 0 |
| **Dasatinib** | Intracellular signaling | TKI specific for Src family kinases | Bristol-Myers Squibb | Phase II |
| **Saracatinib** | Intracellular signaling | TKI specific for Src family kinases | AstraZeneca | Phase II |
| **Everolimus** | Intracellular signaling | mTor kinase inhibitor | Novartis | Phase II |
| **Temsirolimus** | Intracellular signaling | mTor kinase inhibitor | Wyeth | Phase II |
| **HPV-16 vaccine** | HPV-16 | Peptide epitope vaccine | University of Maryland | Phase I |
| **MAGE-A3 HPV-16 vaccine** | HPV-16 | Peptide epitope vaccine | University of Maryland | Phase I |

HNSCC: Head and neck squamous cell carcinoma; mTOR: Mammalian target of rapamycin; STAT3: Signal transducer and activator of transcription-3; TKI: Tyrosine kinase inhibitor.
Adapted from (Fung and Grandis, 2010).

### 1.4.5 Treatment of oropharyngeal squamous cell carcinoma

Oropharyngeal cancer management is in a state of transition at present. As discussed earlier (Section 1.2), the epidemiology of oropharyngeal cancer is changing. Likewise, new treatment modalities are providing a role in the management of these cancers. Oropharyngeal cancers arise from base of tongue, tonsil, valeccula, posterior wall of oropharynx, inferior surface of the soft palate and the uvula. Cervical node metastases are common when tonsillar carcinomas are diagnosed, being present in 66-76% of patients. Contralateral cervical nodes have been reported in up to 22% of patients (Osborne and Brown, 2004, Lin et al., 2005, Weber et al., 2003a). The management of oropharyngeal tumours can be split into management of early (T1 and T2) disease and

management of late (T3 and T4) disease.

Early stage disease has reliably proven amenable to single modality treatment, either radiotherapy or surgery. Both modalities have comparable outcomes in terms of locoregional control and survival at 5 years. A recent publication by O'Hara et al looked at a series of T1-2, N0 oropharyngeal patients. The patients were treated with either surgery (+/-) radiotherapy or chemoradiotherapy. The 5-year disease specific survival rates were 69% and 60%, respectively (p=0.22) (O'Hara and Mackenzie, 2010). Given these equivalent results, primary radiotherapy is now the treatment of choice for several reasons: it has less short term morbidity and mortality; is is associated with better cosmetic results; it treats occult disease and reserves surgery for salvage. Salvage surgery has been shown to improve the overall survival rates of both T1 and T2 oropharygneal tumours (Osborne and Brown, 2004, Hicks et al., 1998). Many institutions are offering patients intensity modulated radiotherapy (IMRT) for the management of their oropharygneal cancer. It has the advantage of being parotid sparing. Ingle et al recently published the results from a small series of oropharyngeal patients treated with IMRT. They demonstrated excellent disease control: 2-year overall survival was 92% and disease specific survival 96%. They also showed that although intermediate toxicity was significant, longer follow-up showed that dysphagia continued to improve with 75% of patients not requiring any form of enteral or oral supplementation (Ingle et al., 2010).

The management of T3 and T4 tumours is more complex, with multimodality management becoming the standard of care for several reasons: first, it has been shown that radiotherapy alone does not provide adequate rates of local control for T3 and T4 lesions; second, although surgery alone provides a better chance of local control than radiotherapy alone, the rates of locoregional recurrence remain unacceptable, even with negative margins; third, several studies have shown that multimodality management is associated with significantly lower rates of local recurrence compared to single modality treatment (Cohan et al., 2009, Osborne and Brown, 2004, Hicks et al., 1998). Although it has been established that multimodality management is best for these malignancies, there are still several questions that need answering: What is the ideal order and aggressiveness of the treatment? Is chemoradiotherapy best given first followed by salvage surgery? Is surgery followed by chemoradiotherapy more effective? The identification of HPV as a predictive prognostic marker in patients with oropharyngeal cancer also offers further potential to optimise treatment (Haigentz et al., 2009).

### 1.4.6 Treatment of laryngeal squamous cell carcinoma

The larynx plays an important role in swallowing, communication and airway protection;

as a result treatment of laryngeal cancer represents one of the most challenging areas of head and neck oncology. Due to the unique functions of the larynx, any treatment will have an impact on these functions and consequently a patient's quality-of-life (McNeil et al., 1981, McLaughlin et al., 1988). Treatment of laryngeal cancer is divided into treatment of early glottic malignancies – i.e. T1 and T2 and treatment of late malignancies i.e. – T3 and T4.

The aim of treatment in patients with early glottic tumours is to cure with voice preservation. Seventy-five percent of glottic tumours present early, due to the effect of the cancer on patient voice quality. These patients do not have any cord fixation, metastasis or extension outside the glottis. Early glottic cancer is potentially curable with single modality treatment, namely radiotherapy or surgery (endoscopic with laser). Both treatment modalities confer similar survival advantages. Early glottic cancer (T1/T2) has an excellent prognosis, with 5-year cancer-specific survival rates between 85-95% (Groome et al., 2001, Shah et al., 1997). Although survival is high, the main cause of treatment failure is locoregional recurrence. As neither treatment has a survival advantage, patients receive treatment based not only on patient factors but also a clinician's experience. Radiotherapy is still more commonly given for the treatment of early glottic cancer, but is not without morbidity. The treatment takes time, patients have to undergo daily treatment for several weeks, and it is associated with significant side effects. In the event of locoregional failure, the only treatment option is major surgery. More recently early glottic cancers are being treated with endoscopic $CO_2$ laser. This has the benefit of being a single treatment, without the associated morbidity with radiotherapy. Voice quality after such a procedure is certainly comparable to that following radiotherapy. If recurrence occurs in this instance, the options for treatment include further endoscopic resection, major surgery or radiotherapy. The main modalities of treatment are either external beam radiotherapy or transoral laser microsurgery, with overall clinical equipoise between the two (Mendenhall et al., 2004). A pooled analysis of 7,600 patients shown no significant difference between the two approaches but there were trends in favour of transoral laser surgery for overall survival and radiotherapy for voice quality (Higgins et al., 2009). In most countries, radiotherapy remains the predominant treatment.

In early glottic SCC, local control generally constitutes disease specific cure. With the obvious importance in avoiding total laryngectomy, the important parameter is local control without laryngectomy. Many factors will influence the selection of either radiotherapy or surgery including: primary tumour site, co-morbidity of patient, availability of expertise in surgery or radiotherapy, patient occupation, preference and compliance, history of previous HNSCC.

An impotant critical area of research in HNSCC has been the evaluation of

chemoradiotherapy for the management of advanced laryngeal or hypopharyhgeal cancer to avoid the need for significant, life changing surgery. The EORTC head and neck cancer cooperative group carried out a phase III trial in patients with stage III and advanced resectable stage IV hypophyarngeal cancer. It demonstrated that neoadjuvent chemotherapy and radiotherapy increased local control to a level close to that achieved with radical surgery and post-operative radiotherapy (Lefebvre et al., 1996). Pignon et al carried out a meta-analysis of three larynx preservation trials showing voice preservation in 67% of surviving patients, 5 years after chemoradiotherapy (Pignon et al., 2000). Forastiere et al published a large multi-institutional randomised trial in which patients were randomised to induction cisplatin plus 5-FU followed by radiotherapy, radiotherapy given concurrently with cisplatin or radiotherapy only. Concurrent radiotherapy with cisplatin produced a statistically significant higher percentage of patients with an intact larynx 2 years after treatment (88% vs 75% vs 70% for concurrent treatment, induction chemotherapy and radiotherapy alone, respectively) and higher loco-regional control (78% vs 61% vs 5% respectively) than the other two regimens (Forastiere et al., 2003). The American Society of Clinical Oncologists published evidence based guidelines regarding treatment of laryngeal cancer, stating that concurrent chemoradiotherapy with surgery reserved for salvage offers potential for larynx preservation without compromising survival (Pfister et al., 2006). It is also recommended that surgical treatment to the neck is carried out for patients with nodal involvement treated with chemoradiotherapy (Pfister et al., 2006).

## 1.5 Prognostic factors in head and neck cancer

Accurate prediction of the prognosis of the newly diagnosed patient with head and neck cancer can assist the physician in patient counselling, clinical decision-making and quality maintenance. Many factors, as discussed below, influence the prognosis of the patient.

### 1.5.1 Staging

The most frequently used classification system for staging cancer is the TNM staging systems of the Union Internationale Centre le Cancer and the American Joint Committee on Cancer, initially devised and implemented by in the 1950s (Greene, 2007). Despite several revisions of the classification system over the years, it remains almost exclusively an anatomical staging system, with no clinical or pathological information used. The TNM classification has three components (Table 1.8). T describes the extent of the primary tumour, N describes the presence and extent of

lymph node metastasis and M describes presence or absence of distant metastasis. If the classification is before treatment it is termed clinical TNM (cTNM). If the classification is performed after histological examination it is termed pathologic TNM (pTNM). Advanced TNM stage is associated with a shorter disease-free survival, decrease in locoregional control and increase in distant metastasis (Dinshaw et al., 2006, Mendenhall et al., 2003). Another staging system used is the American Joint Committee on Cancer stage grouping (Table 1.9). This groups different T,N,M combinations into four stage categories, with increasing stage being associated with decreasing prognosis.

Cancer staging has several purposes: to indicate patient prognosis; to help guide treatment choice; to enable comparison of treatments in same stage patients; and to facilitate the exchange of information between clinicians. There can, however, be considerable variation in treatment response and survival within individual stage groupings. This limitation to the TNM system (Kim et al., 2010) can lead to under- or over-treatment of some patients. This limitation is becoming increasingly important as the addition of chemotherapy to radiotherapy is associated with increased toxicity and there are now several different management options for patients with head and neck cancer. A staging system that improves patients' selection for different treatment modalities would be a significant advance. The staging system should be an instrument that allows a doctor to collect prognostic information to help make treatment decisions. Therefore, there is a need to refine the current staging system to incorporate additional information associated with prognosis: tumour related factors such as histopathologic and molecular biology characteristics (e.g. grade, HPV status); patient related factors such as co-morbid conditions; and environmental factors (e.g. smoking and alcohol use). Predictors of treatment response may also be identified that could be incorporated into the staging system. With the further development of targeted therapies, treatment may be more guided by the biology of individual cancers. The importance of staging will be refined, with benefit to both the clinician and the patient (Takes et al., 2010).

Table 1-7 TNM clinical classification of oropharyngeal tumours

| T- Primary tumour | |
|---|---|
| TX | Primary tumour cannot be assessed |
| T0 | No evidence of primary tumour |
| Tis | Carcinoma in situ |
| T1 | Tumour ≤ 2cm in greatest dimension |
| T2 | Tumour > 2 to 4cm in greatest dimension |
| T3 | Tumour > 4cm in greatest dimension |
| T4a | Tumour invades any of the following: larynx, deep/extrinsic muscle of tongue, medial pterygoid, hard palate, mandible. |
| T4b | Tumour invades any of the following: lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base, carotid artery. |
| N- Regional lymph nodes | |
| NX | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Ipsilateral single ≤3cm |
| N2 | (a) Ipsilateral single >3 to 6cm<br>(b) Ipsilateral multiple ≤6cm<br>(c) Bilateral, contralateral ≤6cm |
| N3 | Metastasis in a lymph node > 6cm in greatest dimension |
| M- Distant metastasis | |
| MX | Distant metastasis cannot be assessed |
| M0 | No distant metastasis |
| M1 | Distant metastasis |

Table 1-8 The American Joint Committee on Cancer stage grouping

| | | | |
|---|---|---|---|
| Stage 0 | Tis | N0 | M0 |
| Stage I | T1 | N0 | M0 |
| Stage II | T2 | N0 | M0 |
| Stage III | T1,T2 | N1 | M0 |
| | T3 | N0, N1 | M0 |
| Stage IVA | T1,T2.T3 | N2 | M0 |
| | T4a | N0,N1,N2 | M0 |
| Stage IVB | T4b | Any N | M0 |
| | Any T | N3 | M0 |
| Stage IVC | Any T | Any N | M1 |

### 1.5.2 Clinico-pathologic factors

#### 1.5.2.1 Age

HNSCC is often regarded as a disease of the elderly, with patients being diagnosed predominantly in the sixth to eight decade of life. HNSCC is rare under 40 years of age when it accounts for about 0.5% of all diagnoses (Schantz and Yu, 2002). However, there is now evidence that the incidence of head and neck cancers is increasing in young people of both sexes (Macfarlane et al., 1992, Robinson and Macfarlane, 2003). There are several series in the literature addressing the issue of age as a prognostic factor. All studies used 40 years as the upper age limit. In multivariate analysis of

large cohorts of patients, age does appear to be a significant factor. Two studies showed that age had prognostic significance for survival, independent of other factors such as smoking, co morbidity, primary site, TNM stage, and nodal disease with older patients having a worse prognosis in terms of survival and recurrence (Lacy et al., 2000, Bhattacharyya, 2003). Another large population based study looked at 931 laryngeal and hypopharyngeal patients. The study showed that advanced age was associated with increased mortality and age at the time of diagnosis was a strong predictor of survival, particularly in the over 70 group (Dikshit et al., 2005).

It has been suggested that the low rate of survival in the elderly may be due to other co-morbidities and difficulty tolerating radical cancer therapy. However, the idea that mortality in the elderly is due to co-morbidity more than the cancer itself may lead to age-related treatment bias, with the elderly receiving less aggressive and therefore less effective treatment (Takes et al., 2010). Age has also been recognised as a prognostic factor for tumour recurrence. A small cohort study identified age as prognostic for tumour recurrence in parotid cancer and the observation was validated using a national and international database, after multivariate correction for treatment type. Older age was also associated with having a more aggressive tumour, having more advanced locoregional disease at presentation, peri-neural growth and high-grade histology (Vander Poorten et al., 1999, Poorten et al., 2009). Nguyen et al looked at the SEER database for 5,535 patients aged 22-99 years (median 58) diagnosed with tonsil SCC between 1974 and 2003. They found that in multivariate analysis, young age was independently associated with 40% relative better cancer specific survival compared with older age (HR 0.60. 95% CI 0.54-0.67). They suggested that the excellent prognosis in this group may be related to HPV associated tumours (Nguyen et al., 2010).

#### 1.5.2.2 Gender

There is evidence that gender affects treatment outcome with females appearing to have a better prognosis. A case series in Oslo looked at 433 patients with HNSCC. The study showed females had a 38% lower risk of mortality compared with males after adjustment for age, stage and tumour site (Faye-Lund and Abdelnoor, 1996). With regards to treatment with radiation specifically, women still appear to have a better prognosis, with better local tumour control and disease-free survival compared to men. In the USA, males have an approximately 2-fold higher mortality rate compared to females (Leborgne et al., 2001, Wang et al., 1996).(Lacy et al., 2000) Although the reasons for this remain unclear, it may result from differences in alcohol and tobacco use, along with the fact that women appear to present with earlier disease than men (McLean et al., 2006).

#### 1.5.2.3 Co-morbidities

Patients treated for head and neck cancer often have co-morbidities in addition to their cancer at the time of diagnosis (Sanabria et al., 2007). The existence of co-morbidities is particularly relevant for head and neck cancer patients as often they have a history of alcohol and tobacco abuse, which cause considerable morbidity in important organs. While the co-morbidity is distinct from the cancer itself, it is an important feature in the head and neck cancer patient. Co-morbidity will directly influence the initial treatment selection, a patient's ability to tolerate treatment and their prognosis. A recent series showed that 37% patients had a modest co-morbidity level and 12% had a high co-morbidity level at diagnosis (Alho et al., 2007). Several studies showed that co-morbidity influences prognosis in HNSCC (Hall et al., 2000) (Hall et al., 2000, Piccirillo and Vlahiotis, 2006, Homma et al., 2010).

A patient's co-morbidity can be obtained without the requirement for additional technology and classified as none, mild, moderate or severe. These four categories can then be combined with the TNM staging system producing an overall combined clinical-severity staging system containing four categories, which was shown to produce better stage groupings and improved survival gradients when compared to the TNM system alone (Piccirillo, 1995, Piccirillo, 2000).

There are a number of validated instruments to code and quantify co morbidity in patients: the Kaplan Feinstein co-morbidity index (KFI), the Charlson co-morbidity index (CCI), the cumulative illness rating scale (CIRS), and the index of coexistent disease (ICED) (Kaplan and Feinstein, 1974, Linn et al., 1968, Charlson et al., 1987, Cleary et al., 1991). Hall et al compared these four instruments and found that the KFI was the most successful in stratifying patients with head and neck cancer (Hall et al., 2002). In 1999, the modified KFI, also known as the adult co-morbidity evaluation (ACE27), was introduced. The ACE27, which has had several revisions and has proven to be a valid tool for quantifying the co-morbidity of HNSCC patients, defines scores according to the highest ranked single ailment: grade 1: mild decompensation; grade 2: moderate decompensation; grade 3: severe decompensation. When two or more grade 2 ailments occur in different organ systems, the overall co-morbidity severity score is designated as grade 3 (Piccirillo and Costas, 2004). Recently, Datema et al published the results of an updated study looking at factors that predict outcome after treatment for HNSCC. They collected the data for 1,371 patients, including primary tumour site, age at diagnosis, gender, TNM classification, and prior malignancies. They also collected co-morbidity information using the ACE27 tool. Co-morbidity was present in 36.4% of their patients. They found that co-morbidity had a

significant impact on overall survival, with significant differences for the different categories of the ACE27. The impact of an ACE27 grade 3 was comparable to the impact of a T4 tumour or an N2 neck (Piccirillo and Costas, 2004). This work highlights the importance of taking co-morbidity into account when making treatment decisions in patients with HNSCC.

#### 1.5.2.4 Haemoglobin

The importance of pre-treatment haemoglobin (Hb) on prognosis was initially identified in cervical cancer patients treated in the 1940s (Evans and Bergsjo, 1965). Anaemia – often defined as a Hb level of <12 g/dL - is commonly found in 40–60% of patients presenting for radiotherapy. Although it can result from the disease itself, it also occurs due to treatment. During radiotherapy the prevalence can increase to 80% (Harrison et al., 2002). There is a positive association between increased Hb levels and increased quality-of-life (Doni et al., 2010, Cortesi et al., 2005).Therefore, the majority of cancer patients will already have anaemia or are at risk of becoming anaemic during their treatment. Generally patients will not have anaemia directed treatment unless their Hb falls below 9–10 g/dL or they are haemodynamically unstable (Hu and Harrison, 2005).

The association between low Hb and survival has been reported extensively over the past 40 years. The incidence of anaemia in HNSCC patients varies depending on its definition. If anaemia is defined as <14.5 d/dL for males and <13g/dL for women, then the incidence of pre-treatment anaemia in several trials has ranged from 41% in a DAHANCA trial, to 56% in the RTOG 79-15 trial up to 64% in the RTOG trial 85-27 (Lee et al., 1995, Fazekas et al., 1989, Overgaard et al., 1998). However, if the World Heath Organisation's definition is used (<12 g/dL), then obviously the incidence of anaemia would be lower (WHO, 2001). Hu et al looked at the prevalence of anaemia in a cohort of their HNSCC patients using the WHO definition. They found that 16% were anaemic before the start of treatment, and this increased to 32% 3-5 weeks after the start of treatment, with the mean decrease in Hb being 1.8 g/dL (Hu and Harrison, 2005).

The incidence of anaemia also appears to vary depending on subsite. Dubray et al reported the incidence of anaemia in a series of patients undergoing radiotherapy for laryngeal cancer. He defined anaemia as Hb <13.5 g/dL for males and <12 g/dL for women. For all patients, 15% were anaemic. However, when it was split into cancer subsite the prevalence changed to 40% for supraglottic laryngeal SCC versus 12% for glottic laryngeal SCC. After radiotherapy, the post treatment incidence of anaemia was 30% for all patients, with 56% for the supraglottic tumours versus 23% for the glottis tumours (Dubray et al., 1996). With the use of concurrent chemotherapy now routine in the management of HNSCC even more patients will become anaemic to some

extent. Hu et al showed that in a series of HNSCC patients receiving chemoradiothearpy, 96% of patients had a Hb <12 g/dL by the end of trewatment (Hu and Harrison, 2005). Other centres have also reported similar experiences (Rosen et al., 2003).

Over the past 35 years, the negative influence of pretreatment anaemia (using Hb thresholds of (9-14.5 g/dL) on locoregional control and overall survival has been consistently shown in many solid tumours including HNSCC (Takeshi et al., 1998, Overgaard et al., 1998, Harrison et al., 2002, Caro et al., 2001). The prognostic significance of pre-treatment Hb has been consistently demonstrated in both univariate and multivariate analysis in HNSCC patients treated with either radiotherapy or surgery, see appendix II (Warde et al., 1998, Stadler et al., 1999, Overgaard et al., 1998, Bush, 1986, Caro et al., 2001, Prosnitz et al., 2005, Stadler et al., 2006, McCloskey et al., 2009). For decades physicians have been aware of the significance of low Hb. In 1984, Blitzer et al reported that low Hb was associated with poor local control in advanced stage HNSCC patients. They demonstrated a ~10% drop in locoregional control for every 2 g/dL drop in Hb (Blitzer, 1984). In 1986, Overgaard et al (Overgaard et al., 1986) also demonstrated that a Hb concentration at the lower limit of normal was associated with poor local control and survival in patients with pharyngeal SCC. Patients with a Hb level >13 g/dL (in females) and >14.5 g/dL (in males) had a significantly better prognosis compared to patients with Hb below this level (Overgaard et al., 1986). In 1996 Dubray et al reported the results of a cohort of 217 HNSCC patients. He found that pretreatment anaemia (men <13.5 g/dL and women <12 g/dL) was associated with a worse 2-year locoregional control (50% vs 76%; $p < 0.00001$) and worse 2-year survival rates (49% vs 77%; $p < 0.00001$) (Dubray et al., 1996). Generally, the results of pretreatment anaemia are consistent, with nearly all studies showing that low pretreatment Hb is a significant adverse prognostic indicator of disease control and survival after potentially curative treatment.

On the basis of the studies described above, it has been suggested that the ideal Hb in the radiation oncology setting should be 12–14 g/dL (Hu and Harrison, 2005). Caro et al systematically reviewed the literature to estimate the effect of anaemia on survival in patients with cancer. He found 10 studies with the relevant clinical information on HNSCC. These patients had an unadjusted HR for anaemia of 2.35, with the adjusted HR of 1.75 (95% CI 1.37-2.23).The relative risk of death increased by 75% in anaemic HNSCC patients. The median survival times for patients with and without anaemia were 30 and 90 months, respectively. This study again confirms the significance of anaemia as a predictor of poor outcome (Caro et al., 2001).

As discussed above, the significance of pretreatment Hb has been reported extensively in the literature. Although not as extensively reported, a drop in Hb during

radiotherapy also appears to affect outcome (van Acht et al., 1992, Skladowski et al., 1999, Tarnawski et al., 1997). van Acht reported the results of 306 patients treated for laryngeal cancer. An Hb drop of more than 1 mmol/L during treatment resulted in significantly lower disease-free survival. Interestingly, the incidence of anaemia did not appear to be related to nutritional status as the incidence of >10% weight loss was similar for both anaemic and non anaemic patients (van Acht et al., 1992). Tarnawski et al reported that anaemia at the end of a course of radiotherapy was a significant predictor for worse local control on multivariate analysis. He noted that the degree of Hb drop was also prognostic, but not as significant as the post-treatment Hb (Tarnawski et al., 1997). Rutkowski et al looked retrospectively at 835 patients treated for advanced laryngeal cancer with surgery and post operative radiotherapy. They found that individual change in Hb concentration during the course of post operative radiotherapy rather than Hb level before or after radiotherapy was most significant for locoregional contol (Rutkowski et al., 2007).

It has been argued that, like cervical cancer, anaemia in HNSCC is an indicator of biologically aggressive disease, possibly caused by cancer cachexia or bleeding from the tumour and therefore not predictive of outcome in this setting (Hu and Harrison, 2005). However, an argument against this is early laryngeal cancer, which is often a small tumour localised to the primary site. There are several retrospective series of early glottic cancer documenting poor outcome (Table 1.10). Warde et al reported on a series of 735 patients with early glottis cancer. Patients who had an Hb <12 g/dL were found to have a 1.8 (95% CI 1.2–2.5) increased risk for locoregional failure in a multivariate analysis (Warde et al., 1998). Fein et al reported on 109 patients with early glottis cancer. He showed that a pre-treatment Hb of <13.0 g/dL was associated with poorer 2-year locoregional control (95% vs 66%; p=0.0016 ) and 2-year overall survival rates (88% vs 46%; p=0.0018) (Fein et al., 1995). However, there have been a few studies that reported no correlation between tumour control and Hb in early glottis cancer, although even in these studies low Hb was associated with a trend for worse survival (Overgaard et al., 1989, Canaday et al., 1999).

The significance of a low Hb for early stage glottic cancer is not just related to those treated with radiotherapy; it also appears to be significant in the surgical setting. Lutterbach et al report a series of T1 larynx tumours that were treated surgically, with clear margins. Preoperative anaemia (Hg <13 g/dL and <12 g/dL in men and women, respectively) was associated with a significantly worse 5-year prognosis (60% vs 85% in patients without anaemia; p=0.002) and high risk of treatment failure (relative risk 3.0). They demonstrated that each 1 g/dl Hb decrease was associated with a relative risk of locoregional relapse of 1.4, suggesting that Hb may be a continuous risk factor even within the accepted range of normal (Lutterbach and Guttenberger, 2000), The

significant prognostic finding of anaemia in small glottic cancers, treated with either radiotherapy or surgery, is interesting because as it implies that anaemia may be linked to more aggressive tumours, and it may be a poor prognostic factor independent of tumour volume or interaction with radiotherapy.

Anaemia is a very relevant risk factor as it is potentially modifiable with various therapeutic techniques. Up until the early 1980s, blood transfusion was the most common treatment for cancer related anaemia (Surgenor et al., 1990). However, blood transfusion have become fairly unpopular among patients and physicians because of risks (e.g., HIV, hepatitis infections, acute/chronic reactions, and immunosuppression), costs and supply shortages (Henry, 1992). There is also some evidence that transfusion to correct a low Hb might adversely affect outcome. Bhide et al reviewed the incidence of anaemia and the effect of a policy of Hb maintenance by blood transfusion on a series of HNSCC patients treated with chemoradiotherapy. In their series of 169 patients, they found that relapse free survival (52% vs. 41%, p = 0.03), disease free survival (71% vs. 66%, p = 0.02), and overall survival (58% vs. 42% p = 0.005) were significantly better for patients who did not have a transfusion vs. those who did, suggesting that blood transfusion may be detrimental (Bhide et al., 2009).

Hoff et al evaluated the prognostic significance of low Hb level and its modification by blood transfusion in HNSCC patients treated with radiotherapy. The study was part of the DAHANCA 5 trial. Patients were randomised to treatment with the hypoxic radiosensitiser nimorazole or placebo. They were sub-randomised to plus or minus transfusion if they had a low Hb (females <13 g/dL; males <14.5 g/dL). Patients with high Hb levels had a significantly better probability of locoregional control, disease-specific survival and overall survival compared to 'low Hb no transfusion' patients. Interestingly, in the group with low Hb, a blood transfusion did not improve outcome (locoregional control, disease-specific survival or overall survival). In a multivariate analyses there were no significant influences of transfusion or Hb level on the outcome endpoints (Hoff et al., 2010).

Several trials explored erythropoietin (EPO) as a treatment for correcting anaemia in HNSCC undergoing chemoradiotherapy. A Cochrane Database Systematic Review was carried out looking at the use of EPO in these patients. Analysis of five randomised controlled trials with a total of 1,397 patients showed addition of EPO to radiotherapy decreased overall survival compared to radiotherapy alone (OR 0.73; 95% CI 0.58 to 0.91; p = 0.005). On the basis of this review it has been suggested that EPO is not administered with radiotherapy outside the experimental setting for patients with head and neck cancer (Lambin et al., 2009).

#### 1.5.2.5 Tobacco use

As discussed previously, tobacco and alcohol use are the dominant risk factors in the development of head and neck cancer (Section 1.4). Unfortunately, up to a third of patients diagnosed with HNSCC continue to smoke after their diagnosis (Ostroff et al., 1995). Several studies have demonstrated that patients who smoke during their treatment have reduced locoregional control and overall survival compared to those who do not (Browman et al., 2002, Chen et al., 2010, Fortin et al., 2009). Farshadpour et al recently published the results of study looking at the influence of continued smoking and drinking on survival. Interestingly they found that HNSCC-specific survival (death due to primary-HNSCC or recurrent HNSCC) and HNSCC/second primary tumour-specific survival (death due to primary-HNSCC or recurrent HNSCC or second primary tumour) were not significantly different for patients who smoked and drank alcohol (HR 1.26; 95% CI 0.86-1.85) compared to those that did not (HR 1.34; 95% CI 0.96-1.88). However, overall survival was significantly affected for patients who smoked and drank alcohol (HR 1.50, 95% CI 1.16-1.93) (Farshadpour et al., 2010).

Although it is clear that smoking during radiotherapy affects outcome, the mechanisms behind this are still not clear. Smoking is thought to contribute to chronic hypoxia, which in turn decreases the efficacy of radiotherapy (Jensen et al., 1991). In an animal model, Grau et al demonstrated that elevated carboxyhaemoglobin, a complex formed when smoke binds to Hb, leads to increased tumour hypoxia, which reduces local control following irradiation (Grau et al., 1994).

#### 1.5.2.6 Site and subsite

Tumours arising in areas very close to each other can display different patterns of behaviour. Over the years people have made clinical observations about the differences in HNSCC subsites. Lindberg et al were one of the first groups to observe differences in subsites. They looked at the frequency of lymph node metastasis at presentation and noted a very wide range in frequency; the range was as high as 71% in T1 tonsil cancers down to 8% in T1 soft palate cancers (Lindberg, 1972). He also noted that for some cancers (e.g. tongue base) increasing T stage did not impact the rate of lymph node metastasis, where as for other sites (e.g. floor of mouth) rate of lymph node metastasis was directly associated with T stage (Lindberg, 1972). Survival rates also differ widely in HNSCC. Lip SCC has the highest 5-year survival rate at 90%, although most of these cancers present early without regional or distant metastasis. Laryngeal cancers are generally associated with a better outcome than oropharyngeal cancer. In 2003, the US national cancer statistics showed that the

death rate per 100,000 population for laryngeal versus oropharyngeal was 2.36 versus 4.06 respectively (Jemal et al., 2007). Carvalho et al used the SEER database to look at the issue of site further and they demonstrated that the difference in survival is not due to clinical stage at presentation, and these site specific differences in survival remain across stages (Carvalho et al., 2005). Such site specific differences are also seen in the management of HNSCC, with larynx and orophayrnx cancer more commonly treated with chemoradiotherapy while oral cavity tumours are still treated predominantly with surgery (Forastiere et al., 2003). Hypopharyngeal cancers have the poorest 5-year survival rates at 29%. Johansen et al demonstrated that the region of origin was highly significant for nodal recurrence, with the best prognosis in glottic tumours, then oropharynx, then supraglottis. The hypopharynx had the highest relative risk of developing nodal recurrence (Johansen et al., 2004).

#### 1.5.2.7 Tumour volume

Many studies have confirmed that tumour volume is a prognostic factor for outcome after treatment with radiotherapy and chemoradiotherapy (Takes et al., 2010, Pameijer et al., 1997, Nathu et al., 2000). Success in controlling a tumour with radiotherapy will depend on the ability to kill all the clonogenic cells in the tumour, so that no cells remain that are capable of causing tumour regrowth. It has been reported that the number of clonogenic cells increases linearly with tumour volume (Brenner, 1993, Dubben et al., 1998). However, some authors have argued that the effect is more complex than a simple increase in clonogenic cells and suggest that other volume related factors such as hypoxia, and clonal radioresistance, may also be involved (Bentzen and Thames, 1996).

The imaging of tumours has improved dramatically over the past decade, with precise and accurate measurements now easy to obtain using CT or MRI. Tumour volume measured using MRI is reported to be equal or smaller than volumes measured using CT (Rasch et al., 1997). Studer el al used volumetric cut-offs to define different prognostic subgroups in a large series of patients with head and neck cancer. He demonstrated that using the cut-off values of 15 and 70 $cm^3$ was superior to TNM in predicting patient outcome (Studer et al., 2007). Chen et al used a cut-off volume of 30 $cm^3$ in a series of patients with advanced hypopharyngeal cancer and showed a strong correlation between tumour volume and outcome (Chen et al., 2009). There is subsite variability, with tumour volume for laryngeal cancer being a better predictor than for tosillar or tongue base cancer. This may be related to differences in radiosensitivity between subsites (Mendenhall et al., 2003).

## 1.6 Molecular diagnostics/biomarkers for head and neck cancer

### 1.6.1 Cancer biomarkers

There has been a significant increase in the knowledge of the molecular biology of cancer over the past two decades. This increase has led to the development of potential biomarkers that might provide information on diagnosis, prognosis, treatment selection and recurrence risk.

A biomarker is any characteristic that is objectively measured and evaluated as an indicator of a normal biological process, pathogenic process or pharmacologic responses to a therapeutic intervention (Lesko and Atkinson, 2001). Biomarkers can be DNA, mRNA, proteins, metabolites or measure processes such as apoptosis, angiogenesis or proliferation (Hayes et al., 1996). Biomarkers can be either produced by the tumour itself or from surrounding tissue in response to the tumour. There are different types of biomarkers. A diagnostic (screening) biomarker is used to detect and identify a particular type of cancer. Although there are no such biomarkers in HNSCC, they are used for detection of other malignancies - the presence of Bence-Jones protein in urine is a strong diagnostic indicator of multiple myeloma. A prognostic biomarker is used once a malignancy has been diagnosed. It is used to indicate a patient's likely prognosis and, therefore, helps guide the aggressiveness of treatment. Again, in HNSCC no such biomarker exists in routine management, but there are prognostic biomarkers used in other malignancies, e.g., human chorionic gonadotrophin and alfa-fetoprotein levels provide prognostic information for patients with testicular teratoma. A stratification (predictive) biomarker is a marker that can predict response to a particular treatment before it has started; such markers can classify patients into likely responders and non-responders.

### 1.6.2 The need for biomarkers in head and neck cancer

There is a need for biomarkers that could guide treatment decisions or predict response for head and neck cancer patients. At present there are no such markers in HNSCC, but HPV is an emerging potential marker. (see Section 1.8.2).

Patients with early stage head and neck malignancy have excellent survival rates. However, often patients present with late stage disease, which has a very poor survival rate. Hence, any biomarker that could aid in the early diagnosis of HNSCC would be key to improving survival outcome. Head and neck cancer, like all cancers, develops in a stepwise process, characterised by genetic alterations causing inactivation of tumour suppressor genes and activation of oncogenes (Ha et al., 2003). The accumulation of genetic changes is related to increasing histological abnormalities and a model of genetic progression in HNSCC has been suggested (Califano et al., 1996, Braakhuis et al., 2004). The model suggests head and neck cancer occurs in precursor fields, areas

of genetically altered epithelial cells. These fields of abnormality will often have p53 pathway disruption (Braakhuis et al., 2005, Braakhuis et al., 2003, Tabor et al., 2004). The fields can be several centimetres in size and are often without clinical symptoms or signs, only a small proportion of patients demonstrate leukoplakia or erythoplakia in the mucosa. A pathologist will assess these areas microscopically categorising them as mild, moderate or severe. The difficulty is that the grading is subjective and not all precancerous areas may be identified. When resecting a tumour these fields are not visible to the naked eye, so they will not be completely removed. Histological grading of the tumour margins is unable to consistently predict if recurrence if likely to occur, therefore relapses may arise unexpectedly (Tabor et al., 2002, van Houten et al., 2004, Pateromichelakis et al., 2005). The detection of patients with precancerous fields would considerably improve the early diagnosis of HNSCC if a suitable biomarker was found.

Another major clinical problem in the management of HNSCC is local recurrence of a tumour that has histologically clear margins at the time of surgery. Salvage surgery is a likely option for the few patients with potentially resectable locoregional recurrence (Weber et al., 2003b). A biomarker that could be used to assess surgical margins and predict recurrence would significantly aid management of HNSCC.

The management of HNSCC focuses on primary disease and neck, with the presence of neck node metastasis being the most important prognostic factor for survival. However, often patients may present with a clinically negative neck, which may harbour occult metastasis (Argiris et al., 2008). The treating clinician must decide whether to treat a clinically negative neck or watch and wait. Thirty to forty percent of patients with a clinically negative neck have neck node metastasis, and a biomarker that could help predict these patients would be particularly useful.

A further clinical problem is the development of distant metastasis without loceregional recurrence, which occurs in around 10-25% of HNSCC patients. The most common sites for these metastases are lungs, liver and skeleton, and at present there is no effective systemic treatment (Argiris et al., 2008). Again, a biomarker that could predict the likelihood of distant metastasis would be useful.

### 1.6.3 Protein biomarkers

Biomarkers can be DNA, mRNA, proteins, metabolites or measuring processes such as apoptosis, angiogenesis or proliferation (Hayes et al., 1996, Sidransky, 2002). A biomarker could be a single molecule, a combination of multiple molecules or a specific molecular profile. Biomarkers should allow easy and reliable analysis, with the test being cost effective with a high degree of analytical sensitivity and specificity. Biomarkers must also contribute to a decision in clinical practice that is going to

improve upon current patient care (Ludwig and Weinstein, 2005). Over the past decade there has been a steady growth in the field of high-throughput, large scale biology. There have been significant advances in genomic and proteomic technologies, along with the discovery of new oncogenes and tumour suppressor genes. These advances along with new, powerful bioinformatics tools will have a large impact on biomarker research. Previously, biomarker discovery was very much based on chance observation. Nowadays, with modern technology, they can help to distinguish patterns and panels of potential biomarkers (Kulasingam and Diamandis, 2008). Proteins are attractive biomarkers. Many regulatory processes occur post-transcriptionally including protein activation, post-translational modification of proteins, and protein transport to the organelle. Proteins can be detected by immunohistochemistry (IHC) in tissue or by enzyme-linked immunosorbent assay (ELISA) in body fluids. Such techniques are quick, easy, and cost-effective, and can easily be applied to routine clinical practice (Schaaij-Visser et al., 2010).

### 1.6.4 *CDKN2A*

*CDKN2A* (also known as *CDKN2AINK4a and* hereafter referred to as *CDKN2A )*is a tumour suppressor gene on chromosome 9p21. It inactivates the function of cdk-4 and cdk-6-cyclin D complexes. A critical substrate of the G1 specific cdk complex is the release of E2F via phosphorylation of the retinoblastoma (pRb)-E2F protein. The function of pRb is negatively regulated by *CDKN2A* through suppression of Rb phosphorylation, and pRb also negatively regulates *CDKN2A* expression (Li et al., 1994). Loss of this cell-cycle inhibitor by deletion, point mutation or promoter methylation has been demonstrated in HNSCC (Olshan et al., 1997, Shintani et al., 2001). HPV16 has been shown to induce *CDKN2A* expression during immortilisation. IHC for *CDKN2A* has been demonstrated in dysplastic tonsil epithelium, but not normal tonsil epithelium, (Klussmann et al., 2003) and immunostaining for *CDKN2A* has been demonstrated as specific for HPV positive HNSCC (Table 1.12) (Klussmann et al., 2003, Lassen et al., 2010, Hafkamp et al., 2003, Wiest et al., 2002).

Table 1-9 *CDKN2A* expression in HNSCC

| Site | Pts | Treatment | *CDKN2A* expression associated with | Reference |
|---|---|---|---|---|
| HNSCC | 156 | Conventional RT | ↑ LRC (5-year actuarial values 58% v 28%; p=0.0005), ↑ DSS (72% v 34%; p=0.0006), and ↑OS (62% v 26%; p=0.0003). In MVA, ↑ LRC (HR 0.35; 95% CI 0.19 to 0.64), DSD (HR 0.36; 95% CI, 0.20 to 0.64), and OD (HR 0.44; 95% CI, 0.28 to 0.68). | (Lassen et al., 2009) |
| HNSCC | 331 | RT (66–68 Gy in 33–34 fx, 5 fx/wk) + nimorazole or placebo (DAHANCA) | Improved outcome (HR 0.41, 95% CI 0.28-0.61). In subgroup *CDKN2A* -ve, LRF more frequent in the placebo group than in the nimorazole group (HR 0.69, 95% CI 0.50-0.95). Patients with *CDKN2A* +ve tumours treated with nimorazole had a LRC similar to patients given placebo (HR 0.93, 95% CI 0.45-1.91). | (Lassen et al., 2010) |
| Stage III or IV oropharyngeal | 185 | TROG 02.02 phase III trial, concurrent RT + cisplatin +/- tirapazamine | ↑ 2-year OS (91% v 74%; HR 0.36; 95% CI, 0.17 to 0.74; p=0.004) and FFS (87% v 72%; HR 0.39; 95% CI, 0.20 to 0.74; p=0.003). On MVA: HR 0.45; 95% CI, 0.21-0.96; p=0.04. | (Rischin et al., 2010b) |
| Oropharyngeal SCC | 239 | IMRT | DFS, DSF and OS in MVA | (Lewis et al., 2010) |
| Locally advanced HNSCC | 55 | Cisplatin and radiotherapy | ↑ DFS (p=0.0001) and OS (p=0.001) at 5 years | (Lau et al., 2010) |
| Tonsil SCC | 92 | Surgery +/- CRT | Favourable prognoses in univariate analysis (p=0.004) and MVA (P=0.01). | (Kuo et al., 2008) |
| HNSCC | 301 | Surgery + RT, surgery, RT | *CDKN2A* –ve associated ↓DSS (HR 2.0, 95% CI 1.0–3.9) and recurrence (adj.HR 3.6, 95% CI 1.6–8.2); | (Smith et al., 2008) |
| Tonsil SCC | 51 | RT | *CDKN2A* +ve group, 94% had a complete RT response. *CDKN2A*+ve had a favourable prognosis. | (Mellin Dahlstrand et al., 2005) |
| Tonsil SCC | 34 | Surgery + CRT | ↑ DFS (p=0.02) | (Klussmann et al., 2003) |

DSD – disease specific death; OD – overall death; FFS – failure free survival.

## 1.7 Hypoxia

Tissue hypoxia develops from an inadequate supply of oxygen that is required for biologic functions (West, 1999). Hypoxia has been an area of interest in radiation biology for many years. Early in the last century Mottram published one of the first papers about tumour hypoxia; he proposed a role for hypoxia in increasing tumour resistance to radiotherapy in mammalian cells (Mottram, 1936). However, it was the landmark paper by Gray et al in 1953 that demonstrated the radioresistance of hypoxic cells (Gray et al., 1953). Thomlinson and Gray in 1955 then gave us the theory of how hypoxia develops in tumours. They demonstrated, with histological specimens of

lung cancer, that there was a constant distance across tumour tissue between necrosis and blood vessels. The distance, a figure still quoted today, of 100-150 μm was consistent with the known oxygen diffusion distance in tissue. They suggested that areas of hypoxia would be adjacent to necrosis and that the radioresistant hypoxic cells would be a cause of failure following radiotherapy (Thomlinson and Gray, 1955). Since this observation hypoxia has been demonstrated in a number of human malignancies including cervical, head and neck, pancreatic, brain, breast and sarcomas There is considerable evidence that tumour hypoxia, acting directly or indirectly, contributes to resistance of radiotherapy, chemotherapy, chemoradiotherapy and surgery, with resultant worse clinical outcomes (Harrison and Blackwell, 2004, Brizel et al., 1997).

### 1.7.1 Development of tumour hypoxia

In normal tissue, oxygen supply meets oxygen demand. However, tumour hypoxia develops as a result of an imbalance between the oxygen supply and oxygen consumption of the tumour cells (Vaupel et al., 2002). Two types of tumour hypoxia exist: “diffusion-limited hypoxia” and “perfusion- limited hypoxia.” The distinction is primarily how long the cells are hypoxic but also indicates their different causes. Diffusion-limited or chronic hypoxia is caused by tumour cells outgrowing their vasculature resulting in an inadequate oxygen supply. Beyond the diffusion distance, typically 100-150 μm, the cells become starved of essential nutrients (Horsman, 1998). Chronic hypoxia is frequently called diffusion-limited hypoxia as the oxygen concentration decreases progressively with the distance from the blood vessel. Cells change their phenotype as they adapt slowly to decreasing oxygen tension (Janssen et al., 2005). In addition to an increase in the diffusion distance, chaotic vascular geometry, the development of plasma channels and rheological effects on red cell deformability also lead to chronic hypoxia (Dewhirst, 1998).

Perfusion-limited or acute hypoxia is often transient and caused by inadequate blood flow in vessels. It arises as a result of the structural and functional abnormalities of the vascular network; elongated and tortuous shape of the vessels, dilatations and incomplete endothelial linings. This results in blocking of vessels by circulating blood and tumour cells, collapse of blood vessels from high interstitial pressure and interruption of blood flow to the tumour (Vaupel, 1996). This interruption in blood flwo deprives surrounding cells of oxygen. As the blood flow changes, oxygen levels will decrease in the surrounding cells (Janssen et al., 2005). These flow changes have been demonstrated in murine and human tumours (Trotter et al., 1989, Janssen et al., 2002). The change in blood flow can have biological consequences. Reynolds et al used a tumourigenic cell line, and demonstrated that exposure of the cultured cells to

hypoxia produced an elevated mutation frequency and a mutation pattern similar to that seen in the tumours. This suggests that the conditions within solid tumours are mutagenic and a fundamental mechanism of tumour progression in vivo is genetic instability induced by the tumour microenvironment (Reynolds et al., 1996).

As the degree of hypoxia is variable on a continuous scale, there is no strict cut-off value to distinguish hypoxic from normoxic cells. As Fig 1.6 demonstrates, important metabolic processes stop working at different $pO_2$ levels. Radioresistance may already occur below 25–30 mmHg (Hockel and Vaupel, 2001). In most studies, values from 0.5-10 mmHg have been used as a cut-off value to discriminate normoxic from hypoxic tumour cells (Rademakers et al., 2008).

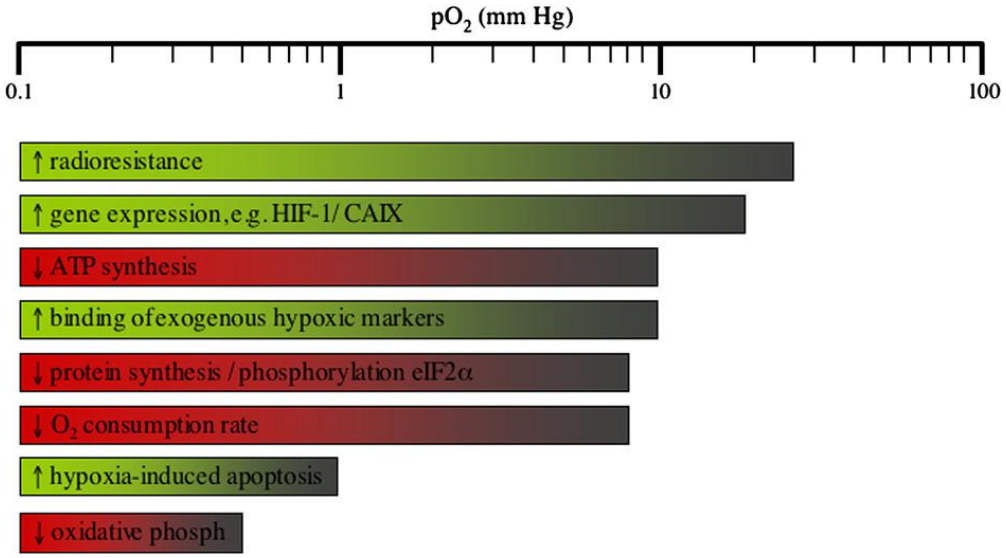



Figure 1-5 Cellular adaptation to hypoxia. The bars show the approximate oxygen levels at which cellular responses gradually change. Taken from (Rademakers et al., 2008).

### 1.7.2 Clinical consequences of tumour hypoxia

#### 1.7.2.1 Tumour progression

Malignancy is characterised by a tumour's ability to spread both locally and to distant sites. The hypoxic microenviroment in solid tumours can cause tumour cells to respond in two ways. It may act as a stressor that prevents growth and promotes death of the cell, by not allowing repair of damaged DNA. It can also promote malignant progression. Treatment resistance and enhanced malignant progression are signs of hypoxia induced proteomic and genomic changes within the tumour (Vaupel et al., 2001b, Vaupel et al., 2001a). Vaupel et al has suggested that fluctuating ($pO_2 \leq 7$ mmHg ) or sustained (>8 hours) hypoxic stress can lead to alterations in gene-expression and post-transcriptional or post-translational modulations that result in

changes in protein expression (Vaupel et al., 2001a).

Anoxia/hypoxia-induced proteomic changes may, in turn, lead to growth stasis or impairment through molecularly mediated cell-cycle arrest, differentiation, programmed cell death (apoptosis) or necrosis (Fig 1.7) (Huang et al., 2007). Under anoxia, most cells are arrested immediately, regardless of their position in the cell cycle. Hypoxia-inducible factor one alpha (HIF-1α) activates the cyclin-dependent kinase inhibitors p21 and p27 causing hypoxia induced cell cycle arrest at the $G_1S$ check point (Goda et al., 2003). $pO_2$ values of 0.2–1 mmHg lead to disproportionately lengthening of the $G_1$ phase or $G_1$ arrest (Amellem et al., 1994).

Hypoxia can induce programmed cell death in tumour cells (Riva et al., 1998). Exposure to severe hypoxia leads to the accumulation of p53 which can in turn lead to rapid apoptosis. When p53 does build up in a hypoxic environment, it activates apoptosis with Apaf-1 and caspase-9 being triggered downstream (Soengas et al., 1999). Hypoxia also stimulate p53-independent apoptosis pathways involving genes of the Bcl-2 family and others (Shimizu et al., 1995). Hypoxia may then result in necrotic cell death. The proteome changes induced by hypoxia cause cell-cycle arrest, apoptosis, necrosis and differentiation, which may explain why some tumours have delayed recurrence and dormant micrometastasis (Hockel and Vaupel, 2001).

Hypoxia induced proteome changes may also help tumour cells to adapt to a hostile environment, adapt to nutritional deprivation or facilitate proliferation, invasion and metastatic spread (Vaupel and Harrison, 2004). HIF-1α is a chief promoter of tumour cell adaption to hypoxia, which increases when cellular oxygen decreases. HIF-1α triggers the transcription of a large number of genes involved in oxygen deliver (e.g. erythropoietin), energy preservation (glucose transporters, GLUT1 and GLUT3), angiogenesis (VEGF) and processes involved in cell proliferation and spread (see Fig 1.7) (Vaupel and Harrison, 2004). Angiogenesis is particularly important for tumour growth and survival as tumours are unable to grow beyond 1 mm in diameter without a blood supply (Folkman, 1995).

As well as the proteomic changes a cell will undergo in hypoxic conditions, genomic changes occur also. Tumour aggressiveness is dependent on mutations in oncogenes and/or tumour suppressor genes. Hypoxia ($pO_2 \leq 0.7$ mmHg) promotes genomic instability, thereby increasing the number of mutations (genetic variants) (Hockel and Vaupel, 2001). In the hypoxic microenviroment, tumour cells that have adaptions for a hypoxic environment will survive and expand over the tumour cells that haven't adapted. These adapted cells as they grow exacerbate tumour hypoxia causing a vicious circle (Fig 1.8). Clinically this will result in local recurrence, metastasis and resistance to treatment (Hockel and Vaupel, 2001).

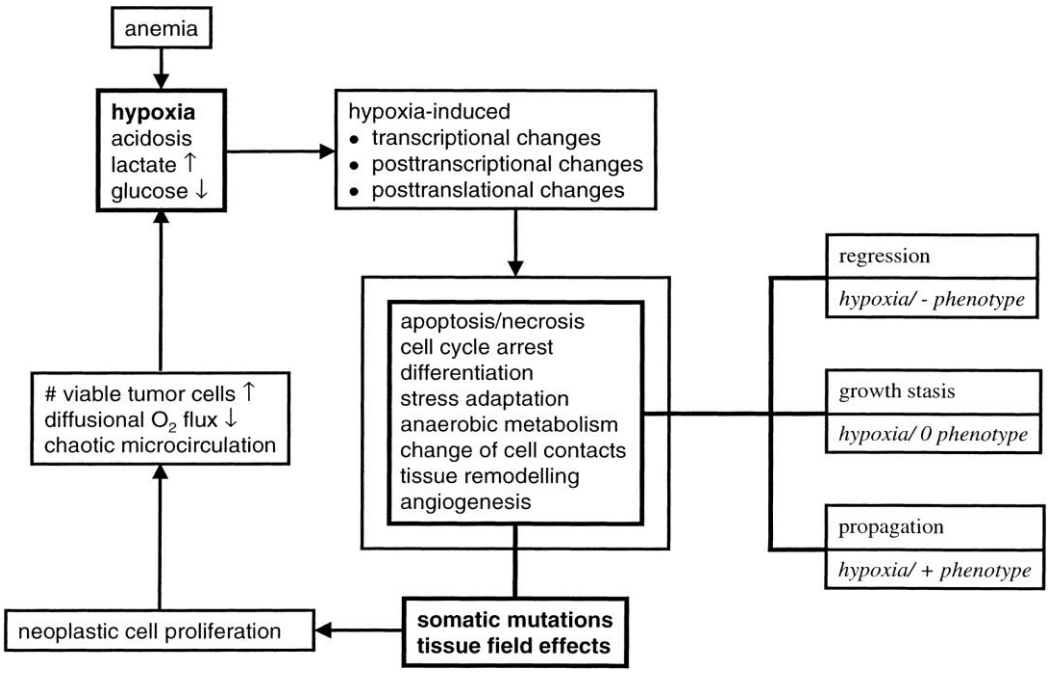



Figure 1-6 Hypoxia-induced proteome changes (i.e., changes in the set of proteins within a cell at a given time) in tumour and surrounding stromal cells influence cancer growth. Taken from (Hockel and Vaupel, 2001)

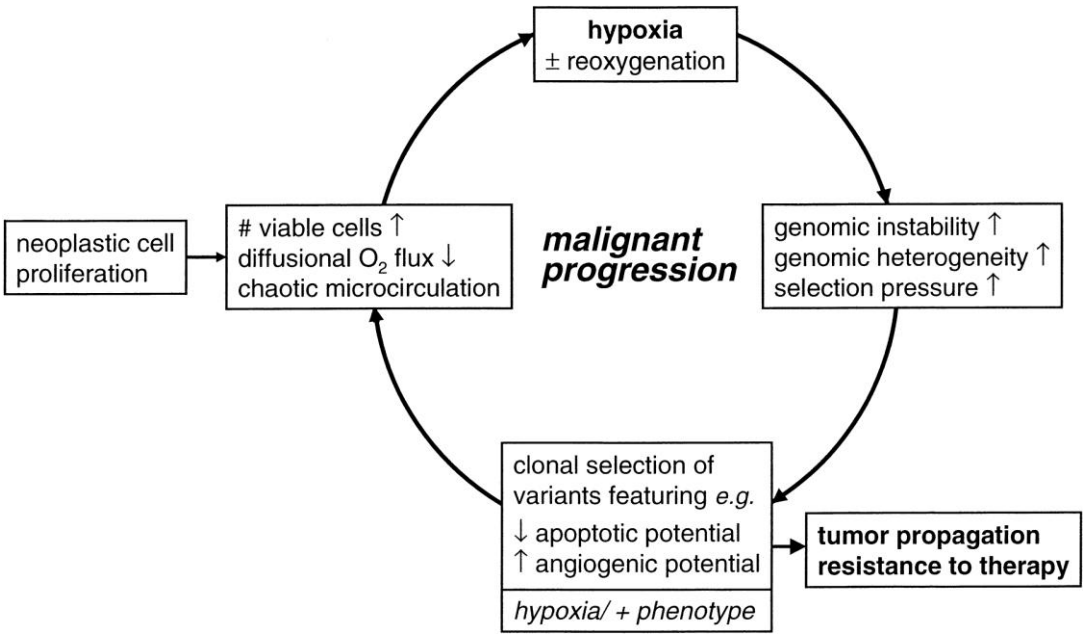



Figure 1-7 Schematic representation of the importance of hypoxia in the malignant progression of solid tumours. Progression occurs through progressive genome changes and clonal selection of hypoxia tolerant cells. Taken from (Hockel and Vaupel, 2001).

#### 1.7.2.2 Tumour resistance to radiotherapy

Radiotherapy is integral to the treatment of many tumours, with chemotherapy often used as an adjunct, for enhanced tumour control and to target occult metastasis. In head and neck cancer, radiotherapy is integral to the management of these patients. However, unfortunately a number of patients will develop recurrence following radiotherapy, with a very poor likelihood of long-term survival. There is evidence to suggest that tumour hypoxia, either directly or indirectly, can contribute to radioresistance. One of the key ways that hypoxia acts directly is by reducing the half-lives of DNA damaging oxygen radicals generated when radiation interacts with tissues. Sensitivity to sparsely ionising radiation declines when oxygen tension falls below 25-30 mmHg (Vaupel et al., 2001b). Oxygen is a potent radiosensitiser and stabilises highly reactive free radicals produced when ionising radiations interact with tissue that cause DNA damage (Molls et al., 1998). As a result of this oxygen enhancement effect, the dose of single fraction ionising radiation required to achieve the same cell survival fraction is two to three times higher under hypoxic conditions, i.e., radiotherapy is two to three times less effective in destroying hypoxic compared to normoxic cells (Vaupel and Harrison, 2004).

Hypoxia can also act indirectly to cause treatment resistance by inducing proteomic and genomic changes, causing an increase in tumour cells with diminished apoptotic potential, increased metastatic or proliferative potential or increased levels of repair enzymes. Moeller et al looked at how radiotherapy affected HIF. They irradiated tumour xenografts with 5, 10 and 15 Gy, which induced HIF-1, leading to increased expression of VEGF and basic fibroblast growth factor, which prevents radiotherapy induced cell death. They also showed that HIF-1 effects were greatest 48 hours after radiotherapy and gave evidence to show that radiation-induced reoxygenation of hypoxic tumour cells results in the production of reactive oxygen species that induce HIF-1 activity (Moeller et al., 2004). Harada et al found similar results using xenografts of human tumour cell lines showing: HIF-1 activity peaked two days after radiotherapy; and suppression of HIF-1 activity caused reduced angiogenesis and significant enhancement of the suppression of tumour growth with radiotherapy (Harada et al., 2007). Table 1.13 shows the many processes that are mediated by HIF-1 which may influence tumour radiosensitivity.

Table 1-10 HIF-1 modulated processes

| HIF-1-mediated effect | Potential impact on radiosensitivity | Reference |
|---|---|---|
| Cell cycle arrest | Decreased | (Goda et al., 2003) |
| Proapoptotic | Increased | (Carmeliet et al., 1998) |
| Antiapoptotic | Decreased | (Piret et al., 2004) |
| Enhanced glycolysis | Increased | (Semenza et al., 1994) |
| Angiogenesis | Decreased | (Gorski et al., 1999) |
| Migration | Increased | (Sullivan and Graham, 2007) |
| Invasion | Increased | (Chan and Giaccia, 2007) |

#### 1.7.2.3 Tumour resistance to chemotherapy

As for radiotherapy, the effectiveness of chemotherapy can be reduced by hypoxia, via both direct and indirect mechanisms. The efficacy of some chemotherapy agents (e.g. cyclophosphamide, carboplatin and doxorubicin) have been shown to be oxygen dependent under both in vivo and in vitro conditions (Teicher, 1994). Acute and chronic hypoxia (see Section 1.7.1) can cause erratic and diminished distribution of chemotherapy agents, which can affect their efficacy (Durand, 2001). First, the inner regions of a solid avascular tumour are often chronically hypoxic, which leads to quiescence and increased chemoresistance. Second, hypoxia leads to anaerobic glycolysis and increases acidity. The uptake of some drugs, such as anthracyclines, is reduced when pH drops. Third, the increasing distance from blood vessels reduces the drug concentration in these hypoxic areas conferring an environmental resistance (Silva and Gatenby, 2010). Fourth, for agents that interact with DNA directly or indirectly, hypoxia can lead to chemoresistance via several mechanisms: it reduces the generation of free radicals, limiting DNA damage by agents such as bleomycin and anthracyclines; it can reduce the number of double strand breaks with topoisomerase 2 inhibitors (e.g. epirubicin); it can reduce the number of DNA cross links formed by agents such as cisplatin (Ebbesen et al., 2009).

Unruh et al used mouse embryonic fibroblasts deficient in HIF-1α to show a reduced ability to respond to hypoxia increased sensitivity to carboplatin, etoposide and radiation (Unruh et al., 2003). Agents that did not cause DNA double strand breaks (e.g. DNA synthesis inhibitors) were equally cytotoxic towards HIF-1α positive and negative cells. There was decreased repair of a fragmented reporter gene in normoxic HIF-1α deficient cells, suggesting that basal HIF-1α expression is required for the expression of genes involved in the repair of DNA double strand breaks, although the

detailed mechanism was not understood in these mouse embryonic fibroblasts. Song et al used human non-small cell lung cancer cell lines and silenced HIF-1α expression by RNAi, which decreased resistance to cisplatin and doxorubicin (Song et al., 2006). The study confirmed the work by Unruch et al and demonstrated the importance of HIF-1α in chemoresistance.

### 1.7.3 Measuring tumour hypoxia

Currently there are several different techniques available for measuring tumour hypoxia, both direct and indirect. Unfortunately, at present there is no quick, easy, reliable and practical way of measuring tumour hypoxia on a routine clinical basis. A workshop convened by the National Cancer Institute agreed that none of the current approaches of measuring hypoxia represents a clear gold standard (Tatum et al., 2006). One of the difficulties when measuring hypoxia is that there is extreme spatial and temporal heterogeneity in tissue oxygen levels due to the complex nature of cellular oxygen consumption and blood supply, and no method can accurately measure this heterogeneity (Le and Courter, 2008).

#### 1.7.3.1 Direct oxygen measurement in tissue

The most direct method for assessing tumour hypoxia is by intratumoural polarographic measurement of oxygen partial pressure ($pO_2$). The polarographic needle is an electrochemical method of quantitative analysis based on the relationship between an increasing current passing through the tumour being analysed, and the increasing voltage used to produce the current. The development of the Eppendorf polarographic oxygen electrode system made it feasible to measure tumour oxygenation in the clinic. One of the first measurements in HNSCC patients using oxygen electrodes was reported by Gatenby et al. They measured $pO_2$ in cervical lymph node metastasis, and measured response to radiotherapy as changes in tumour volume 90 days after treatment. On completion of treatment the mean $pO_2$ was 20.6 (+/- 4.4) mmHg in the complete responder group and 4.7 (+/- 3.0) mmHg in the non-responder group ($p < 0.001$) (Gatenby et al., 1988). Hockel et al first published data suggesting hypoxia (measured using the Ependorf system) could be a prognostic factor. In a series of 31 cervical cancer patients he demonstrated that those with hypoxic tumours had significantly lower recurrence free and overall survival, with a follow up study on 103 patients confirming these results (Hockel et al., 1996, Hockel et al., 1993).

The Eppendorf machine uses a fine needle and automated stepper system for multiple measurements of oxygen concentration in a tumour (Milosevic et al., 2004). The approach avoids tissue compression and bleeding artefacts associated with previously used oxygen electrodes. Multiple measurements are made from several

tracks in the tumour, enabling a histogram of oxygenation to be produced. Oxygen concentrations within normal tissue are in the range 24-66 mmHg. They are significantly lower in solid tumours, ranging from 10-30 mmHg (Hockel and Vaupel, 2001). The Eppendorf machine has been used to demonstrate tumour hypoxia in a number of malignancies, including cervical, prostate and sarcomas (Movsas et al., 2000, Milosevic et al., 2001, Brizel et al., 1996, Nordsmark et al., 2001).

Evidence that hypoxia is important in head and neck cancer comes from studies using polarographic $pO_2$ measurements. As described above, Gatenby was one of the first to demonstrate hypoxia in head and neck tumours. In 1996 Nordsmark et al was the first to report the significance of a low pre-treatment $pO_2$ in patients with head and neck cancer. They studied 35 patients with advanced head and neck cancer treated with radiotherapy. They measured pre-treatment oxygenation status using Eppendorf polarographic needles, with the primary oxygenation endpoint being the fraction of $pO_2$ values less than 2.5 mmHg (HP2.5). Patients with an HP2.5 <5% had significantly higher rates of local control than those with tumour HP2.5 values >15% (p=0.013) (Nordsmark et al., 1996). The authors then repeated the study and confirmed their findings (Nordsmark and Overgaard, 2000). Several studies have also shown the prognostic significance of hypoxia in HNSCC (Table 1.11) (Nordsmark et al., 1996, Brizel et al., 1996, Brizel et al., 1997, Stadler et al., 1999, Rudat et al., 2000, Terris, 2000).

In most of the studies carried out, oxygen tension levels were made in metastatic lymph nodes as access with the Eppendorf probe is easier. Becker et al compared oxygenation measurements of the primary tumour compared to metastatic lymph nodes. He found comparable measurements of median $pO_2$ between the different sites, suggesting that the oxygenation status in any anatomical site is sufficient to estimate a tumours oxygen status (Becker et al., 1998). Brizel et al looked at the change in oxygenation status during treatment with radiotherapy. He performed a repeat assessment of tumour oxygenation after 10 to 15 Gy and found that the values were unchanged compared to the pretreatment baselines (Brizel et al., 1999). Dietz et al re-measured tumour oxygenation one week after induction chemoradiotherapy and found that reoxygenation correlated with a worse outcome (Dietz et al., 2003). Although the Eppendorf directly measures tissue oxygenation it does have some drawbacks. Its use is limited to accessible tumours, like HNSCC or cervix cancer. It does not distinguish necrotic tissue from viable tumour. It also has been shown to have large inter-observer variability (Nozue et al., 1997).

Table 1-11 Measurements of $pO_2$ using needle oxygen electrode and outcome in HNSCC

| Stage | No. of patients | Treatment | Associations with Hypoxia and Outcome | p value | Reference |
|---|---|---|---|---|---|
| IV | 28 | RT once daily (2 Gy/day to 66–70 Gy) or twice daily (1.25 Gy BID to 70–75 Gy) | DFS 78% vs 22% in patients with median $pO_2$ >10 mmHg and <10 mmHg | 0.009 | (Brizel et al., 1997) |
| Tx-T4/N1-3 | 63 | RT once daily (2 Gy/day to 66–70 Gy) or twice daily (1.25 Gy BID to 70–75 Gy) | Median $pO_2$ > 0 mmHg significantly related to ↑ survival; ↑ DFS and ↑ duration of local control in MVA. Median $pO_2$ <10 mmHg adversely affected 2-year LRC (30% vs 73%), DFS (26% vs 73%) and OS (35% vs 83%). | LRC 0.01<br>DFS 0.005<br>OS 0.02 | (Brizel et al., 1999) |
| Advanced | 31 | RT 2 Gy/fraction, 5.5-6.5 wks | 2 year LRC 90% vs 45% for tumours with %$pO_2$ <2.5 mmHg < or > median (15%) | 0.04 | (Nordsmark et al., 1996) |
| IV | 59 | Fractionated RT or hyperfractionated RT chemoradiotherapy with mitomycin-C/5-FU/conventional or hyperfractionated RT | UVA: $pO_2$ < 5 mmHg, $pO_2$ < 2.5 mmHg, and hypoxic subvolume were of prognostic significance. MVA: hypoxic subvolume significant | 0.01 | (Stadler et al., 1999) |
| Without distant metastasis | 134 | Primary RT or chemoradiotherapy ≥ 60 Gy | % $pO_2$ values <2.5 mmHg significant predictor of survival in MVA | 0.004 | (Rudat et al., 2001) |
| HNSCC | 397 | RT or pre or post operative RT or chenoradiotherapy | $pO_2$ values ≤2.5 mmHg assoc with poor OS | 0.006 | (Nordsmark et al., 2005) |

RT=radiotherapy; BID=twice daily; DFS=disease-free survival; LRC=locoregional control; OS=overall survival; UVA=univariate analysis; MVA=multivariate analysis.

#### 1.7.3.2 Imaging

There are several imaging techniques that are being explored for their potential to assess oxygenation status in tissues. MRI, CT and PET are the most widely studied approaches. For example, one method is blood oxygen level dependent magnetic resonance imaging (BOLD MRI). The image contrast is derived from the balance between paramagnetic deoxyhaemoglobin and diamagnetic oxyhaemoglobin and the effect of the latter on the MRI signal (Howe et al., 2001). As the oxygenation of Hb is directly proportional to the oxygenation of blood, BOLD MRI provides a sensitive index of oxygenation status of tissues immediately adjacent to perfused microvessels. BOLD MRI has several advantages; it does not require injection of a contrast agent or radioactive isotopes, it can be repeated as often as required, it is sensitive to the presence of changing oxygenation in tissue and it could potentially predict radiation response. However it also has disadvantages as its signal can also be influenced by blood flow, $CO_2$ tension and haematocrit (Tatum et al., 2006). Hoskin et al investigated the use of BOLD MRI as a potential non invasive measure of tumour hypoxia. They injected prostate carcinoma patients undergoing radical prostatectomy with pimonidazole. Patients also underwent MRI imaging using gradient echo sequences without and with contrast, to map tissue oxygenation. They found that images had a high specificity for defining tumour hypoxia when compared to the pimonidazole staining (Hoskin et al., 2007). Newbold et al investigated the use of dynamic contrast-enhanced MRI (DCE MRI) and perfusion CT as surrogate markers of intramural hypoxia as defined by pimonidazole and CA9 staining, in a small series of head and neck cancer patients. There was a significant correlation between DCE MRI and pimonidazole staining, and a weak correlation with CA9. There were no significant correlations between perfusion CT parameters and pimonidazole staining or CA9 expression (Newbold et al., 2009).

Another imaging technique is $^{19}F$ MRI, which uses perflurocarbons (PFC). These injectable compounds are highly hydrophobic but offer exceptional oxygen solubility. The $^{19}F$ nuclear magnetic resonance spin lattice relaxation rate is highly sensitive to oxygen; therefore injection of PFCs allows measurement of vascular oxygenation and hence tissue oxygenation (Mason, 1994). However, this technique requires local injection of PFCs directly into the tumour for imaging.

Another promising method for non-invasive measurement of hypoxia is the use of positron emission tomography (PET), which allows for the collection of data in real time. The extensive use of PET and the development of suitable hypoxic tracers have allowed the spatial visualisation of hypoxia. Several different hypoxia specific tracers have been developed for use with PET. Many tracers have been developed to image hypoxic tissue. Nitroimidazoles work by entering the cell by passive diffusion, undergoing reduction forming a reactive species. When oxygen is present, the

compound is reoxygenated and leaves the cell. However, when oxygen is not present i.e. hypoxia, further reduction occurs binding the compound covalently to macromolecules and thereby 'trapping' it inside the cell (Lapi et al., 2009). This process requires enzymatic activity, therefore it will only occur in viable hypoxic cells (Rajendran and Krohn, 2005). Nitroimidazole compounds can either be detected by e.g. immunohistochemistry or fluorinated radioactive nitroimidazoles detected by Positron Emission Tomography (PET). $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO) was one of the first tracers developed and is the most commonly used (Rajendran et al., 2006). A few studies have been carried out comparing $^{18}$F-FMISO PET, FDG PET and direct oxygen measurements, giving contrasting results (Table 1.12).

The prognostic value of these imaging techniques has also been assessed. Thorwarth et al describes a small series of patients that were assessed pretreatment with FDG and $^{18}$F-FMISO PET. They demonstrated that maximum uptake of FMISO showed borderline significance for stratifying the patient group (p=0.045) (Thorwarth et al., 2006). Rischin et al enrolled 45 patients with HNSCC into a hypoxic imaging study. Pretreatment and midtreatment $^{18}$F-FMISO PET was performed. Hypoxia on $^{18}$F-FMISO PET was associated with a high risk of locoregional failure and patients with hypoxic tumours benefited most from hypoxia-modifying therapy (Rischin et al., 2006). The advantage of these imaging techniques is that they are non-invasive, allowing repeated measurements of the same tumour, which can allow changes in hypoxia to be seen. However, further work is required to establish if they can be used routinely to assess tumour hypoxia.

Table 1-12 Imaging and correlation with hypoxia

| Site | No Patients | Measurement of $O_2$ status | Finding | Reference |
|---|---|---|---|---|
| **Soft tissue and HNSCC** | 18 | $^{18}$F-FMISO PET imaging followed by Eppendorf $pO_2$ electrode measurements | No correlation observed. In general tumors were more hypoxic based on Eppendorf $pO_2$ measurements as compared to $^{18}$F-FMISO PET. | (Mortensen et al., 2010) |
| **Sarcoma** | 13 | $^{18}$F-FMISO PET imaging followed by Eppendorf $pO_2$ electrode measurements | $^{18}$FMISO PET did not correlate with $pO_2$ measurements. | (Bentzen et al., 2003) |
| **HNSCC** | 24 | fluorodeoxyglucose positron emission tomography (FDG PET), $^{18}$F-FMISO PET and $pO_2$ | FDG uptake was not correlated with $pO_2$ - polarography. $^{18}$F-FMISO PET showed strong correlation with $pO_2$ - polarography. | (Zimny et al., 2006) |
| **HNSCC** | 38 | FDG PET, $^{18}$F-FMISO PET and $pO_2$ | Significant correlation between $^{18}$F-FMISO T/M and the fraction of $pO_2 \leq 2.5$, 5 and 10 mmHg | (Gagel et al., 2007) |

### 1.7.4 Exogenous markers/ injectable markers

Exogenous hypoxia markers are drugs that, when given to a patient, are bioreduced in hypoxic tissue to form stable adducts that can be detected in biopsy specimens. Hypoxia detection with 2 or 5-nitroimidazoles was initially suggested in the 1970s when they were originally developed as hypoxic-cell radiosensitisers. The drugs are reduced by nitroreductases into reactive products that rapidly bind to proteins and DNA. The first one-electron reduction step is quickly and efficiently reversed in the presence of oxygen. The 2-nitroimidazoles, pimonidazole and EF5 have been approved for clinical use as hypoxic markers (Evans et al., 2000, Varia et al., 1998). Bioreduction occurs when $pO_2$ levels fall below 10 mmHg. These injectable compounds form stable adducts with intracellular macromolecules, but binding is inhibited as oxygen concentration increases (Kaanders et al., 2002b). The bound adducts can be detected using specific antibodies used for detection by immunohistochemistry or immunofluorescence. These compounds are given intravenously to the patient prior to tumour resection and require biopsy. Both EF5 and pimonidazole have been shown to be reliable hypoxic markers with good correlation with the radiobiologically hypoxic fraction (Lee et al., 1996, Raleigh et al., 1999) and $pO_2$ (Raleigh et al., 1999).

Several studies have shown the prognostic value of 2-nitroimidazoles in the clinical setting. In head and neck cancer, pimonidazole and EF5 staining has been demonstrated when biopsies are taken after intravenous administration of the drug (Evans et al., 2000, Wijffels et al., 2000). A correlation between hypoxia, as estimated by 2-nitroimidazole binding and locoregional control and event free survival has been demonstrated (Kaanders et al., 2002b, Evans et al., 2007). Bennewith et al developed an oral form of pimonidazole. They gave the drug orally to tumour bearing mice. They demonstrated that as pimonidazole exposure was increased from 3-96 h, the fraction of hypoxic tumour cells and the relative number of pimonidazole adducts in these cells increased (Bennewith et al., 2002). The FDA have approved its use, which would make administration of pimonidazole much easier in the clinical setting (Rademakers et al., 2008).

#### 1.7.4.1 Endogenous markers

It is known that under hypoxic conditions, several proteins are unregulated. It has been estimated that up to 1.5% of all genes are hypoxia responsive (Denko et al., 2003). These proteins are detectable using immunohistochemistry, and therefore could serve as a method for hypoxia assessment, using a biopsy specimen. One of the most

extensively studied oxygen response pathways is that mediated by HIF-1. HIF-1 regulated genes that are responsible for angiogenesis, invasion, cell metabolism, metastasis and apoptosis. HIF-1 and its downstream targets (CA9, VEGF, Glut-1) have been studied as prognostic markers in head and neck cancer. One of the advantages of these endogenous markers is that the protein levels can be assessed on archived material, allowing correlations with outcome in large series. Tissue markers on biopsy specimens allow the assessment of hypoxia in the pre-treatment clinical setting, thus avoiding the need for invasive polarographic needles, or intravenous drug administration. However, despite the avoidance of invasive techniques, endogenous markers are not without problems; a single biopsy specimen may not be truly representative of the tumour, the proteins being assessed may also be up regulated by other factors and the markers may not be expressed by all tumour types. In addition, there is very little correlation between intensity of endogenous marker staining and polarographic electrode measurements. Various studies have looked at biopsy specimens taken along the track of the polarographic electrode. The tissue stained for HIF-1α, CA9 and Glut-1, but there was no correlation between the tumour $pO_2$ and the staining pattern (Mayer et al., 2004, Mayer et al., 2005a, Mayer et al., 2005b).

#### 1.7.4.2 HIF-1α

Cellular oxygen concentration controls most physiological functions of the cell. Cancer cells are involved in the adaptive response; the normal feedback mechanism has been disrupted by mutations and epigenetic changes so as a result adaption to low oxygen levels promotes many aspects of cancer progression (Semenza, 2010b). An important mediator of cell response to reduced oxygen levels is HIF-1 (Fig. 1.8) (Semenza, 2010b).

HIFs are sequence specific DNA-binding proteins. HIF-1 is a heterodimer, made up of an oxygen sensitive alpha subunit (HIF-1α) and a constitutively active beta subunit (HIF-1β). HIF-1α is rapidly degraded under normoxic conditions (Rademakers et al., 2008). When HIF-1 is activated it binds to hypoxia responsive elements (HREs) in its target genes, promoting their transcription to stimulate, for example, angiogenesis (e.g. VEGF) and glucose transport (e.g. GLUT1). There are cofactors that are involved in the transcriptional regulation of the target genes. Two inhibitory pathways of HIF-1α are essential under normoxic conditions. In the first pathway, prolyl hydroxylases (PHDs) hydroxylate proline sites in the oxygen dependent degredation domain (ODD) of the HIF-1α protein. This allows the von Hippel Lindau (VHL) protein to bind, causing proteasomal degradation of HIF-1α (Fig 1.8). When oxygen levels are low, the hydroxylation does not occur, leading to the accumulation of HIF-1. In the second main pathway, factor inhibiting HIF-1 (FIH-1) hydroxylates the C-terminal transactivation

domain (CAD) of HIF-1α, which in turn stops binding of p300/CBP to the HIF-1 complex, which is necessary for transcription. Other than the oxygen dependent activation of HIF-1 α, certain receptors of the tyrosine kinase family, like insulin-like growth factor receptor (IGFR), EGFR and HER2/nue, can all activate HIF-1α independent of oxygen, via the P13K/Akt/mTOR pathway (Semenza, 2000).

HIF-1α is overexpressed in a number of malignancies (Zhong et al., 1999). Its significance as a prognostic factor for aggressive tumour behaviour has been shown in various types of cancer: bladder, sarcoma, ovarian, gastric, breast, lung, rectal, cervical and head and neck (Moon et al., 2007). However some studies have shown an opposite effect (Beasley et al., 2002, Fillies et al., 2005). Increased expression of HIF-1α is generally associated with a poor prognosis (Table 1.13). Given the widespread overexpression of HIF-1α in many cancers, and its control of many cellular functions, it could be a promising therapeutic target. Therapies that are directed at the HIF-1 pathway have so far had limited success (Semenza, 2010b).

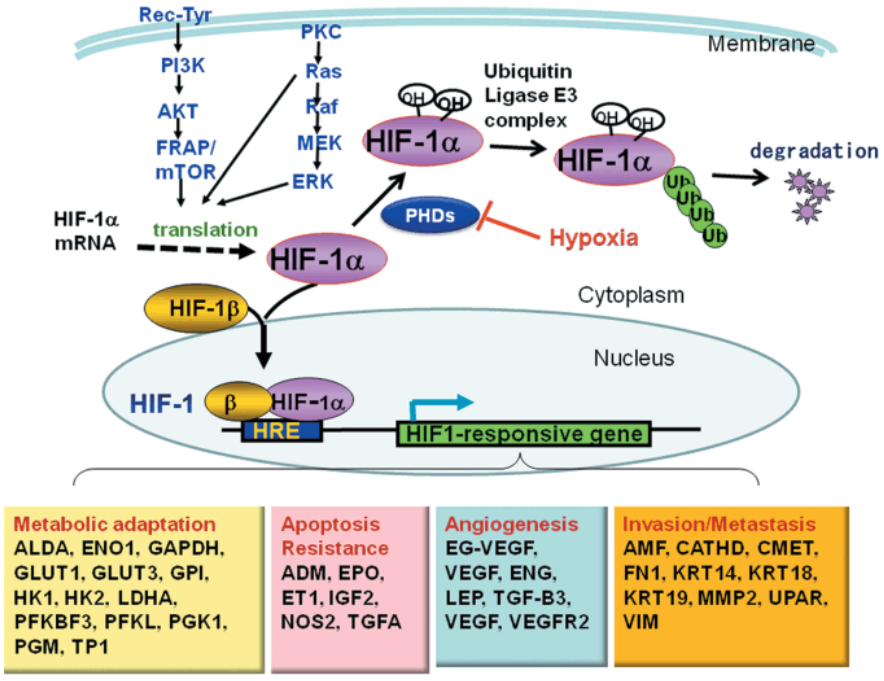



Figure 1-8 Schematic representation of the HIF pathway. Taken from (Kizaka-Kondoh and Konse-Nagasawa, 2009).

Table 1-13 HIF-1α expression and outcomes in head and neck cancer.

| Site | Stage | Pts | Treatment | HIF-1α expression associated with | Reference |
|---|---|---|---|---|---|
| Oropharynx | I-IV | 98 | RT | Lack of complete remission of primary tumour, lymph node metastases, ↓LFRS, ↓DFS & ↓OS | (Aebersold et al., 2001) |
| Oral cavity, oropharynx, larynx & hypopharynx | I-IV | 79 | Surgery | Improved DFS & OS | (Beasley et al., 2002) |
| Nasopharynx | II-IV | 90 | CRT or RT alone | CA9 & VEGF expression. | (Hui et al., 2002) |
| Pharynx, larynx, maxillary antrum & nasopharynx | Tx/N2b-3 or T3-4/Nx | 75 | Concurrent carboplatin CRT | High MVD, bone involvement, poor complete response rate to RT, poor relapse free survival & ↓OS | (Koukourakis et al., 2002) |
| Head & neck | - | 45 | Surgery | MVD | (Koukourakis et al., 2004) |
| Hypopharynx, larynx, oral cavity & oropharynx | I-IV | 151 | Surgery +/- post-op RT | ↓DSS & DFS | (Winter et al., 2006) |
| Oral cavity, oropharynx, hypopharynx, larynx & unknown primary | II-IV | 34 | RT or surgery + post-op RT | HIF-1α expression associated with 4.1 fold ↑ in mortality compared to negative expression of HIF-1α, CA9 & OPN | (Bache et al., 2006) |
| Head & neck | III-IV | 67 | RT & nimorazole | CA9 expression, ↓ LRC | (Nordsmark et al., 2007) |
| Nasopharynx, larynx, epilarynx, oropharynx, hypopharynx & neck | III-IV | 39 | HypoARC | ↓ LRFS and OS | (Koukourakis et al., 2008) |
| Oral SCC | | 82 | Surgery | ↓ DSS. HIF-1α expression associated with 3.5 fold ↑ tumour-related death compared to negative or weak expression of HIF-1α | (Eckert et al., 2010) |
| Oropharyngeal | | 79 | Radiotherapy | In MVA, ↓ LRC (HR 7.10; 95% CI 3.07-16.43) and CSS (HR 9.19; 95% CI, 3.90-21.6). | (Silva et al., 2008) |

Abbreviations: LRFS = local relapse free survival; CRT = chemoradiotherapy; DFS = disease free survival; OS = overall survival; RT = radiotherapy; VEGF = vascular endothelial growth factor; MVD = microvessel density; post-op = post-operative; DSS = disease specific survival; LRC=locoregional control; HypoARC = hypofractionated and accelerated radiotherapy with cytoprotection.

#### 1.7.4.3 CA-9

Carbonic anhydrases act as catalysts in the reversible reaction in which carbon dioxide is converted into carbonic acid (Wykoff et al., 2000). They therefore act to regulate intracellular pH (Hoogsteen et al., 2007a). There are 14 different types of carbonic anhydrase, which can be grouped by where they localise in the cell (Hoogsteen et al., 2007a). Carbonic anhydrase 9 (CA9) is a transmembrane glycoprotein which is expressed in some types of normal tissue, such as duodenal, jejunal, hepatic and pancreatic tissue (Beasley et al., 2001). CA9 is expressed in a number of tumours including HNSCC and cervical, ovarian, renal and bladder carcinomas (Beasley et al., 2001, Hoogsteen et al., 2007a).

CA9 is regulated by HIF-1α and its expression is increased by hypoxia (Hoogsteen et al., 2007a, Vordermark and Brown, 2003). Wykoff et al in 2000 showed that CA9 was induced by hypoxia in a number of different cell lines; they also demonstrated CA9 was expressed more strongly in tumour cells adjacent to necrotic areas, suggesting CA9 as a useful marker of hypoxia (Wykoff et al., 2000). Tumour cells known to express CA9 have been shown to be more resilient to and less likely to die following exposure to ionizing radiation than those which do not express CA9 (Hoogsteen et al., 2007a). A number of studies have been carried out to investigate tumour CA9 expression, hypoxia and outcome in head and neck cancer. High CA9 expression was associated with reduced treatment response, poorer local control rates or reduced survival rates in patients with head and neck cancer in seven of the series. However Jonathan et al, in 2006 showed an association between CA9 expression and improved local control. Three studies showed no association between CA9 expression and local control (Table 1.14).

Table 1-14 CA9 expression and outcomes in head and neck cancer

| Site | Stage | Pts | Treatment | CA9 expression associated with | Reference |
|---|---|---|---|---|---|
| Oral cavity, oropharynx, larynx & hypopharynx | I-IV | 79 | - | ↑MVD & advanced stage | (Beasley et al., 2001) |
| Pharynx, larynx, maxillary antrum & nasopharynx | Tx/N2b-3 or T3-4/Nx | 75 | CRT | ↓MVD, ↓necrosis, ↓ response rate to CRT, LRFS & OS | (Koukourakis et al., 2001) |
| Nasopharynx | II-IV | 90 | CRT | ↑ CA9 & HIF-1α with ↓PFS | (Hui et al., 2002) |
| Glottic, supraglottic, hypopharynx, oropharynx, oral cavity & nasopharynx | I-IV | 67 | RT +/- CT | ↑ CA9 & Glut-1 with ↓ LC, RC & DFS | (De Schutter et al., 2005b) |
| Oral cavity, oropharynx, hypopharynx, larynx & unknown primary | II-IV | 34 | RT or surgery + post-op RT | 4.1 fold ↑ mortality | (Bache et al., 2006) |
| Oral cavity, oropharynx, hypopharynx & larynx | III-IV (& II-HP only) | 58 | ARCON | Improved LRC & absence of DM. CA9 expression observed adjacent to necrotic zones. | (Jonathan et al., 2006) |
| Larynx, pharynx, nasopharynx & oral cavity | I-IV | 198 | RT | ↑CA9 associated with ↓ 5 yr LRC & OS | (Koukourakis et al., 2006) |
| Hypopharynx, larynx, oral cavity & oropharynx | I-IV | 151 | Surgery +/- post-op RT | CA9 expression not associated with OS, DFS or DSS | (Winter et al., 2006) |
| Head & neck | III-IV | 101 | CRT or surgery+RT | ↓CSS & OS | (Le et al., 2007) |
| Head & neck | III-IV | 67 | RT & nimorazole | ↑HIF-1α, unrelated to LRC | (Nordsmark et al., 2007) |
| Supraglottic & pharynx | - | 320 | RT + nimorazole or placebo | No association with LRC,DSS in either nimorazole or placebo group | (Eriksen and Overgaard, 2007) |
| Nasopharynx, larynx, epilarynx, oropharynx, hypopharynx & neck | III-IV | 39 | HypoARC | ↓LRFS | (Koukourakis et al., 2008) |

Abbreviations: LRFS = local relapse free survival; CRT = chemoradiotherapy; DFS = disease free survival; OS = overall survival; RT = radiotherapy; VEGF = vascular endothelial growth factor; MVD = microvessel density; post-op = post-operative; DSS = disease specific survival; LRC=locoregional control; HypoARC = hypofractionated and accelerated radiotherapy with cytoprotection.

#### 1.7.4.4 Secreted hypoxia markers

A marker that could identify hypoxia in a blood sample would make a valuable contribution towards hypoxia detection. There are an increasing number of studies looking at the role of OPN as a predictive marker in HNSCC. Le et al demonstrated a significant relationship between osteopontin (OPN) levels and tumour $pO_2$ (Le et al., 2003). A further study by Nordsmark et al confirmed this result and also demonstrated OPN was an independent and significant predictor of treatment outcome in HNSCC patients (Nordsmark et al., 2007). The DAHANCA study also confirmed these findings in a cohort of patients treated with radiotherapy +/- nimorazole. Interestingly, only the patients with high pretreatment OPN appeared to benefit from the nimorazole, suggesting the OPN could be used to select patients for hypoxia targeting (Overgaard et al., 2005). Snitcovsky et al demonstrated that low pretreatment plasma OPN level is associated with treatment response and better survival, in a series oh HNSCC treated with chemoradiotherapy (Snitcovsky et al., 2009) Hui et al showed that in a series of patients with locoregional nasopharyngeal carcinoma receiving curative radiotherapy, high pretreatment plasma OPN was a significant predictor of poor response to radiotherapy (p=0.009), which remained significant in multivariate analysis (Hui et al., 2008). The advantages of secreted hypoxia markers are that they are non-invasive, inexpensive and allow for serial measurements. However, they have drawbacks, including the lack of method standardisation and regulation by factors other than hypoxia.

### 1.7.5 Hypoxia and anaemia

Anaemia is common in cancer patients (see Section 1.5.2.4). Anaemia is known to be significantly associated with a poor prognosis in head and neck cancer, treated not only with radiotherapy but also surgery (Table 1.10). It is believed that anaemia decreases the oxygen-carrying capacity of blood, increases tumour hypoxia and so reduces locoregional control after radiotherapy. The dose of radiation needed to kill hypoxic cells is two to three times that of normoxic cells (see Section 1.4.2). Becker et al measured pre-treatment $pO_2$ levels on 30 patients with biopsy proven HNSCC, using a polarographic needle electrode. He demonstrated that there was a weak, but significant correlation between the median $pO_2$ of the primary tumour and Hb levels ($p$ = 0.003; p=0.017) (Becker et al., 1998). In a follow up study they found that tumour oxygenation was significantly associated with severe anaemia (p<0.0001). In a multivariate analysis, including Hb and smoking status, a Hb level <11 g/dL was found to be the strongest predictor for poor tumour oxygenation, with smoking also having a marginal influence on tumour $pO_2$ (Becker et al., 2000). Stadler et al. also showed a

correlation between hypoxic volume and Hb concentration in a series of head and neck cancer patients ($p<0.0005$) (Stadler et al., 1998). Brizel et al also showed an association between tumour hypoxia defined as <10 mm Hg and anaemia ($p=0.04$) (Brizel et al., 1999), However, Nordsmark et al did not find a correlation between tumour hypoxia and anaemia, although they were both independent factors for predicting worse local control and survival (Nordsmark and Overgaard, 2000). The lack of consistency in the findings is probably due to small numbers of patients included in individual studies and because many factors influence the development of hypoxia in tumours.

Animal studies have demonstrated that acute changes in Hb alter intra-tumoural hypoxia making tumours radioresistant (equivalent to a 5-20 fold increase in hypoxic fraction) (Hirst et al., 1984). However, the animal studies demonstrated that mice with chronic anaemia had radiosensitive tumours close to mice with normal Hb. A DAHANCA study demonstrated that correcting low Hb can enhance the radiosensitivity of tumours, maintaining the theory that there is a relationship between anaemia and hypoxia (Overgaard et al., 1998). Anaemia is certainly a clinically relevant risk factor for patients undergoing radiotherapy and one which is potentially modifiable (see Section 1.5.2.4). Degner et al demonstrated with mathematical modelling that an increase of Hb by 20% produced a theoretical decrease in hypoxic tissue volume of 30% (Degner and Sutherland, 1988).

### 1.7.6 Hypoxia modifying therapy

Since the discovery of hypoxia in solid tumours in the 1950s, considerable effort has been devoted to developing treatments that can target hypoxia indirectly or directly. Although no strategy has gained general acceptance, a meta-analysis of trials using hypoxic cell sensitisers or hypobaric oxygen demonstrated a small but statistically significant benefit with regards to locoregional control and survival (Overgaard and Horsman, 1996).

#### 1.7.6.1 Correction of anaemia

In clinical practice, anaemia is not corrected unless the patient is symptomatic. Some studies have shown that correcting anaemia with blood transfusions was beneficial whereas other studies have demonstrated that there was no positive effect (see Section 1.5.2.4). To avoid multiple transfusions and the risks associated with this, recombinant erythropoietin (EPO) has been used to increase Hb levels. EPO stimulates red blood cell production in bone marrow and is produced by the kidneys in response to hypoxia. Several studies have shown an increase in Hb levels when EPO is given with radiotherapy. However, a phase III randomised study of radiotherapy +/-

EPO in HNSCC demonstrated that patients who received EPO had worse locoregional progression free survival (Henke et al., 2003). As a result, a recent Cochrane review has advised that EPO is not used in patients to correct anaemia when undergoing radiotherapy (Lambin et al., 2009).

#### 1.7.6.2 Hyperbaric oxygen/ARCON

Another approach to targeting hypoxia is administration of oxygen at pressures higher than 1 atm. This increases blood oxygen levels, both bound to plasma and Hb, causing larger oxygen diffusion distances within tumours, therefore reoxygenating previously hypoxic cells. Overgaard et al performed a meta-analysis of hypoxia modification in radioresistant tumours with oxygen and radiosensitisers. The improvement in local control of solid tumours with the use of hyperbaric oxygen and radiotherapy was 10% (Overgaard and Horsman, 1996). However, giving hyperbaric oxygen to patients undergoing radiotherapy is difficult and potentially dangerous, making it a difficult procedure to implement into routine clinical practice. Another more realistic alternative involves the administration of carbogen and nicotinamide. Nicotimamide is a vasodilator and carbogen is a gas mixture of 3-5% $CO_2$ with oxygen. The use of accelerated radiotherapy with carbogen and nicotinamide (ARCON) increases blood oxygen levels, therefore reducing diffusion limited hypoxia. Kaanders et al demonstrated a 3 year local control rate of 80% for T3/4 larygneal and oropharyngeal cancers using ARCON (Kaanders et al., 2002a). However, the EORTC group did not replicate these findings in their series of patients (Bernier et al., 2000). The results of a phase III trial is testing the efficacy of ARCON in laryngeal cancers (Kaanders et al., 2002a) were recently reported showing no benefit for local control but a statistically significant improvement in the 5-year regional control rate (86% for accelerated radiotherapy vs. 93% for ARCON, p=0.04) (Kaanders, 2010).

#### 1.7.6.3 Hypoxic cell radiosensitisers

Another strategy that has been widely investigated is the use of hypoxic radiosensitisers. These drugs are characterised by a high affinity for electrons, allowing them to mimic the effect of oxygen therefore making cells more radiosensitive. The meta-analysis by Overgaard et al in 1996 looked at 83 randomized trials designed to modify hypoxia, with over 10,000 patients. Although a number of the trials showed no benefit, the meta-analysis showed that modification of tumour hypoxia significantly improved locoregional tumour control after radiotherapy, with an odds ration of 1.21 (95% CI 1.12-1.30). The benefit was most significant for HNSCC with an odds ratio of 1.31 (1.19-1.43). The overall survival rate also improved with an odds ration of 1.13 (1.05-1.21) (Overgaard and Horsman, 1996). The hypoxic cell radiosensitiser

nimorazole is used clinically. Xenograft studies have shown significant radiosensitisation with nimorazole in tumours without enhancing normal tissue toxicity (Brown, 1975, Sheldon et al., 1974). Nitroimidazole compounds have been extensively studied within both the RTOG and DAHANCA groups with mixed results (Lee et al., 1989, Lee et al., 1995, Overgaard et al., 1989). In a large phase III study conducted by the DAHANCA group, the addition of nimorazole to radiotherapy significantly improved local control in larynx and pharynx cancer patients (Overgaard et al., 1998). The toxicity of nimorazole is mild and it is now given as routine treatment in Denmark for patients with head and neck cancer.

Recently DAHANCA published the results of a study looking at HPV-associated *CDKN2A*-expression and the response to hypoxic modification of radiotherapy in HNSCC. They obtained the pre-treatment blocks of 331 patients that were in the DAHANCA 5 trial and stained then for *CDKN2A* expression, with the view of assessing the influence of *CDKN2A* expression and the response to nimorazole. They showed that overall, patients treated with nimorazole had significantly better loco-regional control than did those given placebo (HR 0.70, 95% CI 0.52-0.93). The positive expression of *CDKN2A* also significantly improved patient outcome after radiotherapy (HR 0.41, 95% CI 0.28-0.61). When they looked at the group of patients with *CDKN2A* negative tumours, loco-regional failure was more frequent in the placebo group than in the nimorazole group (HR 0.69, 95% CI 0.50-0.95), yet the *CDKN2A* positive patients treated with nimorazole had a loco-regional control rate similar to patients given placebo (HR 0.93, 95% CI 0.45-1.91). These results show that nimorazole does improve outcome when used with radiotherapy. However, they also show that hypoxia modification does not benefit *CDKN2A* positive tumours, suggesting that hypoxic radioresistance may not be clinically relevant in these patients with HPV associated HNSCC (Lassen et al., 2010).

#### 1.7.6.4 Hypoxic cell selective agents

An important strategy in the targeting of hypoxic cells is the use of bioreductive drugs. These drugs become preferentially reduced to their toxic metabolite in the absence of oxygen. They differ from radiosensitizers, which are not cytotoxic in the absence of radiotherapy. It has also been demonstrated that killing hypoxic cells is more effective than radiosensitising them (Brown and Koong, 1991). Tirapazamine (TPZ) is a widely studied drug that is bioreduced under hypoxic conditions (Wouters et al., 1999). A one-electron reduction in TPZ produces a very reactive DNA damaging radical (Brown, 1993). TPZ was investigated in a randomised phase II trial in patients with locally advanced HNSCC. The combination of TPZ, cisplatin and radiotherapy was superior

to 5-FU, cisplatin and radiotherapy (Rischin et al., 2005). However, the results of the recent phase III trial (TROG 02.02, HeadSTART) cast doubt on the use of TPZ. Rischin et al recruited 861 patients with untreated stage III or IV HNSCC. Patients were randomised to receive definitive radiotherapy (70 Gy in 7 weeks) concurrently with either cisplatin (100 mg/m$^2$) on day 1 of weeks 1, 4, and 7 or cisplatin (75 mg/m$^2$) plus TPZ (290 mg/m$^2$/d) on day 1 of weeks 1, 4, and 7 and TPZ alone (160 mg/m$^2$/d) on days 1, 3, and 5 of weeks 2 and 3 (TPZ/cisplatin). The 2-year overall survival rates were 65.7% for cisplatin and 66.2% for TPZ/cisplatin (TPZ/cisplatin – CIS: 95% CI, −5.9% to 6.9%). There were no significant differences in failure-free survival, time to locoregional failure, or quality of life as measured by Functional Assessment of Cancer Therapy–Head and Neck. They concluded that there was no benefit from adding TPZ to chemoradiotherapy in patients with HNSCC (Rischin et al., 2010a).

## 1.8 Human Papilloma Virus

### 1.8.1.1 HPV and cervical cancer

For a number of years there was a large volume of epidemiologic evidence to link high risk Human Papilloma Viruses (HPVs) with the development of cervical cancer, and in 2008 Zur Hausen was awarded the Nobel Prize for medicine for his work in the 1980's showing the carcinogenic role of HPV in cervical cancer (Zur Hausen, 2009). In 1995, on the basis of epidemiological evidence and molecular studies, the International Agency for Research on Cancer concluded that HPV16 and HPV18 are carcinogenic to humans (WHO, 1995). It is estimated that 85% of humans will have some form of HPV infection in their lifetime (Schiffman et al., 2007).

The foundation for the role of HPV in malignant transformation of epithelial cells comes from cervical cancer literature. HPV is a ubiquitous, nonenveloped, double stranded DNA virus (Schiffman et al., 2007). There are about 120 known types of HPV. They are a family of small DNA viruses that all appear to be epitheliotropic; they infect epithelial cells of the skin, anogenital tract and oropharyngeal mucosa. There does not appear to be any evidence to suggest HPV infects the gastrointestinal tract. HPVs are divided into two groups; low risk and high-risk types. Low risk types include HPV 6 and 11, and have been shown to induce benign hyperproliferation of the epithelium, causing papillomatous warty lesions that very rarely progress to cancer. High-risk types include HPV 16, 18, 31, 33 and 35. They are associated with the development of lesions that often undergo carcinogenic progression.

The molecular progression of HPV associated malignancy has been extensively researched in cervical cancer. If a woman is immunocompetent then the majority of HPV cervical infections are cleared by the immune response of the female with

resultant cellular transformations rare. However, even with a functioning immune system a subset of females will develop a chronic HPV infection that can lead to cellular transformation (Schiffman et al., 2007). HPV infection is necessary and can lead to the development of cervical cancer (Munoz et al., 2003). HPV 16 and 18 in particular are known to be capable of causing malignant transformation of normal cervical epithelial cells (McDougall, 1994). These high risk HPVs are also associated with the development of penil, anal and vulvar cancers (Frisch et al., 1997). This work has been repeated in the HNSCC setting putting together a case for the role of HPV as an aetiological agent in a subset of HNSCC (Strati and Lambert, 2007, Kreimer et al., 2005, Ragin and Taioli, 2007, Allen et al., 2010).

#### 1.8.1.2 Epidemiology of HPV and oropharyngeal cancer

The surveillance epidemiology and end results data suggest that the incidence of tonsil and tongue base cancers have increased by 2.1% and 3.9% per year, respectively, between 1973 and 2001, among white women and men, aged 20-44 years in the USA (Shiboski et al., 2005). There is also similar evidence of an increase in the UK (see section 1.2.1). The increase in orophayrngeal cancer is particularly apparent in the under 45 age group. This age group has seen a 2% per year increase in base of tongue cancer and a 4% per year increase in tonsil cancer between 1973 and 2004 (Sturgis and Cinciripini, 2007). A study from Sweden looked at archived tonsil cancer specimens over a number of years. HPV DNA was isolated in 23% of the specimens from the 1970s, 28% from the 1980s, 57% from the 1990s and 68% from specimens since 2000 (Hammarstedt et al., 2006). Licitra et al (Licitra et al., 2008) published a study showing the change in demographics of head and neck cancer patients in 15 European countries. The analysis was conducted on 29,265 cancer patients diagnosed between 1988 and 2002. The HPV unrelated HNSCC sites had an age-standardised incidence higher than the HPV related HNSCC cases (3.8 versus 2.5/100,000 per year). The incidence of HPV related HNSCC increased compared to HPV unrelated HNSCC. The three year survival rates were better for the HPV related cancer patients compared to the HPV unrelated cancer patients (Licitra et al., 2008). These epidemiological trends imply that HPV related oropharyngeal cancer is becoming more common at a time when the overall incidence of HNSCC is decreasing (Gillespie et al., 2009).

#### 1.8.1.3 Method of HPV Transmission

The current evidence suggests that oral HPV infection is sexually transmitted, with transmission by oral genital contact transmitting HPV into the upper aerodigestive tract (D'Souza et al., 2007). Husbands of women with in-situ and invasive cervical cancer

and individuals with a history of HPV associated anogenital cancer are more likely to develop HPV associated oropharyngeal cancer (Schwartz et al., 2001, Frisch and Biggar, 1999, D'Souza et al., 2007). HIV positive patients and immunocompromised patients, such as post transplant patients, also have an increased risk of developing HPV associated head and neck cancer (Swoboda and Fabrizii, 1993). A small study of 62 patients and 248 controls demonstrated that the presence of HPV DNA in the oral cavity was significantly associated with a younger age of first sexual intercourse and increasing numbers of lifetime sexual partners (Anaya-Saavedra et al., 2008). D'Souza et al carried out a hospital based case control study looking at 100 newly diagnosed oropharygneal cancer patients and matched them with 200 control patients with no history of cancer. He showed that a high (i.e., 26 or more) lifetime number of vaginal-sex partners, and six or more lifetime oral sex partners was associated with the subsequent development of oropharyngeal cancer with an odds ratio of 3.1 and 3.4 respectively (D'Souza et al., 2007).

#### 1.8.1.4 Methods of HPV detection

There are currently several methods used to detect HPV status, based upon HPV DNA, mRNA transcripts or translated proteins (Table 1.15). As mentioned previously (Section 1.8.2.1) only transcriptionally active HPV associated malignancies are biologically and clinically relevant. To detect for true HPV associated tumours, high risk E6/E7 mRNA or protein would have to be detected. *CDKN2A* protein expression status, which has been shown to be expressed in HPV-positive specimens, is determined by immunohistocemistry (Reimers et al., 2007, O'Regan et al., 2008, Shi et al., 2009, Klussmann et al., 2003, Fischer et al., 2010). There are a number of studies that have shown a statistically significant correlation between *CDKN2A* and HPV DNA when measured either by in situ hybridization (ISH) or polymerase chain reaction (PCR) in HNSCC (Reimers et al., 2007, Shi et al., 2009, Klussmann et al., 2003, Fakhry et al., 2008, Hafkamp et al., 2008). When looking in detail at biologically active HPV, that express E6/E7 transcripts, specificity appears to be reduced as some tumours do not express *CDKN2A* (Wiest et al., 2002, Shi et al., 2009). In addition, *CDKN2A* appears to be over expressed in a small group of tumours that lack HPV DNA or E6/E7 transcript levels (Smeets et al., 2007). Specificity of *CDKN2A* for HPV activity is therefore questionable. However, as most HPV positive tumours express *CDKN2A*, sensitivity is high.

PCR is another method for detecting HPV RNA. PCR is an extremely sensitive method of detecting HPV DNA, by amplifying a single sequence of DNA across several orders of magnitude, allowing just one copy of HPV DNA to be detected from a cell (McKaig et al., 1998). DNA primers are used to detect and amplify a region of DNA for

which the sequence is known. So when detecting HPV in a sample, you can either use a primer that detects a region of DNA common to all HPV subtypes, or you can use a primer that amplifies a region of DNA unique to one particular subtype (Innis et al., 1988). However, the detection of HPV DNA by PCR only tells us the DNA is present, it does not provide information on the physical status of the DNA or the expression of HPV genes. In addition, when a tumour sample is used, it is impossible to say if the DNA detected is from the cancer cells or the surrounding nonneoplastic cells unless laser capture microdissection is used. The method of specimen preparation is also important. PCR can be performed on formalin fixed paraffin embedded (FFPE) tissue or fresh frozen tissue (Specht et al., 2001). It is more efficient when using fresh frozen tissue. Smeets et al used fresh frozen HNSCC tumours, with HPV DNA and E6/E7 oncogene RNA positives as a control, to show that half of the same tumour population was PCR positive in the FFPE specimens. Conversely, he showed PCR positivity in a group of the HPV DNA and E6/E7 mRNA negative on fresh frozen tissue, showing false positives (Smeets et al., 2007). This information shows that PCR has a high sensitivity, especially when using fresh frozen tissue. The disadvantage of PCR is that it has a low specificity for HPV biologic activity, and it can produce false positives. However, according to three large meta-analysis, PCR appears to be the most common method of detection for HPV DNA, either when used alone or with another modality (Termine et al., 2008, Kreimer et al., 2005, Ragin and Taioli, 2007).

A further method of detecting HPV DNA is the use of ISH. ISH allows you to determine if HPV DNA is present, and if it is, where exactly in the specimen it is. Nucleic acid probes, which have been labelled with fluorescent dye or radioactive labelled bases allow the HPV DNA to be localised and visualised using a microscope. Like PCR, ISH probes can detect sequences of HPV DNA specific to an individual subtype of HPV or they can detect a sequence of DNA common to many subtypes (Lizard et al., 2001). Smeets et al evaluated the value of ISH in HNSCC. He demonstrated that ISH had a high specificity for biologically active HPV in FFPE tumours samples; however ISH had a decreased sensitivity, as a few of the tumours that were negative on ISH FFPE were positive for HPV DNA and E6/E7 mRNA in FFPE specimens (Smeets et al., 2007). Shi et al also published comparable data (Shi et al., 2009).

All these above technique assess a specimen for the presence or activity of the HPV virus on the molecular level making use of DNA, RNA or protein detection assays. It has also been suggested that changes at a cellular level following HPV infection may produce a distinct phenotype that can be seen on histological examination. El-Mofty et al inspected a large series of oropharygneal tumours and correlated their HPV status with morphology. HPV 16 positive tumours were non-keratinising, with well defined

sheets or nests with little stroma, composed of oval to spindle-shaped, basaloid cells with ill-defined borders and hyperchromatic nuclei. Excessive mitosis, cells undergoing apoptosis and comedo-type necrosis were all characteristic. These findings all support the suggestion of a nonkeratinising HPV-positive oropharyngeal tumour as a different subtype of HNSCC, however classifying tumours based on their microscopic appearance is not specific for HPV activity and this technique has not been validated for interobserver reliability (El-Mofty and Patil, 2006). However, they did go on to demonstrate that nonkeratinising morphology alone predicts strong *CDKN2A* expression with a positive predictive value of 100% (Chernock et al., 2009).

All the above information shows that at present there is no ideal test to detect biologically active HPV infection in HNSCC specimens (Shi et al., 2009, Braakhuis et al., 2009). The ideal test which is presently described in the literature, the use of PCR to detect E6/E7 transcript levels using fresh frozen tissue is cumbersome and requires fresh tissue. As a result, authors have suggested using *CDKN2A* IHC and HPV ISH as the standard detection technique for HPV in oropharyngeal SCC (Shi et al., 2009).

Table 1-15 Methods of HPV detection

| **Detection Method** | **Advantages** | **Disadvantages** |
|---|---|---|
| PCR | High sensitivity* | Low specificity† |
| | Widely available | Cumbersome |
| DNA ISH | High specificity | Low sensitivity |
| E6/E7 mRNA | High sensitivity | May require fresh frozen tissue |
| | High specificity | Cumbersome |
| E6/E7 protein IHC | High specificity | Questionable sensitivity |
| | | Technically difficult |
| *CDKN2A* IHC | Very high sensitivity | Questionable specificity |
| | Widely available | |
| Morphology‡ | Always available | Imperfect correlation§ |
| | No expense | Questionable reproducibility |

*, † Sensitivity and specificity refer to detection of biologically active HPV.
‡ Nonkeratinising histology.
§ Between morphology and biologically active HPV.
PCR = polymerase chain reaction; ISH = in situ hybridization; IHC = immunohistochemistry.
Adapted from Allen et al (Allen et al., 2010).

#### 1.8.1.5 Molecular evidence for role of HPV in HNSCC

The link between HNSCC and HPV was suggested over 20 years ago by Syranen et al (Syrjanen et al., 1983). In 1989, Brandsma el at were the first to report HPV16 DNA presence in 2 of 7 tonsil SCCs. This was the first of many papers that have been published reporting the presence of HPV DNA in tonsillar carcinoma. Many case

control studies have linked the presence of oral HPV infection with an increased risk of oral cavity and oropharyngeal cancer, independent of tobacco and alcohol consumption (Smith et al., 1998), (D'Souza et al., 2007).

The molecular progression of HPV associated malignancy has been extensively researched in cervical cancer. When initially infected with the DNA, it presents as a nuclear episome with a low copy number in the basal layer of the stratified epithelium. Viral particles are then formed as the basal cells differentiate, which are then released when the cells reach the outer layer (Doorbar, 2006). The HPV viral genome encodes for two viral oncoproteins E6 and E7. The E7 oncoprotein results in loss of the retinoblastoma (Rb) tumour suppressor protein thus precipitating neoplastic transformation. The loss of Rb protein causes intracellular accumulation of *CDKN2A*. *CDKN2A* would usually inhibit cell cycle progression via cyclin D1 and CDK4/CDK6-mediated events; however the E7 oncoprotein overrides this cell cycle control, pushing cells from G1 into S phase. The E6 oncoprotein causes degradation of the tumour suppressor protein TP53 therefore preventing the cell from undergoing programmed cell death in response to genetic mutations. When the virus infects a patient initially, the HPV DNA exists in its episomal form with E6 and E7 expressed at low levels. If the virus is not cleared from the host tissue the virus DNA can become integrated into the host genome. This disrupts the regulation of E6 and E7 causing their expression to increase (Zerfass et al., 1995). However, an increase in E6 and E7 alone is not enough to cause malignant progression. The additional genetic events that are required for the development of malignancy are currently not known.

#### 1.8.1.6 HPV and prognosis

Recently it has become apparent that HPV plays a role in a distinct subset of HNSCCs, in particular oropharyngeal SCC. HPV DNA has been isolated from tumours throughout the head and neck region. Three large metaanalysis have shown that proportion of HNSCC positive for HPV16 ranged from 0-86%, with controls ranging from 0-38% (Termine et al., 2008, Kreimer et al., 2005, Ragin and Taioli, 2007). However, there was no consistency with regards to the type of tissue or the method of detection used in each study, making it difficult to come to a single figure. When stratified by anatomical site the association was strongest for tonsil, intermediate for oropharynx, and weakest for oral cavity and larynx (Hobbs et al., 2006). Another recent meta-analysis found that HPV DNA was detected in 26% of all HNSCCs using sensitive PCR based methods of detection. When looking specifically at site, oropharynx is consistently the most common place for presence of HPV DNA (Fakhry and Gillison, 2006, Allen et al., 2010). Despite there being reports of a high prevalence of HPV16 DNA in HNSCC, detection of HPV16 DNA per se does not suggest a causal

role.

There is growing evidence that HPV-associated HNSCC are associated with a better prognosis compared to stage matched HPV negative HNSCC in the majority of studies, see appendix III. Allen et al recently evaluated the role of HPV positivity on survival in head and neck cancer patients. He identified 50 studies that have been published examining the effects of HPV positivity on survival. Of the 50, 23 analysed only oropharyngeal tumours. In these studies, 17 revealed a significant survival advantage associated with HPV positivity, while six did not reveal any difference survival probability according to HPV status (Table 1.20) (Allen et al., 2010). Ragin et al carried out a meta-analysis in 2007, showing that patients with HPV associated HNSCC had a 38% reduced risk of disease failure and an 18% reduced risk of dying compared to those with HPV negative tumours. However, a statistically significant association was only seen in the oropharyngeal subgroup (Ragin and Taioli, 2007). Oropharyngeal cancer associated with HPV has a significantly better chance of survival compared to HPV negative cancer.

In a prospective cohort of 96 patients with stage III and IV laryngeal and oropharyngeal cancer treated with chemoradiotherapy, patients with HPV DNA positive tumours had an improved 2-year survival rate compared to those with HPV negative disease (95% vs 62%, p=0.005) (Fakhry et al., 2008). Patients with HPV positive HNSCC are younger, by an average of 5 years, compared to HPV negative patients. With regards to gender, both a male and female predominance have been reported. HPV positive SCCs are more likely to develop in non-smokers and non-drinkers compared to the HPV negative SCCs. With regards to oropharyngeal SCC specifically, non-smokers were been found to be 15-fold more likely to have a HPV positive tumour than smokers (Lindel et al., 2001).

There have also been several studies reporting an inverse association between HPV status and the use of alcohol. There is a small amount of evidence suggesting a synergistic effect with regards to cancer risk for HPV positivity, alcohol and smoking, however there is also evidence suggesting no such relationship (Luginbuhl et al., 2009, Hafkamp et al., 2008). More importantly, the absence of the two main risk factors (smoking and alcohol) for head and neck cancer, in HPV positive oropharygneal cancers supports a causal relationship with HPV infection and a particular subset and oncogenic oropharyngeal SCCs.

Table 1-16 Comparison of HPV positive and HPV negative patients

| **Type of HNSCC** | **HPV positive** | **HPV negative** |
|---|---|---|
| Clinical Factors | Predominantly oropharynx (tonsil and tongue base)<br>Better Survival<br>More radiosensitive | All head and neck sites<br>Worse survival<br>Radiation response unpredictable |
| Epidemiological factors | Never smokers<br>Mild/mod alcohol intake<br>High marijuana exposure<br>Intact dentition<br>High oral sex exposure<br>Younger age (<45 yrs)<br>Higher socioeconomic status<br>Increasing incidence | Heavy smokers<br>Heavy alcohol intake<br>Low marijuana exposure<br>Poor dentition<br>Low oral sex exposure<br>Older age (> 50 yrs)<br>Lower socioeconomic status<br>Decreasing incidence |
| Molecular factors | P53 wild-type present<br>HPV E6 and E7 RNA<br>HPV DNA (type 16)<br>D cyclin underexpressed<br>*CDKN2A* overexpressed<br>Rb down regulated | P53 mutational loss common<br>Rb up-regulated<br>*CDKN2A* underexpression<br>D cyclin overexpression<br>No HPV DNA/RNA |

#### 1.8.1.7 Implications of HPV HNSCC

There are significant implications associated with the rising number of HPV positive HNSCC. As discussed above, HPV related head and neck cancer appears to be a distinct cancer from non-HPV HNSCC in terms of epidemiology and molecular biology. Patients with HPV positive disease are usually younger and more likely to be employed. They are more likely to survive the cancer, but survive with the often significant co morbidities of treatment. This will in turn have an impact on health and social services.

Although having an HPV positive tumour gives patients a survival advantage, assumptions about a causal role for HPV in cases of oropharyngal cancer have psychosocial implications (Allen et al., 2010). Many patients, especially those without a significant tobacco and alcohol history will want to know the aetiology of their cancer. Patients with a negative alcohol and tobacco history will require counselling when diagnosed, and talking about the role of HPV can be difficult and upsetting for the patient and their relatives due to the social stigma surrounding HPV infection (Adelstein et al., 2009). There is evidence that if one partner has persistant high risk oral HPV infection then this is a significant risk factor for high risk oral HPV infection for the other partner (Adelstein et al., 2009, Rintala et al., 2006). Haddad et al reported on the case of a synchronous HPV positive oropharyngeal cancer in a husband-wife couple with the same strain of HPV (Haddad et al., 2008). The US National Cancer Institute State of Science meeting recently discussed patient counselling in the context of patients having a newly diagnosed HPV positive oropharyngeal cancer. They recommended that counselling should suggest no change in sexual behaviours for monogamous partners and the use of barrier protection to prevent HPV transmission in new partners (Adelstein et al., 2009).

## 1.9 Aims of the research

The management of HNSCC has become increasingly complicated in recent years. The choice of primary treatment modality is generally between surgery and radiotherapy (with or without chemotherapy). Deciding between the two has become more difficult since the introduction of synchronous chemoradiotherapy as a realistic option for patients with locally advanced head and neck cancer (as an alternative to radical surgery) and the popularisation of conservation surgery (as an alternative to radiotherapy) for early stage disease in many sites. The ability to select treatment based on measurement of tumour biology that would predict resistance/sensitivity to

radiotherapy is very relevant and important clinically.

The above issues are particularly pertinent for the two HNSCC sites investigated in this thesis. There is increasing evidence that chemoradiotherapy is as effective as radical surgery with reconstruction for patients with oropharyngeal SCC. However, in the event of a tumour proving to be recurrent or resistant after chemoradiotherapy, it is often unsalvageable surgically. Therefore, knowing the radioresponsiveness of such tumours has immense clinical relevance, if this can be predicted (currently it cannot).

Similarly with glottic cancer, the standard treatment for decades has been external beam radiotherapy. However, increasingly, many early glottic tumours are being managed with transoral surgical laser excision. There is clinical equipoise between radiotherapy and transoral surgery for the treatment of early glottic cancer. One factor, which would be useful to determine treatment, is the intrinsic radioresponsiveness of a given tumour, something for which we have no measure of presently. The rationale behind the work carried out, therefore, was the need to investigate aspects of tumour biology that might, in the future, enable an increased individualisation of treatment in patients with HNSCC.

The overall hypothesis underlying the work was that molecular profiles can be developed that predict response to radiotherapy versus surgery in patients with head and neck cancer. The specific aims of the thesis were:

1. To investigate markers of hypoxia (CA9 and HIF-1α) in early glottis cancer, and look at a subset of T2 glottic cancer patients and the role of hypofractionated radiotherapy in their managment.
2. To investigate markers of hypoxia (CA9 and HIF-1α) and viral infection in oropharyngeal cancer, and in particular to test for an association between hypoxia markers and viral infection.
3. To investigate biomarkers of response to primary surgery in HNSCC and explore whether HIF-1$\alpha$, CA9 are associated specifically with radioresponsiveness or a general poor prognosis.

### 1.9.1 Submission of thesis in alternative format

This thesis is presented in the alternative format permitted by the Faculty of Medicine of The University of Manchester. The alternative format involves division of a thesis into chapters that have been published, are under review for publication or can be submitted for consideration for publication.

# 2 Expression of hypoxia markers in glottic cancer

## 2.1 Abstract

Purpose: There is clinical equipoise between radiotherapy and transoral surgery for the treatment of early glottic cancer. Currently, most management decisions are based on clinical parameters with little appreciation of patient differences in underlying tumour biology. The identification of biomarkers that predict response to radiotherapy would be clinically useful in determining optimal management. Here, we investigate the prognostic significance of tumour expression of carbonic anhydrase 9 (CA9) and hypoxia inducible factor 1-α (HIF-1α) and clinicopathological features in a homogeneous series of patients with early stage glottic cancer who received radiotherapy.

Methods and Materials: From 1999-2005, 423 patients were identified who underwent radiotherapy for early glottic cancer. Pretreatment biopsy samples were available for 310 patients. Immunohistochemistry was performed on formalin fixed, paraffin embedded material for CA9, HIF-1α. Within this group of patients a subset of 113 patients with T2N0 glottic cancer were further analysed.

Results: Adverse prognostic factors for locoregional control were low pre-treatment haemoglobin (Hb; $p = 0.010$), advancing T stage ($p = 0.001$) and high CA9 expression ($p = 0.032$). Low Hb and high CA9 expression were independent factors on multivariate analysis; and combined predicted locoregional recurrence with an odds ratio of 8.0 (95% CI: 2.7-23.9), or either/or with an odds ratio of 3.3 (95% CI 1.5-7.1). Within the T2N0 series five-year locoregional control following radiotherapy was 82% and cancer specific survival was 90%. Serious morbidity occurred in 1.8% of patients. T stage subdivided by vocal cord movement was significant for local control. Also, the series of T2 glottic cancer patients treated with accelerated hypofractionated radiotherapy had outcomes superior to that of series reported using conventional fractionation.

Conclusions: There are significant differences in radiotherapy outcome within a homogeneous subsite of early glottic cancer related to pre-treatment Hb and tumour expression of CA9. We show that these biomarkers could be used together to identify patients with a high probability of a poor outcome following radiotherapy. Furthermore, in the T2N0 series Subdivision of T2 cancer into 2a and 2b should be considered when treatment decisions are made. Also, modified fractionation, chemoradiotherapy or surgery should be considered, particularly for T2b disease.

## 2.2 Introduction

Cancer of the larynx accounts for 2.4% of cancer cases worldwide (Parkin et al., 2005). Early stage (T1-T2 N0 M0) squamous cell carcinoma (SCC) of the glottic larynx is characterised by low tumour volume and a low rate of regional metastasis. The goals of treatment are: disease cure and preservation of organ function. The main modalities of treatment are with either external beam radiotherapy or transoral laser microsurgery, with overall clinical equipoise between the two (Mendenhall et al., 2004). In most countries, radiotherapy remains the predominant treatment. In early glottic SCC, local cure generally constitutes disease specific cure. With the obvious importance in avoiding total laryngectomy, the important parameter is local control without laryngectomy. Due to a perceived superior voice quality, radiotherapy remains the most common primary treatment, with partial or total laryngectomy in reserve. The 5-year initial local control rates for T1 glottic lesions treated with radiotherapy are reported as 85% to 95%, with ultimate control between 95% and 100% (Mendenhall et al., 2001, Warde et al., 1998, Jorgensen et al., 2002). However for T2N0 lesions treated with radiotherapy the reported 5-year local control rates are often considerably lower with initial local control rates between 65% and 85% with ultimate control between 75% and 95% (Garden et al., 2003, Frata et al., 2005). Despite reported inferior local control rates of only 70% with conventional fractionation, this schedule continues to be widely used. Clinical outcomes may be improved with modified radiotherapy schedules. This analysis was undertaken to examine an accelerated hypofractionation approach and to calculate tumour and normal tissue radiobiological parameters. The main modalities of treatment are with either external beam radiotherapy or transoral laser microsurgery, with overall clinical equipoise between the two (Mendenhall et al., 2004). In most countries, radiotherapy remains the predominant treatment. In early glottic SCC, local cure generally constitutes disease specific cure. With the obvious importance in avoiding total laryngectomy, the important parameter is local control without laryngectomy.

Controversy remains over the best modality for any given patient. A key role for predictive biomarkers in head and neck squamous cell carcinoma (HNSCC) is the prediction of response to radiotherapy, on the basis that treatment can be escalated by using surgery or radiotherapy modification on an individual basis. In early glottic SCC, such a predictive biomarker could be used to inform the difficult choice between radiotherapy and transoral laser microsurgery.

There is a consistently reported relationship between low pre-treatment Hb and a poor prognosis in HNSCC treated with radiotherapy, including early glottic SCC (Lutterbach and Guttenberger, 2000, van Acht et al., 1992, Cho et al., 2004). Intrinsic markers of tumour hypoxia have been shown to predict outcome after radiotherapy in

HNSCC (Nordsmark et al., 2007), although with less consistency. In early glottic SCC, tumour expression of the hypoxia-associated markers HIF-1α and CA9 has been associated with outcome after radiotherapy (Schrijvers et al., 2008). In addition, Bcl-2 expression, as a marker of apoptosis, has previously been shown to be associated with outcome in this group (Condon et al., 2002).

Here, we evaluate clinico-pathologic variables, pre-treatment Hb and immunohistochemical markers (HIF-1α, CA9) in consecutive series of patients who underwent radiotherapy for early glottic SCC. We then look in more detail at a group of T2 glottic cancer patients within this series, and examine the role of hypofractionated radiotherapy in their treatment.

## 2.3 Materials and Methods

### 2.3.1 Patients and Tissue

Ethical approval was obtained from the local research ethics committee (Reference Number: 03/TG/076), appendix IV.

Patients were identified through the radiotherapy database at Christie Hospital, Manchester and were consecutively-treated patients between January 1999 to December 2005. The demographic, clinico-pathologic and outcome patient data were collected retrospectively from the case notes and the radiotherapy proforma used prior to any patient receiving radiotherapy, see appendix V. Pre-treatment Hb levels were obtained from the case notes. Tumour blocks were requested from the referring hospitals in which the diagnostic biopsies were performed. Inclusion criteria were patients included in the study were those that had a biopsy proven glottis cancer, patients treated at the Christie Hospital between January 1999 and December 2005, patients treated with only radiotherapy i.e single modality treatment, patients over the age of 16 years. Exclusion criteria were patients treated palliatively; patients that received surgery or chemotherapy, patients that did not receive radiotherapy. All patients received hypofractionated radiotherapy, delivered using a 4-6 MeV linear accelerator. The radiotherapy beam arrangement used was a single phase anterior oblique pair of fields or a lateral parallel opposed pair of fields using fields sizes predominantly 5.5-7 cm long. All patients received 52.5 Gy in 16 daily fractions over three weeks. A subset of these patients with T2 N0 glottic cancer were further analysed. Inclusion criteria for this was a diagnosis of T2N0 glottic cancer, with subdivision of staging by vocal cord movement (as determined by clinical oncologist). All patients received radiotherapy only. Exclusion was those without subdivision by vocal cord movement.

### 2.3.2 Immunohistochemistry

Immunohistochemical detection of HIF-1α was performed using the Tyramide Signal Amplification System (NEN Life Sciences, Boston), which is based on a streptavidin-biotin-horseradish peroxidase complex formation. Sections 4 μm thick were deparaffinisd. Antigen retrieval was carried out by microwaving the sections in 10 mmol/L citrate buffer for 20 min. The sections were rinsed, and then soaked in TBS (Tris Buffered Saline) for 5 min, followed by immersion in TBS and 30% hydrogen peroxide. The sections were washed, soaked in TBS again, and then TNB (Tris NaCl Blocking) reagent was applied for 30 min. The mouse monoclonal IgG1 (immunoglobulins isotype 1) antibody (BD Biosciences 610958) in TNB reagent was applied to the sections, mouse IgG1 (Dako X0931) was used for the negative control sections. They were incubated overnight at 4oC. The secondary antibody, a biotinylated rabbit anti-mouse immunoglobulin (RAMBO) (Dako E0413) in TCP (Tryptone Casein Peptone), was applied for 30 min followed by SA-HRP (Streptavidin-Horse Radish Peroxidase) from the TSA (tyramide signal amplification) Biotin Kit in TCP for 30 min. Biotinyl Tyramide Amplification reagent was applied to the sections for 8 min, followed by SA-HRP in TNB for 30 min. TNT was used to wash the sections in between applications of the agents. DAB (3,3' diaminobenzidine tetrahydrochloride) chromogen was then applied to the sections for 5 min. Gill's haematoxylin was used as a counterstain, prior to dehydration and cover slipping (Kim et al., 2003). Batch-to-batch variation was assessed by choosing two sections showing high and low HIF-1α expression and running additional sections from these biopsies with each batch (Aebersold et al., 2001).

Imunohistochemical detection of CA9 was performed using the Envision kit system. Sections 4 μm thick were deparaffinisd. Slides were then washed with TBS (Tris Buffered Saline) for 5 min. A hydrogen peroxide block (30%) was applied for 15 min, then a casein block (10%) for 15 min. The primary antibody, mouse monoclonal antibody (M75,1:50, gift from Oxford) was then applied for 30 min. Mouse IgG 2B (immunoglobulins isotype 2B) (Dako 0944) was used for the negative control tissue sections. A secondary antibody was applied to the sections for 30 min and after rinsing, DAB+ (3,3' diaminobenzidine tetrahydrochloride) was applied to each section for 5 min. Both the secondary antibody and the DAB+ were from the Envision kit. Gill's haematoxylin was used to counterstain the sections, which were then rinsed, dehydrated and coverslipped.

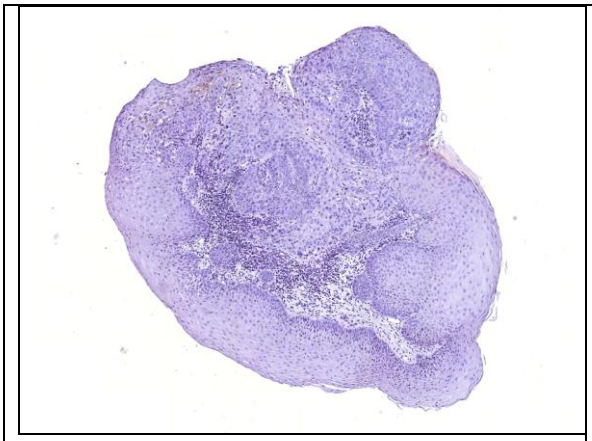
### 2.3.3 Scoring Method

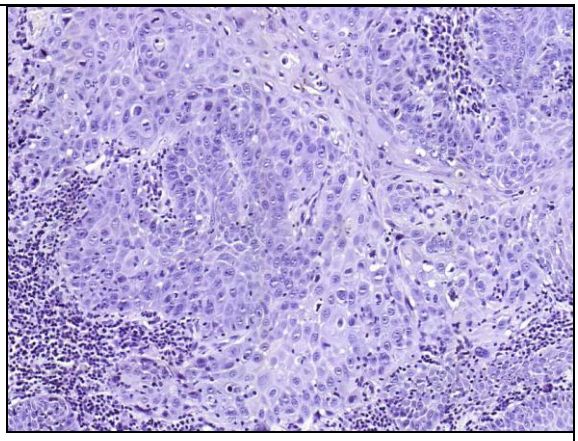
The presence of SCC was confirmed by a senior pathologist (Dr Rachel Hall, Consultant Pathologist). For CA9 the scoring system was as follows: 0, less than 1%

plasma membrane staining; 1, 1-9% plasma membrane staining; 2, 10-30% plasma membrane staining; 3, greater than 30% plasma membrane scoring. For analysis the scoring was further sub divided into low expression ( 0-9%) (see Fig 2.1) and high expression (10->30%) (see fig 2.2). These are similar scoring systems used by previous authors (Loncaster et al., 2001) . The scoring system for HIF 1-α was as follows: 0, no nuclear staining; 1, less than 10% nuclear staining; 2, 10% to 29% nuclear staining; 3, 30% or greater nuclear staining (see Fig 2.3). This is a similar scoring to previous authors (Condon et al., 2002). All scoring was blinded to outcome and performed independently by two scorers and repeated with resolution of any conflicting scores by independent re-scoring followed by discussion and consensus.

### 2.3.4 Statistical analysis

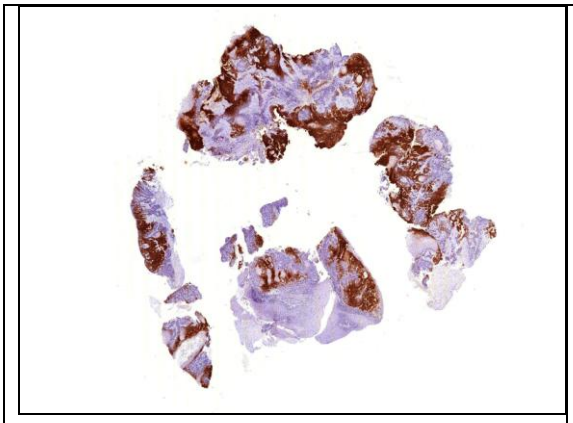
Actuarial estimations of locoregional control and cancer-specific survival were obtained using the Kaplan-Meier method. The survival curves were compared in univariate analysis using the log rank test. The variables analysed for prognostic significance were site, alcohol history, smoking history, age, gender, pre-treatment Hb level, stage, histological grade and tumour CA9 and HIF-1α expression. In the univariate analyses, patients were categorized according to the median Hb level (<13/>13 gdL). Multivariate analysis was performed with a Cox proportional hazards model to study patient and disease variables in relation to loco regional control, and included CA9, Hb and stage. A multivariate model using different combinations of Hb and CA9 was then performed. Spearman's rho test was used for non-parametric correlations.

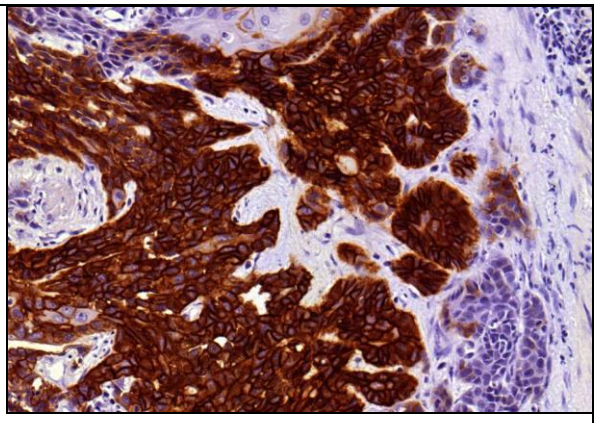




| Low magnification | High magnification X 40 |
|---|---|

Figure 2-1 CA9 staining of glottis biopsy showing low expression.





| Low magnification | High magnification X 40 |
|---|---|

Figure 2-2 CA9 staining of glottis biopsy showing high expression.

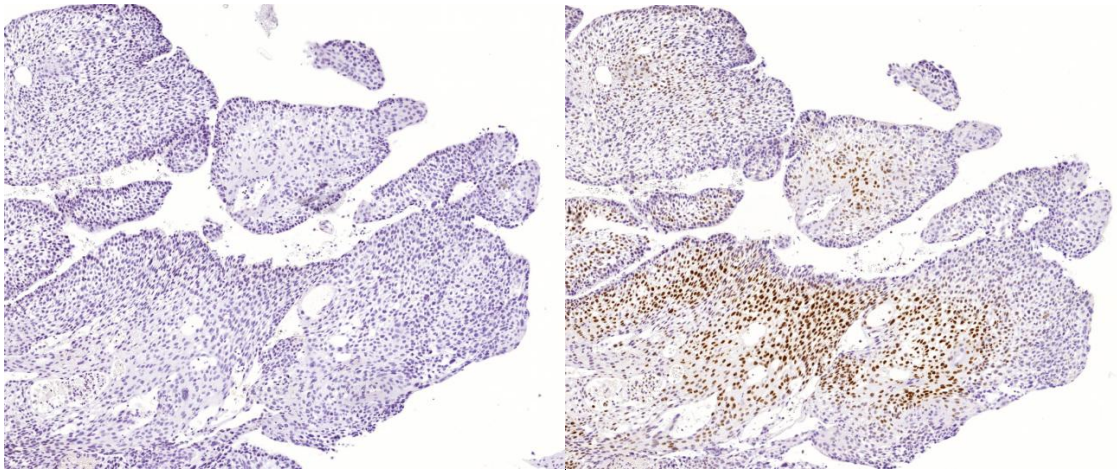


Figure 2-3 HIF-1α staining of glottis biopsy showing negative control (left) and high expression (right)

## 2.4 Results – overall glottis series

### 2.4.1 Patient characteristics

A total of 423 patients were included in the series, 371 of whom were male and 52 female, see appendix VI . Tissue specimens obtained for analysis were available for 310 patients. Pre-treatment Hb levels were available for 361 patients. The characteristics of the 423 patients are summarised in Table 2.1. On scrutiny of the notes, 26 tumours were found to be T3 and 26 Tis. The median duration of follow up for surviving patients was 5.5 (range 1.5 – 9.7) years. Deaths occurred in 128 patients and 17 of these deaths were caused by glottic cancer. Recurrence occurred in 45 patients. 35 patients developed primary failure and in 13 patients regional lymph node failure occurred. The five-year overall survival rate for all patients was 78.6%. The five-year loco-regional control rate was 88.0% and the five-year cancer specific survival rate was 94.5%. Adverse prognostic factors for locoregional control were low Hb ($p=0.010$) and advancing T stage ($p=0.001$). Age, grade, gender, smoking status and alcohol status were not significant for locoregional control (all $p>0.05$; Table 2.2).

### 2.4.2 CA9

CA9 scores were available for 310 of the patients. CA9 expression was categorised as low (<10%) in 201 patients (<1% in 87 patients and 1-9% in 114 patients) and high (≥10%) in 109 patients (10-29% in 59 patients and >30% in 30 patients). No significant associations were found between CA9 expression and the patient variables gender, age, smoking status, alcohol consumption, overall stage and pre-treatment Hb level ($p>0.05$, for all). On univariate analysis, high CA9 tumour expression (≥10%) was significantly associated with lower locoregional control ($p=0.032$) and cancer specific survival ($p=0.040$).

### 2.4.3 HIF-1α

HIF-1α scores were available for 297 patients. HIF-1α expression was categorised as low (<10%) in 162 patients and high (≥10%) in 135 patients. No significant associations were found between HIF-1α expression and the patient variables gender, age, smoking status, alcohol consumption, overall stage and pre-treatment Hb level ($p>0.05$ for all). However, there was a positive correlation between CA9 expression and HIF 1-α expression (Table 2.3). On univariate analysis high HIF-1α (≥10%) was not associated with worse locoregional control ($p=0.74$) or cancer specific survival ($p=0.24$).

Table 2-1 Patient characteristics of the glottic series

| Characteristic | | Value |
|---|---|---|
| Gender | Male | 371 (88) |
| | Female | 52 (12) |
| Age | <65 years | 216 (51) |
| | ≥65 years | 207 (49) |
| Smoking status | Current | 109 (26) |
| | Ex ≤1 year | 99 (23) |
| | Ex >1 year | 169 (40) |
| | Never | 29 (7) |
| | Unknown | 17 (4) |
| Alcohol consumption | Heavy/Medium | 94 (23) |
| | Low | 225 (53) |
| | None | 21 (5) |
| | Previously Heavy | 27 (6) |
| | Unknown | 56 (13) |
| T stage | 1a | 213 (50) |
| | 1b | 38 (9) |
| | 2a | 79 (19) |
| | 2b | 41 (10) |
| | 3 | 26 (6) |
| | In-situ | 26 (6) |
| Tumour grade | Well Differentiated | 108 (26) |
| | Moderately Differentiated | 126 (30) |
| | Poorly Differentiated | 20 (5) |
| | CIS | 26 (6) |
| | Not Classified | 143 (33) |
| Pre-treatment Hb | ≤13 g/dL | 61 (14) |
| | >13 g/dL | 300 (71) |
| | Missing | 62 (15) |
| Tumour CA9 expression | ≤1% | 87 (21) |
| | 1-9% | 114 (27) |
| | 10-29% | 59 (14) |
| | ≥30% | 50 (12) |
| | Missing | 113 (27) |
| Tumour HIF-1α expression | ≤1% | 39 (9) |
| | 1-9% | 123 (29) |
| | 10-29% | 100 (24) |
| | ≥30% | 35 (8) |
| | Missing | 126 (30) |

Abbreviations: Hb=haemoglobin; CA9=carbonic anhydrase 9; HIF-1α = hypoxia inducible factor-1α;. Values are number (percentage).

Table 2-2 Univariate analysis of locoregional recurrence.

| Characteristic | Univariate HR (95% CI) | p |
|---|---|---|
| Smoking | | |
| Smoker | 1 | |
| Non-smoker | 1.94 (0.82-4.57) | 0.13 |
| Alcohol | | |
| Heavy/prev heavy | 1 | |
| Light/never | 1.14 (0.66-2.81) | 0.41 |
| Stage | | |
| Low | 1 | |
| High | 3.59 (2.007-6.42) | <0005 |
| Gender | | |
| Female | 1 | |
| Male | 1.18 (0.47-2.97) | 0.73 |
| Age | | |
| >65 | 1 | |
| <65 | 1.23 (0.70-2.17) | 0.47 |
| Overall stage | | |
| 0 | 1 | |
| 1 | 1.05 (0.25-4.51) | |
| 2 | 2.68 (0.64-11.28) | 0.005 |
| HIF-1α | | |
| Low | 1 | |
| High | 1.11 (0.59-2.10) | 0.74 |
| CA9 | | |
| Low | 1 | |
| High | 2.08 (1.1-3.93) | 0.020 |
| Hb | | |
| >13 g/dL | 1 | |
| ≤13 g/dL | 2.11 (1.06-4.21) | 0.030 |

Table 2-3 Glottic series - Spearman's rho test for non-parametric correlations

| Variables | rho | p-value | N |
|---|---|---|---|
| Age vs Hb Level | 0.17 | p=0.001 | 361 |
| Age vs CA9 Expression | 0.02 | p=0.76 | 310 |
| Hb Level vs CA9 Expression | -0.09 | p=0.13 | 267 |
| CA9 vs HIF-1α | 0.27 | p<0.001 | 291 |

### 2.4.4 Pre-treatment haemoglobin

Hb data were available for 361 patients. The median Hb was 13.0 g/dL (10.2-17.7). Low Hb was significantly associated with the female gender (p=0.001). There was no association between Hb and age, smoking status, alcohol consumption, overall stage, CA9 expression and HIF 1-α expression (Table 2.3). On univariate analysis low Hb (≤13 g/dL) was an adverse prognostic factor for locoregional control (p = 0.027) but had no prognostic significance for cancer-specific survival.

### 2.4.5 Multivariate analysis

The Cox-Proportional hazard model was used to study the patient and disease variables in relation to locoregional control. CA9, Hb and stage remained significant independent prognostic factors for locoregional control (Table 2.4). A multivariate model using different combinations of Hb and CA9 is shown in Table 2.5 and Figure 2.1. The combination of high CA9 and low Hb was associated with a hazard ratio of 7.98 (p=0.002). A multivariate model splitting the patients into two groups - high Hb and low CA9 versus all other patients – showed that patients who did not have high Hb and low tumour CA9 expression had a hazard ratio for locoregional control of 3.26 (p = 0.0027; Table 2.6).

Table 2-4 Multivariate analysis of local recurrence

| Characteristic | | Multivariate HR (95% CI) | p |
|---|---|---|---|
| Stage | Low | 1 | |
| | High | 2.98 (1.43-6.19) | 0.004 |
| Hb | High | 1 | |
| | Low | 2.79 (1.31-5.94) | 0.008 |
| CA9 | Low | 1 | |
| | High | 2.15 (1.06-4.37) | 0.034 |

Table 2-5 Multivariate model for CA9 and Hb combinations for local recurrence

| Characteristic | Recurrence/number | Multivariate HR (95% CI) | p |
|---|---|---|---|
| Low CA9 + high Hb (1) | 9 / 143 | 1 | |
| High CA9 + high Hb (2) | 13 / 82 | 2.60 (1.11-6.08) | |
| Low CA9 + low Hb (3) | 5 / 28 | 3.53 (1.18-10.55) | |
| High CA9 + low Hb (4) | 5 / 14 | 7.98 (2.67-23.89) | 0.002 |
| Groups 2, 3, 4 | 23 / 124 | 3.26 (1.51-7.05) | 0.0027 |

Table 2-6 Glottic series - multivariate model for low CA9 and high Hb + all other patients for local recurrence

| Characteristic combination | Number/Recurrence | Multivariate HR (CI) | p |
|---|---|---|---|
| Low CA9 + High Hb | 143/9 | 1 | |
| All other patients | 124/23 | 3.26 (1.51-7.05) | 0.0027 |

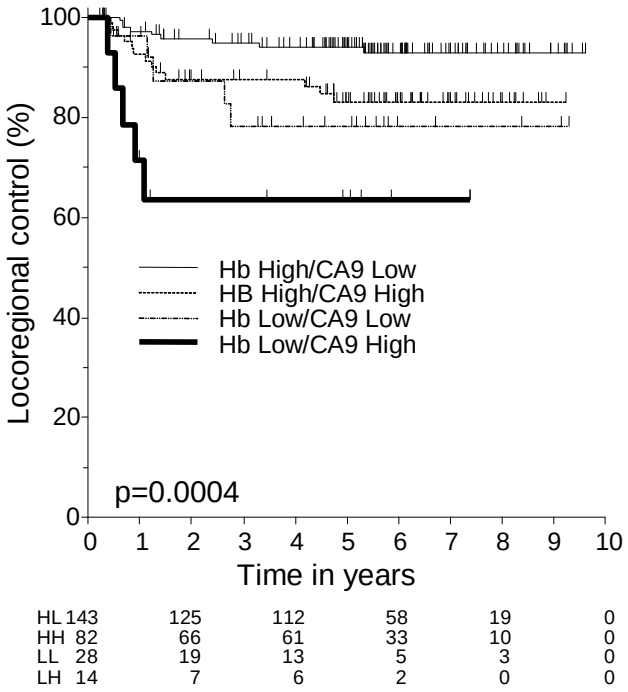



Figure 2-4 Kaplain-Meier survival curve for locoregional control for patients stratified according to Hb and CA9.

Group 1 = Low CA9+ high Hb; Group 2 = high CA9 + high Hb; group 3 = low CA9 and low Hb; group 4 = high CA9 and low Hb.

## 2.5 Results – T2N0 subseries

The median follow up was 5.8 (range 0.1-9.6) years. All but four cases had disease recurrence or at least three or more years follow up. Overall survival at 5 years was 67%. Locoregional control following radiotherapy was 82% and cancer specific survival was 90% at 5 years. Nineteen patients developed recurrence (Table 2.7). There were 18 second cancers (kidney, bladder, and lung). Prognostic factors associated with an adverse effect on locoregional control were age less than 65 years (p=0.003) and T substage (p=0.032; Table 2.8 and Fig 2.4). Serious morbidity (defined as requiring surgical intervention) occurred in 2 (1.8%) patients post radiotherapy: one patient required a tracheostomy; one patient had a total laryngectomy (Table 2.9). Thirteen of the 113 patients had a total laryngectomy (12 for recurrence, 1 for morbidity).

Table 2-7 Recurrence site for T2 larynx cancers

| Site | Number |
|---|---|
| Larynx alone | 11/113 (9%) |
| Nodes alone | 4/113 (3.5%) |
| Larynx and nodes | 4/113 (3.5%) |

Table 2-8 Prognostic factors for 5 year locoregional control in T2N0 series.

| Factor | Group (n) | 5 yr locoregional control (95% CI) | p value* |
|---|---|---|---|
| Sex | Male (99) | 84.8% (75.5-90.8) | |
| | Female (14) | 70.0% (38.3-87.6) | 0.20 |
| Age (yr) | <65 (59) | 73.2% (59.9-82.9) | |
| | >65 (54) | 93.9% (82.3-98.0) | 0.003 |
| Pre treatment Hb g/dL | ≤13 (43) | 89.4% (74.2-95.9) | |
| | >13 (60) | 81.1% (68.3-89.10 | 0.46 |
| Stage | 2a (76) | 88.8% (79.0-94.2) | |
| | 2b (37) | 70.8% (52.4-83.1) | 0.032 |
| Smoker | Current/ex < 1yr (47) | 79.0% (63.6-88.4) | |
| | Ex > 1 yr/never (64) | 87.0% (75.6-93.3) | 0.42 |
| Alcohol | Low/no alcohol (69) | 82.5% (70.6-89.9) | |
| | Heavy/prev heavy (29) | 89.6% (71.0-96.5) | 0.41 |

*Log rank

Table 2-9 Patient morbidity in T2N0 patient series

| | Age at Diagnosis | Morbidity | Morbidity | Smoking status |
|---|---|---|---|---|
| Patient 1 | 59 | Early | Tracheostomy 1 month after completion of radiotherapy | 70 cpd, continued to smoke during/after radiotherapy |
| Patient 2 | 44 | Late | Laryngectomy 13 months after completion of treatment | 25 cpd, continued to smoke during/after treatment |

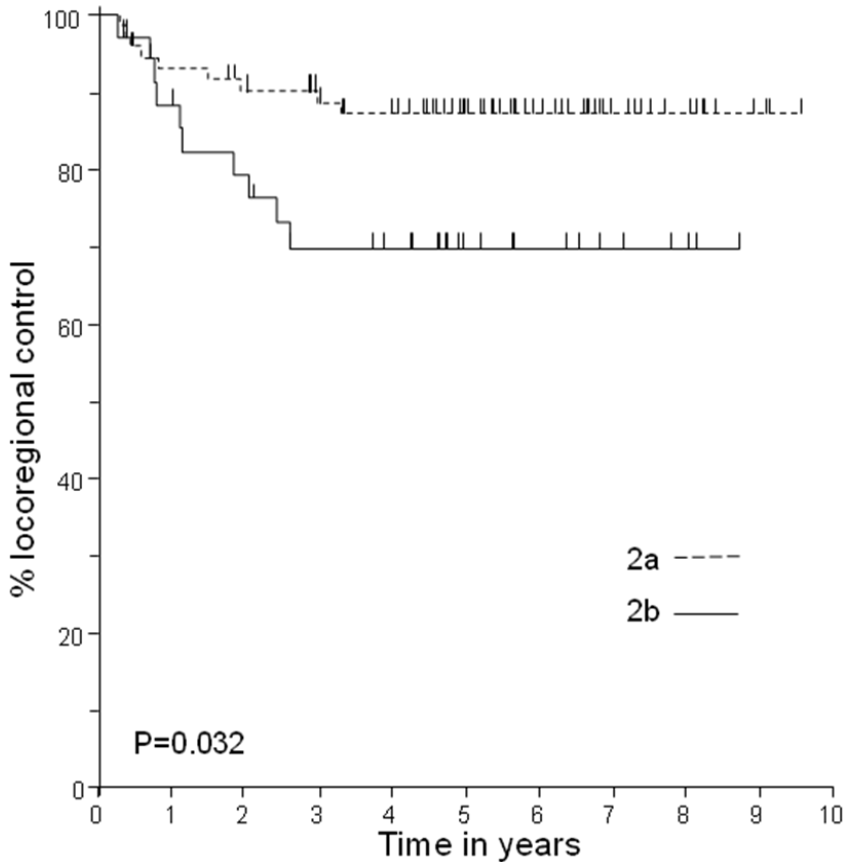



Figure 2-5 Influence of cord mobility on locoregional control in T2N0 series

## 2.6 Discussion

In this study, we evaluated the prognostic effect of three immunohistochemical markers, pre-treatment Hb and standard clinico-pathologic variables in a consecutive series of patients treated with radiotherapy for early glottic laryngeal SCC. In our series of patients, with a 5 year locoregional control rate of 88%, we demonstrated that low pre treatment Hb ($p = 0.008$) and high tumor CA9 expression ($p = 0.034$) were significant independent adverse prognostic factors for locoregional control in multivariate analysis. HIF-1α did not have any prognostic significance in the series of patients studied.

Outcomes in HNSCC following radical radiotherapy are adversely influenced by tumour hypoxia (Brizel et al., 1999, Gatenby et al., 1988, Nordsmark and Overgaard, 2000). Despite early glottic cancers being small, there is experimental evidence demonstrating that tumours as small as 1 mm can have areas of hypoxia (Rockwell, 1997). Oxygen is a potent radiosensitiser which mediates DNA damage, with oxygen tension distribution being an important modifier in radiation response (Okunieff et al., 1996). Hypoxia also promotes a more aggressive phenotype, with cancer cells able to adapt to a hostile, hypoxic environment, contributing to aggressive tumour behaviour

with a greater propensity for angiogenesis, metastasis and escape from apoptosis (Dachs and Chaplin, 1998, Dachs and Tozer, 2000, De Jaeger et al., 2001).

There are many proteins that are known to be up regulated under hypoxia, with up to 1.5% of the human genome hypoxia responsive (Denko et al., 2003). Two hypoxic markers, detectable using standard immunohistochemistry, that have shown good correlation with eppendorf polarographic oxygen status, are HIF-1α and CA9 (Loncaster et al., 2001, Haugland et al., 2002). HIF-1α is the archetypal hypoxia regulated protein. Hypoxia prevents its hydroxylation and therefore HIF-1α levels rise. Its activation up regulates more than 50 genes which are involved in the adaptive response to hypoxia including: cell proliferation, angiogenesis, metabolism, apoptosis, immortilisation and migration (Semenza, 2001). CA9 is regulated by HIF-1α and its expression is increased by hypoxia (Hoogsteen et al., 2007a, Vordermark and Brown, 2003). CA9 has been shown to be expressed more strongly in tumour cells adjacent to necrotic areas, suggesting CA9 as a useful marker of hypoxia (Wykoff et al., 2000). Tumour cells known to express CA9 have been shown to be more resilient to and less likely to die following exposure to ionizing radiation than those which do not (Hoogsteen et al., 2007b). In several series of patients with HNSCC treated with radiotherapy, the over expression of HIF-1α and/or CA9 has been found to be associated with poorer outcome (Aebersold et al., 2001, Silva et al., 2008, Koukourakis et al., 2008) (Koukourakis et al., 2002, Koukourakis et al., 2001, De Schutter et al., 2005a, Bache et al., 2006). However, there is a lack of consistency in these findings, particularly with regard to CA9 (Eriksen and Overgaard, 2007, Kaanders et al., 2002b).

Anaemia is a frequently associated with cancer. Several studies have demonstrated that pre-treatment anaemia is associated with poor locoregional control and survival in HNSCC, especially when treated by radiotherapy (Lee et al., 1998, Dubray et al., 1996). However, the mechanism by which tumour control is compromised by anemia is not clear. Whilst there seems to be a correlation between anaemia and increased hypoxia in solid tumours, it is weak and the precise relationship between Hb levels and tumour hypoxia remains unclear (Becker et al., 2000, Nordsmark et al., 2005, Hoogsteen et al., 2007a). There does not appear to be an association between anaemia and intrinsic pathways of tumour adaption to hypoxia (Koukourakis et al., 2004). In addition, the correction of pre-treatment anemia has not been shown to be beneficial in HNSCC (Henke et al., 2003).

Our finding of an association between pre-treatment Hb and outcome in early glottic laryngeal SCC is consistent with other published series (Lutterbach and Guttenberger, 2000, van Acht et al., 1992, Cho et al., 2004, Warde et al., 1998, Grant et al., 1999, Fein et al., 1995). Our finding of an association between CA9 expression and outcome is also consistant with that of Schrijvers et al in a series of 91 patients with T1-2 N0

glottic SCC (Schrijvers et al., 2008), although we did not find such an association with HIF-1α and outcome. This may be explained, in part, by the differences in immunostaining of the two markers. Our method for CA9 immunostaining is relatively simple, easily reproducible and membranous staining is easily scored. However, the immunohistochemical method for HIF-1α is a two day technique staining the nucleus, technically more complex than and more subjective in grading. This aspect is also important in the clinical translation of these types of studies, as ease and reproducibility are vital. Our findings and those of Schrivers et al regarding CA9 are in contrast to the findings of others who have found no predictive effect in patients treated with radiotherapy, albeit in other HNSCC patient groups (Eriksen and Overgaard, 2007). There are a number of possible explanations for this discrepancy, particularly differences in immunohistochemistry technique used and less intra-tumour variability within smaller tumours. Supporting the former is the finding that our group has found predictive effect of CA9 on outcome after RT for or oropharyngeal SCC (Silva et al.) and our antibody is the same as that used by Schrivers et al with similar technique. The differences between groups highlight the need to develop standardised methods for immunohistochemical marker studies.

The arguments regarding the superiority of radiotherapy or transoral laser surgery for early glottic SCC are complex and remain unresolved with little clinical evidence base (Dey et al., 2002). One key factor in the clinical decision making process could be the use of biomarkers to detect increased risk of recurrence after radiotherapy. The majority of patients who develop recurrence following radiotherapy will go on to require total laryngectomy (Johansen et al., 2002), with a resultant major impact on quality of life and morbidity. At present, there are currently no clinical, pathological or molecular features that are used in clinical practice to predict for radiotherapy failure in early glottic cancer.

There is general agreement that localised small volume tumours (generally T1a) are highly suitable for transoral laser surgery, with comparable voice results (Sjogren et al., 2008, Goor et al., 2007, Delsupehe et al., 1999), at least equal local control and more convenient for most patients than radiotherapy. However, for other early glottic tumours that involve the anterior commissure, have more paraglottic space involvement or are poorly defined, the choice of treatment is much more difficult. Voice outcomes are significantly worse in this patient group(Vilaseca et al., 2008), yet ultimate local control without total laryngectomy better (Steiner et al., 2004). Indeed, a higher laryngeal preservation rate has been demonstrated even in T1a tumours with transoral laser surgery (Schrijvers et al., 2009). Therefore, a biomarker that informs the decision making process would be clinically very useful.

A multivariate model (Table 2.4) using Hb and CA9 expression demonstrated a

hazard ratio of 7.98 (95% CI 2.67-23.89) in patients with low Hb and high CA9 expression (p=0.002), although only 14 patients were in this category. Given the clinical equipoise between the two modalities of treatment for early glottic cancer, an arguably more clinically useful model is to split these patients into 2 nearly equal groups (Table 2.5) - group 1 constituting high Hb and low CA9 and group 2 being all other patients. This gave a hazard ratio of 3.26 (95% CI 1.51-7.05; p=0.0027).

The work described here shows that CA9 and pre-treatment Hb could be used in concert as such a biomarker but this assumes that it is specific for radiotherapy. It might be that patients with such an adverse biomarker profile have a poor outcome probability irrespective of treatment modality. In the context of T1-2 glottic SCC, however, tumours are low volume and (in the absence of field-change) it can be reasonably assumed that complete surgical excision will be curative, irrespective of tumour biology. Whilst associations between intrinsic tumour hypoxia markers (Winter et al., 2006) and anaemia (van de Pol et al., 2006), and outcome in surgically-treated patients have been demonstrated, these have generally been in patients with advanced tumours and in those who require post-operative radiotherapy.

This T2N0 glottic series is helpful in elucidating prognostic factors as The Christie policy is for radiotherapy in almost all cases and the dose delivered was the same for all patients. The assessment of vocal cord movement was performed by clinical oncologists, not otolaryngologists, which is a potential weakness in this series. However, despite this, subdivision of T stage by vocal cord movement was prognostic significance for local control (p= 0.032). The subdivision of T2 tumours should be considered when MDTs are making treatment decisions. Although smokers (current or recently stopped) had an inferior local control to non smokers (long term ex or never smoked), the difference did not reach statistical significance. Pre treatment Hb has been found to be prognostic in other reported radiotherapy series (Warde et al., 1998) but not this series. The only other factor significant for local control was age, a factor not seen in the majority of other glottic series (Warde et al., 1998, Mendenhall et al., 2001). Outcomes from radiotherapy are generally reported to have improved over the past 30 years (Frata et al., 2005, Le et al., 1997, Jorgensen et al., 2002) and this is indeed the case for this series. This is likely to be due to improvements in staging rather than better treatment in the Christie experience, as dose used in the current series is lower than the historical practice (Table 2.10). Nevertheless in many series the local control is 70% or even lower, suggesting that for some institutions their standard practice needs to be changed, by adopting modified fractionation, chemoradiotherapy or partial laryngectomy, particularly for T2b cases. Published local control of T2 glottic cancer by transoral laser or open partial laryngectomy is often superior to that from radiotherapy, albeit patients for surgery are selected and voice

quality is generally acknowledged to be inferior to that from radiotherapy (Table 2.11).

Table 2-10 T2N0 glottic cancer patients treated with radiotherapy at the Christie

| | **(Slevin et al., 1993)** | **This series** |
|---|---|---|
| Time period | 1970-1984 | 1999-2005 |
| No cases/year | 16 | 16 |
| Median age (years) | 64 | 64 |
| 2a/2b | 64/36% | 67/33% |
| Staging | Tomogram | CT scan |
| Tracheostomy pre radiotherapy | 5% | 0% |
| Dose | 55 Gy/16f (commonest) | 52.5 Gy/16f (all) |
| 5-year locoregional control | 76% | 82% |
| 5-year cancer specific survival | 84% | 90% |
| Overall survival | 64% | 67% |
| Serious morbidity | 4.1% | 1.8% |

Table 2-11 Local control of T2 glottic cancer treated with surgery

| **Author** | **Pts** | **Type of surgery** | **Local control (%)** | **5-year local control with larynx preservation** |
|---|---|---|---|---|
| (Giovanni et al., 2001) | 65 | OPL | 92 (5 yr) | 92% |
| (Crampette et al., 1999) | 23 | OPL | 83 | NA |
| (Spector et al., 1999) | 71 | OPL | 93 (5 yr) | 93% |
| (Peretti et al., 2000) | 23 | TLE | 73 ( 5 yr) | 91%[a] |
| (Laccourreye et al., 1994) | 90 (T2a) | OPL | 74 | NA |
| | 31 (T2b) | OPL | 68 | NA |
| (Grant et al., 2007) | 21 | TLE | 93 (5yr) | 95% |

[a] Local control with laser treatment alone; OPL=open partial laryngectomy; TLE= transoral laser excision.

Chemoradiotherapy is now a standard for stageT3 glottic cancers and should be considered for T2b disease, as local control following radiotherapy alone is closely aligned to that for T3 disease. This series is a historical series, but the Christie Hospital now routinely uses 55 Gy in 4 weeks with synchronous chemotherapy for T2b disease. Local control from conventionally (2 Gy) fractionated radiotherapy is about 70%. Trials are currently in progress exploring hypofractionated schedules as well as twice daily hyperfractionation. The linear quadratic equation is the most widely accepted method of fitting the survival of cells following radiation to an equation. The equation is $S(D)=e^{-(\alpha D+\beta D^2)}$ where S is the number of surviving cells following a dose of D, and α and β describe the linear and quadratic parts of the survival curve. The α and β constants vary between different tissues and tumours. The α term describes the linear component to the curve. Therefore, the cell death which results from the α component increases linearly with dose. The β term describes the quadratic part of the curve. As the dose increases, the cell death resulting from the β constant increases in proportion to the square of the dose. A useful term is the α/β ratio. This is the dose, in Gray, when the number of cells killed by the linear component α is equal to the cell kill from the quadratic β constant. Tissues which have a higher α/β ratio (that is, more linear killing than quadratic killing) tend to have a more linear slope when plotted on the logarithmic scale. Tissues with a low α/β ratio generally have a parabolic shape (Fowler, 1989). The biologically effective dose (BED) is an approximate quantity by which different radiotherapy fractionation regimens may be intercompared, assuming that full repair occurs between fractions so that the biological effect of each fraction is the same. The biologically effective dose (BED) if the biologically effective dose ( of a given schedule): the total dose required to give the same log cell kill as the schedule being studied, at an infinitely low dose rate or with infinitely small fractions well spaced out: with an overall time factor for repopulation during continued irradiation. When using BED, the α/β ratio of the tissues needs to be known (Fowler, 2010). Radiobiological modelling of tumour and normal tissue parameters using linear quadratic formulations is undermined by diversity in patient, tumour and particularly treatment characteristics. Moreover, even when homogeneous data are used to characterise tumour control, assumptions have to be made around the values chosen for α/β ratio, duration of lag phase and time factor (Roberts et al., 1997). Accepting these caveats, it can be seen from Figure 2.5 that the dose response gradient for T2 glottic cancer is not as steep as that previously reported for T3 disease (Figure 2.6 from (Wylie et al., 1999)). For this analysis the dose response data were analysed using weighted linear regression based on individual sample sizes. Values for γ/α range from 0.5 to 1 and we chose a mid-range value of 0.75 Gy. Quoted values for T (lag phase) lie between 3 and 4 weeks and we have chosen an intermediate value of

25 days (Roberts et al., 1997). Difference in slope between T2a and T2b conforms to a standard sigmoid dose response curve. Modelling late normal tissue effects is hampered by the lack of “clean” clinical data and by the impact of continued smoking on late effects. Surgical intervention for complications in generally reported in 4 bands, 0% (Warde et al., 1998, Harwood et al., 1981), 1-2% this series and (Mendenhall et al., 2001), 2-3% (Howell-Burke et al., 1990, Garden et al., 2003) and at least 4% (Slevin et al., 1993). The crude dose response data from published studies (Table 2.12) gives an indication of an α/β ratio for larynx late effects of 2.5 Gy.

In summary, this work confirms previous findings of the prognostic effect of pre-treatment Hb concentration in patients with early-stage glottic SCC treated with radiotherapy. It also confirms the findings of Schrivers et al with regard to the prognostic effect of CA9 in this patient group and shows that these two hypoxia-related biomarkers are independent of each other and could be used in in combination as a biomarker for patients with this disease. Given the clinical equipoise overall between radiotherapy and surgery for this group of patients, such a biomarker might be the key in determining which patients require which modality of treatment. Further work is merited to validate and these findings using the same immunohistochemistry method in independent cohorts of patients. Furthermore the ideal treatment for T2 glottic cancer still remains open to debate. Approaches other than conventional radiotherapy fractionation should be considered, including modified fractionation, partial laryngectomy or synchronous chemoradiotherapy, particularly for T2b disease.

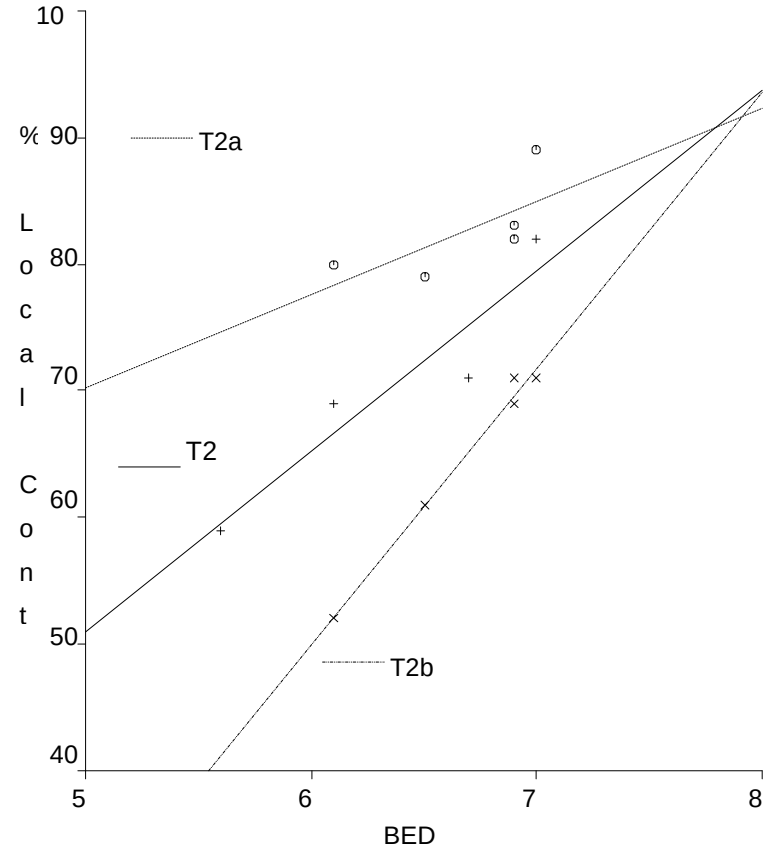



Figure 2-6 Larynx complications with α/β ratio of 2.5 Gy

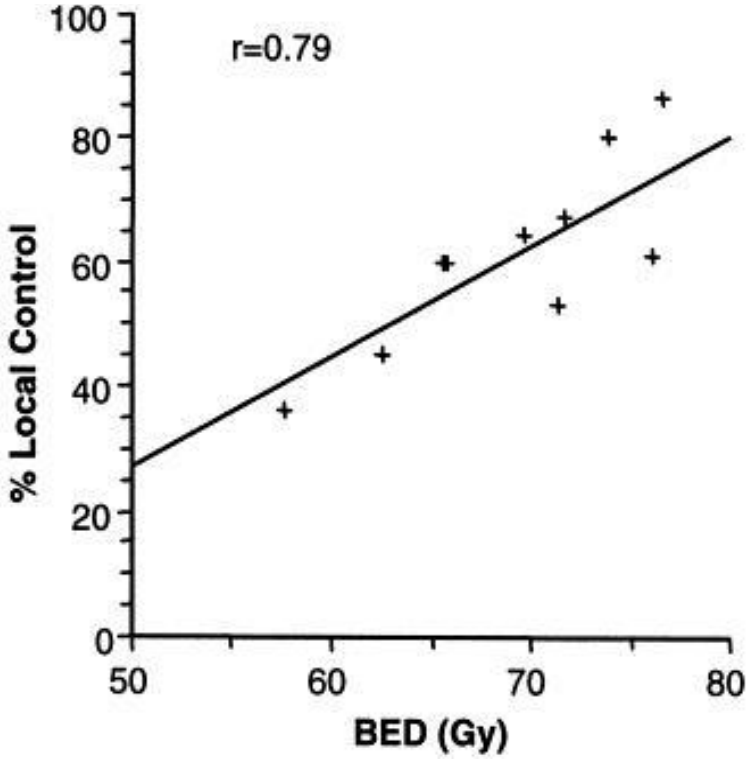



Figure 2-7 Relationship between the biological effective dose (BED) and local control for T3 glottic carcimoma (including Christie data). ('r' represents Pearson's correlation coefficient). From (Wylie et al., 1999).

Table 2-12 Complications

| Author | Total dose (Gy) | Fraction size | Frequency (%) | $BED_{2.5}$ |
|---|---|---|---|---|
| (Le et al., 1997) | 65.5 | 2.25 | 3/161 (1.9%) | 124 |
| This series | 52.5 | 3.28 | 2/113 (1.8%) | 121 |
| (Howell-Burke et al., 1990) | 70 | 2.00 | 3/114 (2.6%) | 126 |
| (Slevin et al., 1993) | 55 | 3.44 | 10/242 (4.1%) | 131 |
| T1* (Gowda et al., 2003) | 50 | 3.13 | 0% | 113 |

*Gowda et al T1 Glottis only.

# 3 Prognostic significance of hypoxia markers and HPV status in oropharyngeal cancer

## 3.1 Abstract

Purpose: The management of head and neck squamous cell carcinoma is complex and often involves multimodality treatment. Currently, most management decisions are based on clinical parameters with little appreciation of patient differences in underlying tumour biology. The identification of biomarkers that predict response to radiotherapy would be clinically useful in determining optimal management. Here, we investigate the prognostic significance of tumour expression of carbonic anhydrase 9 (CA9), hypoxia inducible factor 1-α (HIF-1α), HPV status and clinicopathological features in a homogeneous series of patients with oropharyngeal squamous cell carcinoma.

Material and Methods: From 1999-2005, 279 patients were identified who underwent radiotherapy for oropharyngeal cancer. Pretreatment biopsy samples were available for 167 patients. Immunohistochemistry was performed on formalin fixed, paraffin embedded material for CA9, HIF-1α and *CDKN2A*. PCR was performed to identify the presence of HPV 16.

Results: Features associated with a poor locoregional control were older age ($p=0.002$), tongue base subsite ($p=0.002$), heavy alcohol use ($p=0.004$), heavy smoker ($p=0.0002$), low Hb level ($p=0.001$), advancing T ($p<0.0001$), N ($p=0.001$) and AJC ($p=0.001$) stage, high CA9 expression ($p=0.020$) and high HIF-1α expression ($p<0.0001$). In multivariate analysis T stage ($p=0.003$) and high HIF-1α expression ($p=0.001$) remained significant.

Conclusion: This work highlights the prognostic significance of HIF-1α expression in a homogeneous group of oropharyngeal patients treated with primary radiotherapy.

## 3.2 Introduction

Head and neck cancer is a relatively common malignancy, accounting for 5% of all new cancers diagnosed internationally (Ferlay et al., 2004). Around 40% of patients that present with head and neck cancer have advanced disease at presentation, commonly involving regional lymph nodes. Distant metastasis is uncommon at presentation, arising in only about 10% of patients (Ries, 2006). Treatment decisions are made in the setting of a multi disciplinary team – tumour factors including primary tumour site, stage, resectability and patient factors including comormidities and age – are used to guide appropriate management. Most patients present with squamous cell carcinoma (HNSCC) and their management is complex: radiotherapy and surgery play a pivotal role, and more recently treatment has also incorporated systemic agents.

Treatment decisions are arguably more pertinent now as there have been significant advances in this field. For radiotherapy, advances in imaging of tumours and radiation delivery have changed management approaches. The role of systemic agents has evolved from use only for palliation to being a key component for the potentially curative treatment of locally advanced HNSCC. Concurrent chemoradiotherapy now offers an organ preserving treatment for advanced malignancy. More recently, molecularly targeted agents have been investigated in the head and neck setting. Cetuximab is the first molecularly targeted agent that has been introduced into standard practice (Karamouzis et al., 2007). Surgery is frequently limited by the anatomical extent of the tumour and desire to preserve organ function, but recent advances have made surgery a more feasible option. Advances in both microsurgical free tissue transfers allowing reconstruction of surgical defects and advances in conservative transoral laser surgery make surgery an option for early HNSCC.

As mentioned above, management decisions are based on patient and tumour factors. Patients with advanced malignancy (stage III/IV, accounting for up to 60% of patients) were traditionally treated with surgery followed by post-operative radiotherapy. More recently there has been a shift in thinking and these patients are now commonly managed with chemoradiotherapy. However, despite this change in management, only 50% of patients respond well to chemoradiotherapy suggesting that some of the patients should be offered surgery as the primary treatment modality rather than chemoradiotherapy. The difficulty is predicting which patients are likely to respond to a particular treatment, allowing treatment to be tailored to the individual patient. Biomarkers that allow prediction of management effectiveness are therefore crucial.

Two relevant factors for the development of biomarkers for head and neck cancer are hypoxia and human papilloma virus (HPV). Tumour hypoxia is a common feature of many solid tumours, and has been consistently demonstrated in HNSCC

(Nordsmark et al., 2005). Hypoxia plays a pivotal role in malignancy, influencing biological behaviour, response to treatment and prognosis. Hypoxic tumours are more resistant to killing by ionising radiotherapy and chemotherapy, more invasive and metastatic, more resistant to apoptosis and genetically unstable (Semenza, 2010b). Nevertheless, hypoxia may present an opportunity for selecting therapeutic approaches to management. Identification of HNSCC that are hypoxic might allow therapy to be targeted accordingly as there is evidence that hypoxic tumours benefit most from hypoxia-modifying therapy (Overgaard et al., 1998, Rischin et al., 2006). Hypoxia-inducible factor (HIF)-1α, the master transcriptional factor of the hypoxic response and carbonic anhydrase (CA) 9, one of its downstream target genes, are hypoxia-associated biomarkers. Their expression has been demonstrated to be associated with outcome in a number of malignancies. Regarding HPV, there is abundant evidence now that HPV 16 is an aetiological agent in up to one quarter of HNSCC, particularly oropharyngeal SCC. The aim of this paper was to examine the relationship between hypoxia and HPV status in a homogeneous series of oropharyngeal patients.

## 3.3 Materials and Methods

### 3.3.1 Patients

Ethical approval was obtained from the local research ethics committee (Reference Number: 03/TG/076), appendix IV. A cohort of oropharyngeal patients were identified through the radiotherapy database at the Christie Hospital, Manchester and were a consecutively treated series between January 1999 to December 2005. The demographic, clinico-pathologic and outcome patient data were collected retrospectively from the case notes and the Christie Head and Neck assessment form, see appendix V. Pre-treatment Hb levels were obtained from the case notes. Tumour blocks were requested from the referring hospitals in which the diagnostic biopsies were performed. Inclusion criteria for the study were patients with a had histologically confirmed squamous cell carcinoma of the posterior third of the tongue or the tonsil, patients treated at the Christie Hospital between January 1999 and December 2005, patients over the age of 16 years, patients who had radiotherapy as their primary treatment. As this is a historical cohort of patients, management evolved over the years. Of the 279 patients identified, 141 had radiotherapy only, 74 patients had radiotherapy and an ipsilateral neck dissection, 38 had chemoradiotherapy and 26 had chemoradiotherapy plus ipsilateral neck dissection. Radiotherapy was delivered using a 4-6 MeV photons linear accelerator. The radiotherapy beam arrangement used was a single phase anterior oblique pair of fields or a lateral parallel opposed pair of fields using fields sizes predominantly 5.5-7 cm long. The prescribed radiation dose for posterior tongue tumours was 50 Gy using lateral parallel pair radiation in 16 fractions

over three weeks. The prescribed radiation dose for tonsil tumours was ipsilateral therapy of 52.50 Gy in 16 fractions over three weeks. Exclusion criteria were patients treated palliatively; patients that received surgery as their primary treatment.

### 3.3.2 Immunohistochemistry

Immunohistochemical detection of HIF-1α was performed using the Tyramide Signal Amplification System (NEN Life Sciences, Boston), which is based on a streptavidin-biotin-horseradish peroxidase complex formation. Sections 4 μm thick were deparaffinised and antigen retrieval carried out by microwaving in 10 mmol/L citrate buffer for 20 min. The sections were rinsed, soaked in tris buffered saline (TBS) for 5 min, and immersed in TBS and 30% hydrogen peroxide. The sections were washed, soaked in TBS again, and TNB (Tris NaCl Blocking) reagent applied for 30 min. The mouse monoclonal IgG1 (immunoglobulins isotype 1) antibody (BD Biosciences 610958) in TNB was applied to the sections, with mouse IgG1 (Dako X0931) used as a negative control sections. Sections were incubated overnight at 4$^{o}$C. The secondary antibody, a biotinylated rabbit anti-mouse immunoglobulin (RAMBO) (Dako E0413) in TCP (Tryptone Casein Peptone), was applied for 30 min followed by SA-HRP (Streptavidin-Horse Radish Peroxidase) from the TSA (tyramide signal amplification) Biotin Kit in TCP for 30 min. Biotinyl tyramide amplification reagent was applied to the sections for 8 min, followed by SA-HRP in TNB for 30 min. TNT was used to wash the sections in between applications. DAB (3,3' diaminobenzidine tetrahydrochloride) chromogen was then applied to the sections for 5 min. Gill's haematoxylin was used as a counterstain, prior to dehydration and cover slipping (Kim et al., 2003). Batch-to-batch variation was assessed by choosing two sections showing high and low HIF-1α expression and running additional sections from these biopsies with each batch. (Aebersold et al., 2001).

Immunohistochemical detection of CA9 was performed using the Envision kit system. Sections 4 μm thick were deparaffinised and then washed in TBS for 5 min. A 30% hydrogen peroxide block was applied for 15 min followed by a 10% casein block for 15 min. The primary antibody, a mouse monoclonal anti-human antibody (M75) raised to the external domain of CA9, was a gift from Profs S. Pastorekova and J. Pastorek, Slovak Academy of Sciences, Bratislava, Slovak Republic. The CA9 antibody was applied at a 1:50 dilution for 30 min and mouse IgG 2B (immunoglobulins isotype 2B) (Dako 0944) was used as the negative control. A secondary antibody was applied to the sections for 30 min and after rinsing, DAB+ (3,3' diaminobenzidine tetrahydrochloride) was applied to each section for 5 min. Both the secondary antibody and the DAB+ were from the Envision kit. Gill's haematoxylin was used to counterstain the sections, which were then rinsed, dehydrated and coverslipped.

Immunohistochemical detection for *CDKN2A* was performed using CINtec histology kit (mtm laboratories, Germany). Sections 4 µm thick were deparaffinised and antigen retrieval carried out by microwaving in AR buffer solution for 25 min. After cooling for 20 min, the sections were rinsed and soaked in TBS for 5 min. An endogenous peroxidise block was applied for 5 min. Slides were then washed in water, and then washed in buffer for 5 min. A further block of 10% casein was applied for 10 min. The primary antibody was applied to sections (Mouse anti-human *CDKN2A* ink4a, clone E6H4), with mouse IgG1 (Dako X0931) used for the negative control, and incubated for 30 min. Visualisation reagent was appplied (goat anti mouse polymer HRP conjugated) and slides incubated for 30 min. The sections were washed in buffer and DAB applied for 5 min. Gill's haematoxylin was used as a counterstain prior to dehydration and cover slipping.

### 3.3.3 HPV genotyping

Approximately 6 x 10 µm thick sections from each tissue block were placed in a 1.6 ml Eppendorf tube. To prevent cross contamination, the microtome blade was cleaned with ethanol between blocks. Tissue sections were sent to the Virology Laboratory, Central Manchester University Hospitals NHS Foundation Trust for HPV detection. Tissue sections were de-waxed by adding 1ml of octane to each Eppendorf tube followed by 75 µl of methanol. Tubes were vortexed, incubated at 56˚C for 30 minutes prior to centrifugation at 13,000 rpm for 1 min after which the octane layer was removed using a fine-tipped Pasteur pipette. The tissue pellet was then washed with 1.0 ml of ethanol, centrifuged as before and the ethanol removed. The tubes were then left at 56˚C for 30-45 min to evaporate off residual ethanol. The tissue was digested in 600-1000 µl (depending on size of pellet) of proteinase K lysis buffer containing 10 mM Tris/HCl buffer pH 8.3; 1 mM EDTA, 0.5% Triton X-100, 0.002% SDS and 250 µg/ml of proteinase K. Tissue digestion was carried out at 56˚C for 72 hours with constant agitation. DNA was then extracted from a 200 µl aliquot of digested tissue using the Roche MagNA.

Pure automated extraction system involved an elution volume of 100 µl. A 50 µl aliquot of the extracted DNA was used for the PCR reaction stage of the Roche LA test. The Roche LA test was used according to manufacturer's instructions. Individual oligonucleotide capture probes enabled identification of 37 high-risk (HR) and low-risk (LR) HPV genotypes. In addition, the assay amplifies a portion of the β-globin gene which acts as a control for cell adequacy, extraction and amplification. HPV positive and negative controls provided within the Roche LA test kit were included for every 46 tissue section samples tested.

### 3.3.4 Scoring Method

The presence of SCC was confirmed by a senior pathologist (Dr Rachel Hall, Consultant Pathologist). For CA9 the scoring system was as follows: 0, less than 1% plasma membrane staining; 1, 1-9% plasma membrane staining; 2, 10-30% plasma membrane staining; 3, greater than 30% plasma membrane scoring. For analysis the scoring was further sub divided into low expression ( 0-9%) and high expression (10->30%) (see fig 3.1). These are similar scoring systems used by previous authors (Loncaster et al., 2001) . The scoring system for HIF 1-α was as follows: 0, no nuclear staining; 1, less than 10% nuclear staining; 2, 10% to 29% nuclear staining; 3, 30% or greater nuclear staining (see Fig 3.2). This is a similar scoring to previous authors (Condon et al., 2002). The scoring system for *CDKN2A* was as follows: it was regarded as overexpression if there was strong and diffuse staining (>80% of tumor cells) and was regarded as nonoverexpression if staining was absent or weak (Weinberger et al., 2004) (see Fig 3.3). All scoring was blinded to outcome and performed independently by two scorers and repeated with resolution of any conflicting scores by independent re-scoring followed by discussion and consensus.

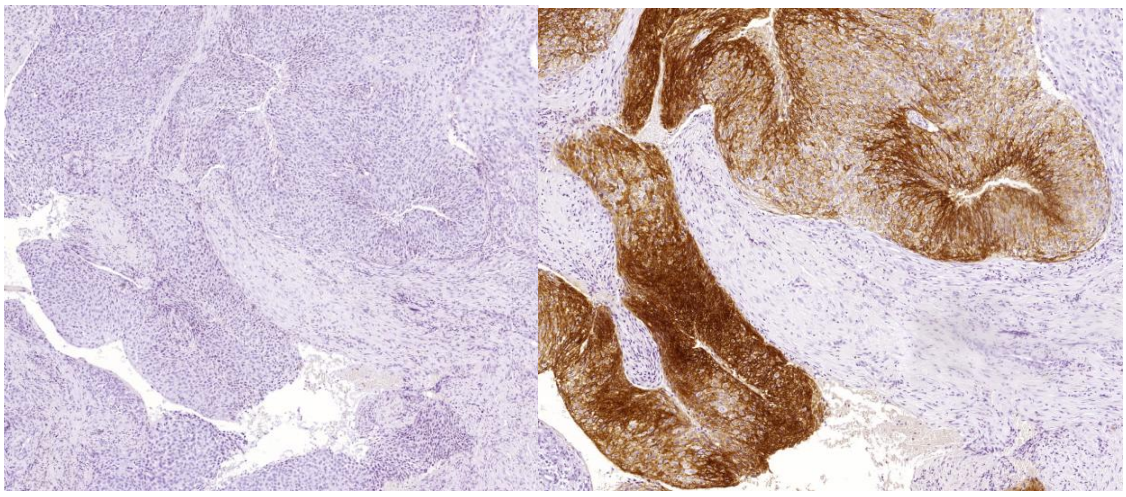


Figure 3-1Oropharyngeal biopsy demonstrating negative control for CA9 (left) and high CA9 expression (right)

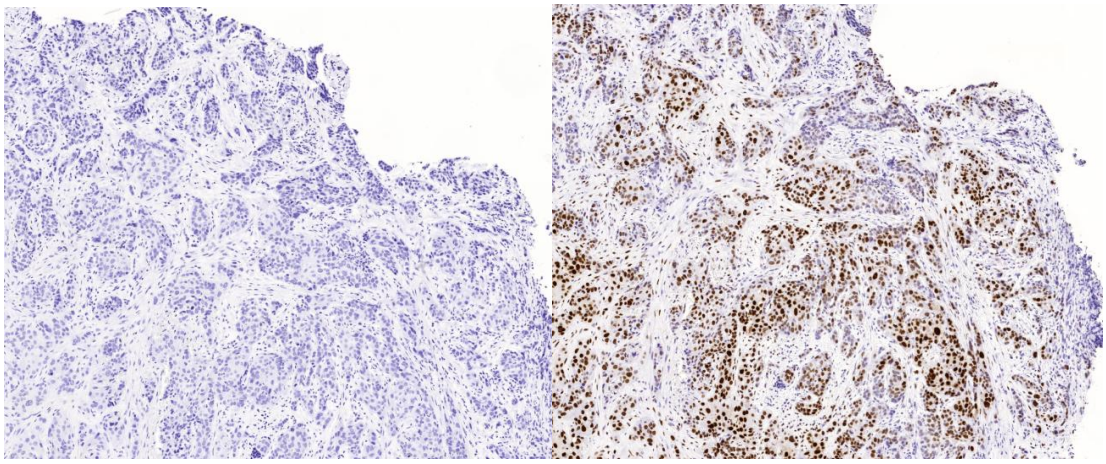


Figure 3-2 Oropharyngeal biopsy demonstrating the negative control for HIF-1α (left) and high HIF-1α expression (right)

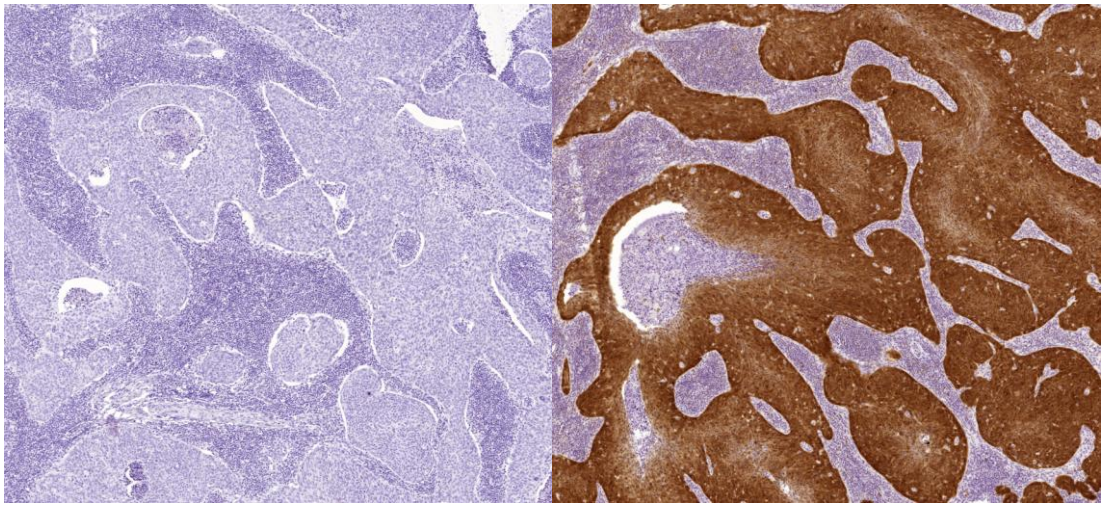


Figure 3-3 Oropharyngeal biopsy demonstrating the negative control for *CDKN2A* expression (left) and positive *CDKN2A* expression (right)

### 3.3.5 Statistical analysis

Actuarial estimations of recurrence, locoregional control and cancer specific survival were obtained using the Kaplan-Meier method. The survival curves were compared in univariate analysis using the logrank method. Analysis was carried out on all patients, and for tonsil and tongue subgroups. The variables analysed for prognostic significance were: smoking history, alcohol history, site, gender, age, pre-treatment Hb level, stage, tumour HIF-1α expression, tumour CA9 expression, tumour *CDKN2A* expression, tumour HPV 16 status.

## 3.4 Results

### 3.4.1 Patient characteristics

The characteristics of the 279 patients are summarised in Table 3.1, see appendix VII. The median duration of follow up was 3.84 (range 0.33-7.99) years. For 15 patients, Hb scores were unavailable.

### 3.4.2 Univariate analysis

The 5 year locoregional control, cancer specific survival and overall survival for the 279 patients were 48%, 52% and 42% respectively. Factors that were associated with a poor locoregional control in all oropharyngeal patients were: age less than 70 years (p = 0.001); tongue base subsite (p = 0.007, Figure 3.1); alcohol intake (p = 0.006); low advancing T (p = <0.0001), N (p=<0.0001) and AJC stage (p = 0.020); high CA9 (p = 0.031) and HIF-1α (p = <0.0001, Figure 3.2), Hb (p = 0.001) and smoking status (p = 0.014) (Figure 3.3).. Adverse prognostic factors for cancer specific survival were age less than 70 years (p=<0.0001), tongue base subsite (p=0.001), smoking status (p=0.009), low Hb (p=<0.0001), advancing T (p=<0.0001), N (p=0.001) and AJC stage (p=0.001), high CA9 expression (p=0.015), high HIF-1α expression (p<0.0001) and positive *CDKN2A* (p=0.0004) (Table 3.2).

Table 3-1 Clinical characteristics of oropharyngeal patients

| **Feature** | | **Number of patients (%)** |
|---|---|---|
| Gender | Male | 215 (77) |
| | Female | 64 (23) |
| Age (y) | Median (range) | 59 (28-92) |
| Smoking Status | No | 42 (16) |
| | Ex | 69 (26) |
| | Current | 152 (58) |
| Alcohol | None | 22 (9) |
| | Low | 101(40) |
| | Medium | 31 (12) |
| | Heavy | 99 (39) |
| Site | Tongue base | 109 (39) |
| | Tonsil | 170 (61) |
| Hb | Median (range) | 13.2 (7.5-16.4) |
| T Stage | T1 | 54 (19) |
| | T2 | 119 (43) |
| | T3 | 44 (16) |
| | T4 | 62 (22) |
| N Stage | N0 | 84 (30) |
| | N1 | 55 (20) |
| | N2 | 122 (54) |
| | N3 | 18 (6) |
| AJC Stage | I | 12 (4) |
| | II | 43 (16) |
| | III | 53 (19) |
| | IV | 171(61) |
| CA9 expression | Low | 118 (86) |
| | High | 19 (14) |
| HIF-1α expression | Absent | 28 (17) |
| | Weak | 70(42) |
| | Moderate | 46 (27) |
| | High | 23 (14) |
| *CDKN2A* | Positive | 64 (61) |
| | Negative | 41 (39) |
| PCR HPV 16 status | Positive | 63 (61) |
| | Negative | 41(39) |

Abbreviations: Hb=haemoglobin; CA9=carbonic anhydrase 9; HIF-1α = hypoxia inducible factor-1α;.

### 3.4.3 Subsite analysis

Table 3.3 shows the univariate analysis for tongue base and tonsil subsites. There was a significant difference in outcome for tongue base compared to tonsil tumours, with 5 year locoregional control rates of 39% vs 54% (p=0.002) and 5 year cancer specific survival rates of 38% vs 60% (p=0.001). T stage, N stage and HIF-1α expression were all significant for both locoregional control and cancer specific survival in both groups (Table 3.4). In the tongue base tumour subgroup, there was a significant association between smoking and locoregional control (p=0.016) and cancer specific survival (p=0.025), which was not seen in the tonsil tumour subgroup. Low Hb had prognostic significance for tonsil tumour patients, for both locoregional control (p=0.002) and cancer specific survival (p=0.001), although this association was not seen for the tongue base tumour subgroup. *CDKN2A* expression was also significant for the tonsil tumour subgroup for both locoregional control (p=0.012) and cancer specific survival (p=0.008), although this was not significant for the tongue base subgroup.

Table 3-2 Treatment outcome analyses for oropharyngeal patients

| **Characteristic** | **All recurrence HR (95%CI)** | **P** | **LRC HR(95%CI)** | **p** | **CSS HR (95%CI)** | **p** |
|---|---|---|---|---|---|---|
| Age | | | | | | |
| 70+ | 1 | | 1 | | 1 | |
| ≤ 50 | 2.02 (1.18-3.46) | | 2.87 (1.43-5.76) | | 2.27 (1.23 – 4.20) | |
| 51-69 | 3.19 (1.68-5.78) | .001 | 4.14 (1.89-9.07) | 0.002 | 4.36 (2.21 – 8.61 ) | <0.0001 |
| Age | | | | | | |
| <59 | 1 | | 1 | | 1 | |
| ≥59 | 1.36 (0.96-1.93) | 0.08 | 1.38 (0.93-2.064) | 0.114 | 1.55(1.06 – 2.26) | 0.021 |
| Sex | | | | | | |
| Female | 1 | | 1 | | 1 | |
| Male | 0.80 (0.52-1.24) | .32 | 1.035 (0.65-1.65) | 0.887 | .81(0.50 – 1.29) | 0.38 |
| Site | | | | | | |
| Tongue | 1 | | 1 | | 1 | |
| Tonsil | 0.61 (0.43-0.87) | 0.007 | 0.52 (0.35-0.79) | 0.002 | 0.52(0.35-0.75) | 0.001 |
| Alcohol | | | | | | |
| No | 1 | | 1 | | 1 | |
| Low | 0.50 (0.25-0.97) | | 0.39 (0.19-0.81) | | 0.54(0.26-1.11) | |
| Mod | 1.27 (0.63-2.58) | | 1.13 (0.53-2.39) | | 1.04(0.47-2.30) | |
| Heavy | 0.83 (0.44-1.57) | 0.006 | 0.62 (0.31-1.24) | 0.004 | 0.89(0.44-1.77) | 0.092 |
| Smoking | | | | | | |
| No | 1 | | 1 | | 1 | |
| Ex | 0.91 (0.48-1.72) | | 0.24 (0.27-1.44) | | 1.23(0.59 – 2.56) | |
| Current | 1.67 (0.97-2.87) | 0.014 | 2.08 (1.09-3.95) | 0.0002 | 2.19(1.15-4.15) | 0.009 |
| Hb | | | | | | |
| <13 | 1 | | 1 | | 1 | |
| ≥13 | 0.53 (0.37-0.77) | 0.001 | 0.51 (0.34-0.76) | 0.001 | 0.46 (0.33– 0.67) | <0.0001 |
| T Stage | | | | | | |
| T1 | 1 | | 1 | | 1 | |
| T2 | 1.47 (0.80-2.70) | | 1.42 (0.71-2.81) | | 1.763 (0.87-3.56) | |
| T3 | 3.46 (1.81-6.62) | | 2.49 (1.15-5.39) | | 4.28 (2.034–9.04) | |
| T4 | 4.73 (2.58-8.69) | <0.0001 | 4.99 (2.54-9.84) | <0.0001 | 6.68 (3.33-13.39) | <0.0001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| N Stage | | | | | | |
| N0 | 1 | | 1 | | 1 | |
| N1 | 0.98 (0.58-1.67) | | 0.87 (0.47-1.61) | | 1.094 (0.60-1.97) | |
| N2 | 1.19 (0.78-1.83) | | 1.13 (0.69-1.83) | | 1.51 (0.95-2.43) | |
| N3 | 3.65 (1.98-6.72) | <0.0001 | 3.55 (1.79-7.03) | 0.001 | 3.65 (1.87-7.12) | 0.001 |
| AJC Stage | | | | | | |
| 1 | 1 | | 1 | | 1 | |
| 2 | 0.98 0.5811.67) | | 1.99 (0.45-8.89) | | 3.26 (0.42-25.46) | |
| 3 | 1.19(0.78-1.83) | | 1.80 (0.41-7.94) | | 4.14 (0.55-31.24) | |
| 4 | 4.44(1.09-18.06) | 0.020 | 3.55 (1.79-7.03) | 0.001 | 8.36 (1.16-60.08) | 0.001 |
| CA9 | | | | | | |
| Low (<30) | 1 | | 1 | | 1 | |
| High (≥ 30 ) | 2.02(1.07-3.85) | 0.031 | 2.45 (1.15-5.23) | 0.020 | 2.21 (1.15-3.69) | 0.015 |
| HIF-1α 1 | | | | | | |
| Absent | 1 | | 1 | | 1 | |
| Weak | 1.39 (0.64-3.06) | | 2.51 (0.74-8.52) | | 1.47 (0.63-3.39) | |
| Moderate | 1.99 (0.88-4.53) | | 4.75 (1.39-16.23) | | 1.89 (0.79-4.58) | |
| High | 9.52 (4.16-21.80) | <0.0001 | 21.86 (6.35-75.18) | <0.0001 | 7.98 (3.31-19.20) | <0.0001 |
| HIF-1α 2 | | | | | | |
| Low (<30) | 1 | | 1 | | 1 | |
| High(≥30) | 6.45 (3.82-10.89) | <0.0001 | 7.83 (4.36-14.08) | <0.0001 | 5.36 (3.10-9.27) | <0.0001 |
| HPV (PCR) | | | | | | |
| Pos | 1 | | 1 | | 1 | |
| Neg | 0.63 (0.33-1.21) | 0.17 | 0.55 (0.24-1.27) | 0.16 | 0.56 (0.28-1.11) | 0.093 |
| *CDKN2A* | | | | | | |
| Pos | 1 | | 1 | | 1 | |
| Neg | 2.55 (1.31-4.96) | 0.006 | 2.16 (0.94-5.01) | 0.07 | 2.72 (1.36-5.41) | 0.004 |

Abbreviations: Hb=haemoglobin; CA9=carbonic anhydrase 9; HIF-1α = hypoxia inducible factor-1α;.

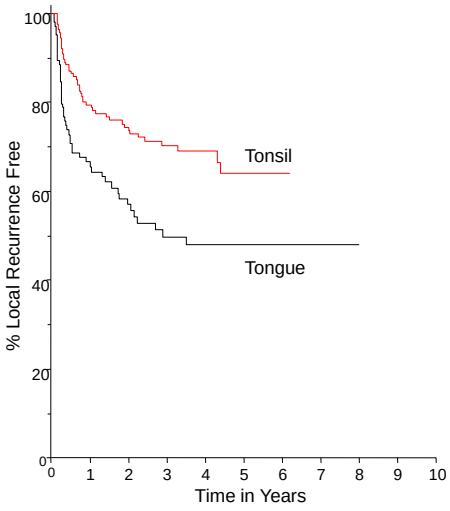



Figure 3-4 Kaplan-Meier survival curves showing the relationship between subsite and locoregional control.

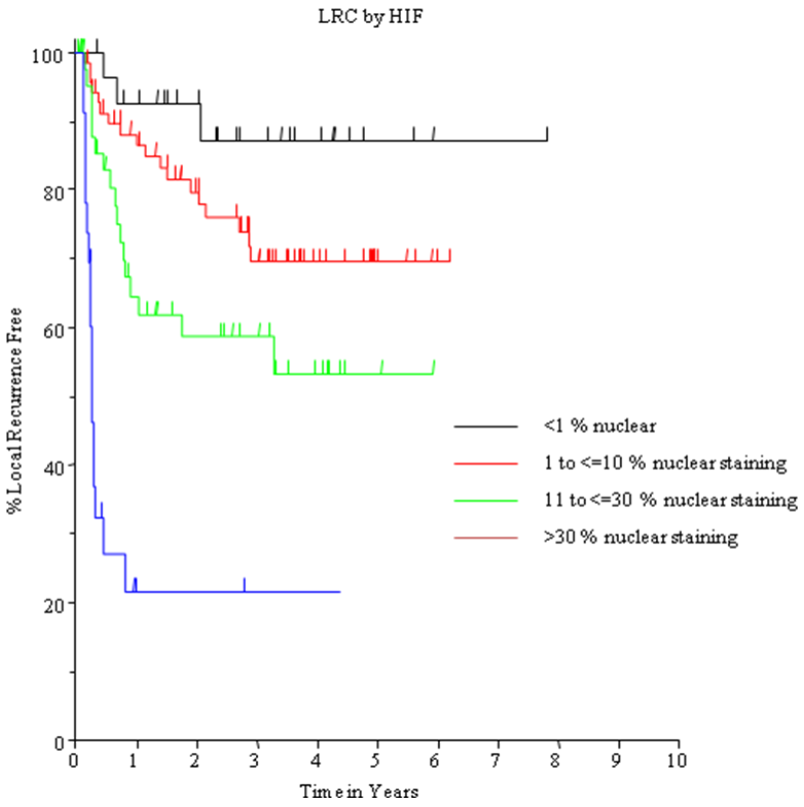



Figure 3-5 Kaplan-Meier survival curves showing the relationship between HIF-1α expression and locoregional control.

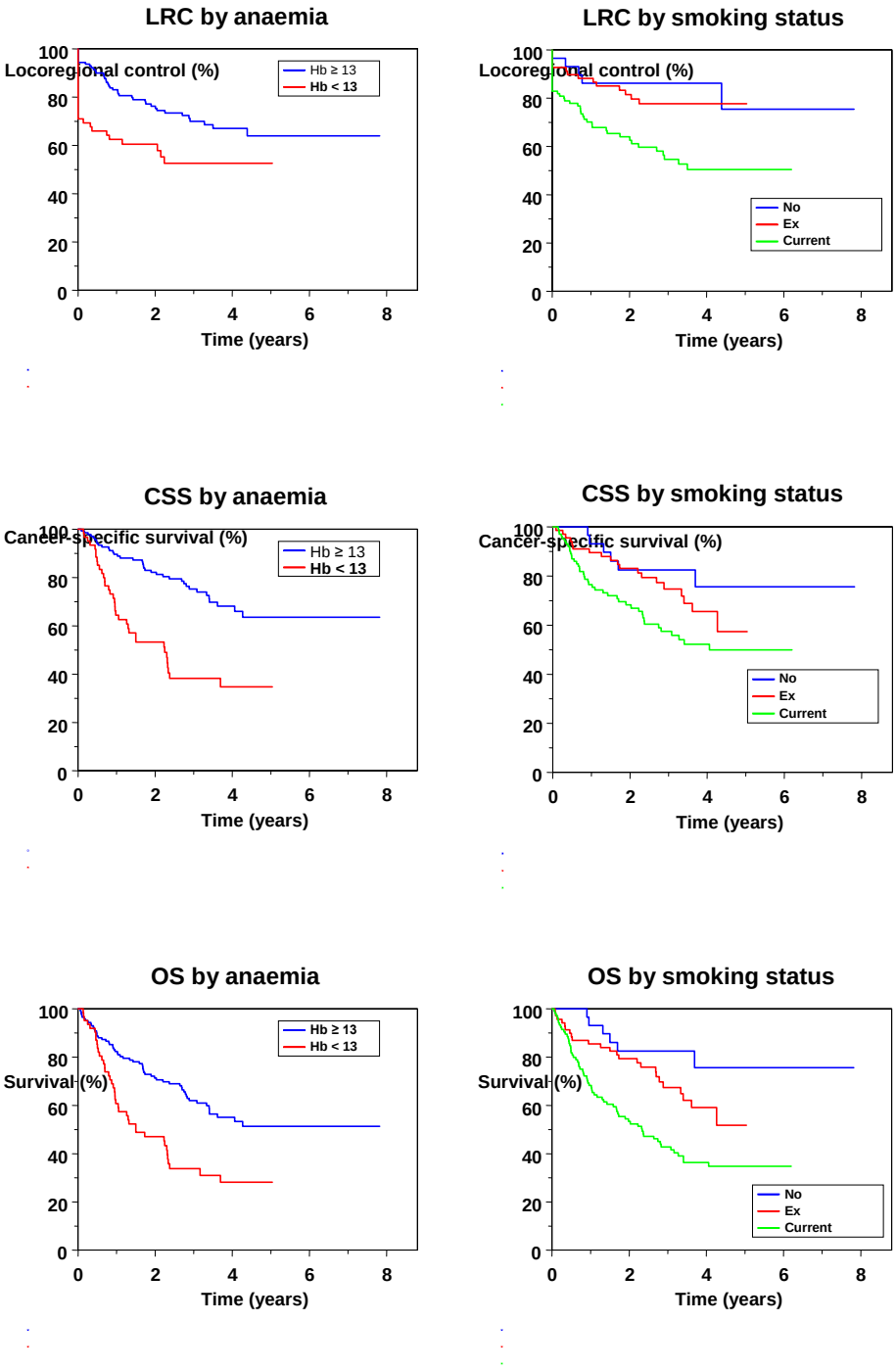



Figure 3-6 Kaplan-Meier curves showing the relationships of anaemia and smoking status with survival in oropharygeal cancer

Table 3-3 Outcome analysis for tongue base and tonsil subgroups

| Characteristic | Tonguebase LRC HR (95%CI) | p | CSS HR (95%CI) | p | Tonsil LRC HR (95%CI) | p | CSS HR (95%CI) | p |
|---|---|---|---|---|---|---|---|---|
| Age | | | | | | | | |
| 70+ | 1 | | 1 | | 1 | | 1 | |
| <50 | 3.26 (1.28-8.32) | | 3.10 (1.21-7.94) | | 1.51 (0.78-2.93) | | 1.82 (0.81-4.11) | |
| 51-70 | 4.27(1.54-11.80) | 0.019 | 4.34 (1.57-12.01) | 0.018 | 2.51 (1.12-5.63) | 0.077 | 4.11 (1.63-10.36) | 0.006 |
| Age | | | | | | | | |
| <59 | 1 | | 1 | | 1 | | 1 | |
| ≥59 | 1.40 (0.83-2.36) | 0.208 | 1.51 (0.88-2.61) | 0.130 | 1.22 (0.77-1.97) | 0.418 | 1.39 (0.82-2.38) | 0.215 |
| Sex | | | | | | | | |
| Female | 1 | | 1 | | 1 | | 1 | |
| Male | 0.89 (0.47-1.73) | 0.749 | 0.94 (0.48-1.83) | 0.863 | 0.78 (0.43-1.41) | 0.416 | 0.76 (0.39-1.47) | 0.422 |
| Alcohol | | | | | | | | |
| No | 1 | | 1 | | 1 | | 1 | |
| Low | 0.62 (0.23-1.68) | | 0.60 (0.22-1.64) | | 0.40 (0.16-0.98) | | 0.49 (0.17-1.38) | |
| Mod | 1.42 (0.48-4.18) | | 1.81 (0.461-4.13) | | 1.16 (0.45-2.95) | | 0.87 (0.27-2.74) | |
| Heavy | 0.82 (0.31-2.19) | 0.325 | 0.86 (0.32-2.30) | 0.348 | 0.82 (0.35-1.92) | 0.031 | 0.94 (0.35-2.49) | 0.283 |
| Smoking | | | | | | | | |
| No | 1 | | 1 | | 1 | | 1 | |
| Ex | 0.48 (0.12-1.91) | | 0.47 (0.11-1.90) | | 1.09 (0.51-2.34) | | 2.02 (0.75-5.45) | |
| Current | 2.08(0.88-4.90) | 0.016 | 1.95 (0.83-4.61) | 0.025 | 1.27 (0.62-2.60) | 0.752 | 2.13 (0.81-5.58) | 0.294 |
| Hb | | | | | | | | |
| ≥13 | 1 | | 1 | | 1 | | 1 | |
| <13 | 0.67 (0.40-1.14) | 0.339 | 0.61 (0.36-1.05) | 0.212 | 0.46 (0.28-0.76) | 0.002 | 0.38 (0.22-0.66) | 0.001 |
| T Stage | | | | | | | | |
| T1 | 1 | | 1 | | 1 | | 1 | |
| T2 | 0.78 (0.29-2.11) | | 0.83 (0.31-2.24) | | 2.13 (0.94-4.82) | | 3.97 (1.20-13.13) | |
| T3 | 2.66 (1.10-6.43) | | 2.15 (0.86-5.33) | | 3.47 (1.31-9.16) | | 7.86 (2.11-29.20) | |
| T4 | 2.65(1.14-6.15) | 0.002 | 2.74 (1.18-6.37) | 0.006 | 7.02 (2.91-16.96) | 0.000 | 16.23 (4.71-55.88) | 0.000 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| N Stage | | | | | | | | |
| N0 | 1 | | 1 | | 1 | | 1 | |
| N1 | 1.35 (0.61-2.96) | | 1.69 (0.74-3.84) | | 0.76 (0.36-1.58) | | 0.70 (0.28-1.72) | |
| N2 | 1.49 (0.76-2.90) | | 1.79 (0.88-3.64) | | 0.99 (0.56-1.76) | | 1.31 (0.69-2.47) | |
| N3 | 8.55(3.07-23.80) | 0.000 | 6.94(2.48-19.41) | 0.003 | 2.79(1.28-6.11) | 0.021 | 2.83(1.15-6.96) | 0.051 |
| AJC Stage | | | | | | | | |
| 1 | 1 | | 1 | | 1 | | 1 | |
| 2 | 0.6 9(0.04-11.13) | | 0.71 (0.04-11.39) | | 3.69 (0.48-28.11) | | 3.25 (0.41-25.46) | |
| 3 | 2.98 (0.38-23.17) | | 2.44 (0.31-19.34) | | 2.70 (0.30-21.62) | | 4.14 (0.54-31.24) | |
| 4 | 3.87(0.53-28.16) | 0.181 | 3.92 (0.54-28.56) | 0.122 | 4.70 (0.64-34.18) | 0.210 | 8.35 (1.16-60.08) | 0.001 |
| CA9 | | | | | | | | |
| Low (<30) | 1 | | 1 | | 1 | | 1 | |
| High (≥ 30 ) | 2.20(0.82-5.88) | 0.173 | 1.81 (0.68-4.84) | 0.348 | 1.76 (0.73-4.23) | 0.204 | 2.31 (0.94-5.66) | 0.067 |
| HIF-1α | | | | | | | | |
| Low (<30) | 1 | | 1 | | 1 | | 1 | |
| High(≥30) | 8.11(3.42-19.23) | 0.000 | 7.21 (3.00-17.34) | 0.000 | 5.84 (3.01-11.34) | 0.000 | 4.58 (2.24-9.36) | 0.000 |
| HPV (PCR) | | | | | | | | |
| Pos | 1 | | 1 | | 1 | | 1 | |
| Neg | 0.28 (0.81-1.02) | 0.055 | 0.25 (0.07-0.93) | 0.039 | 0.85 (0.39-1.85) | 0.685 | 0.73 (0.32-1.67) | 0.463 |
| *CDKN2A* | | | | | | | | |
| Pos | 1 | | 1 | | 1 | | 1 | |
| Neg | 1.78 (0.57-5.53) | 0.319 | 1.88 (0.60-5.87) | 0.276 | 2.87 (11.25-6.56) | 0.012 | 3.27 (1.36-7.86) | 0.008 |

Abbreviations: Hb=haemoglobin; CA9=carbonic anhydrase 9; HIF-1α = hypoxia inducible factor-1α;.

### 3.4.4 HIF-1α Expression

Tissue blocks suitable for HIF-1α analysis were obtained for 167 of the 279 patients; this discrepancy represents logistical difficulties in obtaining the original biopsy material for all patients. Expression of HIF-1α was high in 23 patients, moderate in 46 patients, weak in 70 patients and absent in 28 patients. Expression was predominantly nuclear, with variable cytoplasmic staining. HIF-1α was a highly significant factor for recurrence (p=<0.0001), lococregional control (p<0.0001; Figure 4.2) and cancer specific survival (p<0.0001). As the HIF-1α curves split rougly into two groups, low (absent, weak and moderate; n = 144) and high (high; n=23) HIF-1α was used for further analyses. HIF-1α expression was not associated with any of the following patient characteristics: age (Mann-Whitney (MW) p=0.18); gender (MW p=0.99); subsite (Chi-squared (CS) p=0.96); alcohol consumption (CS p=0.32) or smoking status (Fishers exact test (FE) p=0.48). HIF-1α expression was not associated with any of the following markers: CA9 (CS p=0.45); HPV 16 (CS p=0.43) and there was no correlation between HIF-1α and CA9 (Spearman's *r* =0.152, p=0.089). There was an association between high HIF-1α expression and postive *CDKN2A* (CS p=0.010). There was also a significant association between increased HIF-1α expression and advancing T stage (CS p<0.0001), advancing N stage (CS p<0.0001) and decreased Hb (MW p=0.003). There was a significant inverse correlation between HIF-1α and Hb (Spearman's *r* =-0.241, p=0.002).

### 3.4.5 *CDKN2A*

Tissue blocks suitable for *CDKN2A* analysis were obtained for 105 of the 279 patients; this discrepancy represents logistical difficulties in obtaining the original biopsy material for all patients. Expression was positive in 64 patients and negative in 41 patients. *CDKN2A* expresion was highly significant for recurrence (p=0.006) and cancer specific survival (p=0.004), but not locoregional control (p=0.07). *CDKN2A* expression was not associated with age (MW p=0.179), T stage (MW p=0.057), AJC stage (MW p=0.193), site (CS
p=0.694) and Hb (MW p=0.074), however there was an association with low alcohol consumption (CS p=0.033) and non smokers (CS p=0.005). There was no association between *CDKN2A* and CA9 expression (CS p=0.30), but there was an association with HIF-1α (CS p=0.010).

### 3.4.6 Multivariate Analysis

The Cox-proportional hazard model was used to study the clinical variables of sex, alcohol and smoking status, subgroup, T, N and AJC stage, pretreatment Hb, alcohol and smoking status, and tumour HIF-1α, CA9, *CDKN2A* expression and HPV status in relation to locoregional control and cancer specific survival. T stage, HIF-1α and

*CDKN2A* remained a significant independant prognostic factor for cancer specific survival, but only T stage and HIF-1α remained significant for locoregional control (Table 4.4).

Table 3-4 Multivariate cox regression analysis for oropharyngeal patients

| **Characteristic** | **LRC HR (95%CI)** | **p** | **CSS HR (95%CI)** | **p** |
|---|---|---|---|---|
| T Stage | | | | |
| 1 | | | | |
| 2 | 1.13 (0.22-5.96) | | 1.57 (0.42-5.87) | |
| 3 | 2.01 (0.28-14.34) | | 4.71 (1.16-19.05) | |
| 4 | 7.91 (1.60-38.95) | 0.003 | 5.67 (1.45-22.15) | 0.010 |
| HIF-1α | | | | |
| <30 | | | | |
| ≥30 | 9.76(2.68-35.61) | 0.001 | 3.48 (1.08-11.15) | 0.036 |
| *CDKN2A* | | | | |
| Pos | | | | |
| Neg | | NS | 2.29 (1.00-5.26) | 0.05 |

## 3.5 Discussion

Many clinicopathological variables and molecular markers that are of prognostic value have been identified in HNSCC but wide heterogeneity in clinical outcomes is still seen. The ability to predict the probability of successful treatment would allow for more individualised treatment in the hope of reducing toxic side effects and treatment failure. Although HNSCC has been extensively researched for the optimum treatment regimens and the ability to predict a successful response to treatment, there is an unmet need for identification of biomarkers that will guide treatment decisions. The aim of this series was to establish if certain markers were related to outcome in a homogeneous group of oropharyngeal patients, investigating in particular the hypoxic markers HIF-1α and CA9 and markers for HPV status HPV PCR and *CDKN2A*. At the time of doing this research, *CDKN2A* positivity was an acceptable surrogate marker for HPV positivity. However, more recently it has been established that *CDKN2A* positivity alone is not sufficient to establish HPV positivity. Now the gold standard for HPV detection is *CDKN2A* positivity plus positive insitu hybridisation for HPV 16 (Robinson et al., 2010, Shi et al., 2009).

In this series of patients, age, alcohol intake, smoking status and stage all had prognostic significance, consistent with other publications (Bhattacharyya, 2003, Marron et al., 2010, Greene, 2007). In this series, tonsil tumour patients had a significantly better outcome compared to tongue base tumour subsite, consistent with other reported series (Pedruzzi et al., 2008, Silva et al., 2008). Low Hb was also

associated with a worse outcome, particularly in the tonsil tumour subset of patients. The prognostic significance of anaemia has been widely reported in HNSCC (Fortin et al., 2008, McCloskey et al., 2009). The means by which low Hb influences locoregional and cancer specific survival is not clearly understood. The importance of pre-treatment Hb on prognosis was initially identified in 1965 in a group of cervical cancer patients (Evans and Bergsjo, 1965). Since then, numberous studies have confirmed its prognostic significance in HNSCC (Fortin et al., 2008, McCloskey et al., 2009, Stadler et al., 2006). It is not clear whether low Hb levels are associated with poor survival because they indicate advanced disease or because they indicate poor tumour oxygenation. In this series low Hb was associated with both poor locoregional control (p=0.001) and cancer specific survival (p=<0.0001).

When broken down by subsite, Hb remained significant for tonsil tumours, for both locoregional control (p=0.002) and cancer specific survival (p=0.001), but not for the tongue base tumours, implying that these two subsites are biologically different tumours. This is further supported by the fact that positive *CDKN2A* expression was significant for locoregional control (p=0.012) and cancer specific survival (p=0.008) in the tonsil tumours, but was not significant in the tongue base tumours. Becker et al looked at the relationship between Hb concentration and the pre-treatment tumour oxygenation status in a series of head and neck cancer patients. He showed that low Hb concentration was a predictor for decreased local control following radiotherapy. He also demonstrated a link between low Hb level and low tumour oxygenation (p<0.0001) suggesting that the association between Hb and outcome is mediated through tumour hypoxia (Becker et al., 2000). However Nordsmark, in a study of 357 head and neck cancer patients found no association between Hb and oxygenation levels (Nordsmark et al., 2005). Nordsmark also demonstrated that low Hb was an adverse prognostic factor independent of tumour hypoxia (Nordsmark and Overgaard, 2004). We found a significant association between low Hb and high HIF-1α expression (p=0.003), consistent with Silva et al (Silva et al., 2008). This shows that although there may be a relationship between low Hb and hypoxia, if is far more complex than simply low Hb equals hypoxic tumour. It is well known that anaemia occurs in chronic disease and the correlation between low Hb and poor prognosis may reflect the poor general condition of the patient when entering treatment. This study was retrospective and so investigation into the cause of anaemia in the patients studied was not possible, but this relationship must be taken into account when interpreting survival results.

Smoking also emerged as a significant prognostic factor in the univariate analysis for both locoregional failure (p=0.0002) and cancer-specific survival (p=0.009). When broken down by subsite, smoking was only significant for tongue base tumours (LRC

p=0.016, CSS p=0.025), not tonsil tumours, suggesting that some other aetiological factors may be involved. In a study investigating an association between smoking and tumour oxygenation, 116 out of 133 patients were smokers and the median $pO_2$ in tumours tended to be lower in smokers than in non-smokers (Becker et al., 2000). Smokers inhale small amounts of carbon monoxide which reacts with Hb to form carboxyHb (CO-Hb). Overgaard et al. demonstrated in head and neck cancer patients that for the range of 0-12% CO-Hb the oxygen utilisation decreased from 70% to 52% (Overgaard et al., 1992). This means a relative reduction of 25% of the amount of oxygen available to the tumour which could interfere with the oxygen-dependent effects of radiation. These results show that that the pre-treatment correction of low Hb and the maintenance of a sufficient Hb level during treatment, supported by smoking abstinence could be a meaningful way to improve outcome.

High CA9 expression was a significant prognostic factor in univariate analysis for locoregional control (p=0.020) and cancer specific survival (p=0.015), but not in multivariate analysis. CA9 has been studied extensively as a potential marker of tumour hypoxia and its role as a prognostic marker for radiotherapy outcome has produced contradictory results. Some studies have shown high tumour CA9 is associated with a poor outcome (Eriksen and Overgaard, 2007), others a favourable outcome (De Schutter et al., 2005b, Koukourakis et al., 2006). When the analysis was further down broken down by subsite the prognostic significance of CA9 was lost. It has been well demonstrated that CA9 is induced by hypoxia and growing evidence points to a fundamental role for hypoxia in promoting metastatic progression.

In contrast to our finding in laryngeal cancer where CA9 was a better prognostic marker than HIF-1α (Chapter 3), in oropharyngeal cancer HIF-1α appeared to be superior to CA9. Winter et al reported similar results, demonstrating that HIF-1α, and not CA9 expression correlated with outcome in a series of HNSCC patients (Winter et al., 2006). HIF-1α has been studied widely as a potential marker of tumour hypoxia. A number of studies show high HIF-1α expression is associated with poor locoregional control and survival in HNSCC (Hui et al., 2002). Our results show high HIF-1α expression to be significant for locoregional control and cancer specific survival in both univariate and multivariate analysis (Table 4.2), in agreement with several other published series (Bache et al., 2006, Koukourakis et al., 2008, Eckert et al., 2010). Novel therapies are currently being investigated to target HIF-1α and HIF-1α expression might have a role in stratifying patients for molecularly targeted treatment (Semenza, 2010a). Further work is required to explore whether tumour HIF-1α expression might predict benefit from a particular intervention. The now well documented association between HPV positivity and good outcome in HNSCC can affect interpretation of other potential clinical and biological prognostic markers

(Gillison, 2006). Oropharyngeal tumours are traditionally associated with alcohol and tobacco use (Lubin et al., 2009). However, is has become apparent over recent years that there is a subset of oropharyngeal patients who do not have the traditional risk factors of smoking and tobacco use. HPV positive tumours differ from HPV negative tumours in many aspects including: risk factors, differentiation, histological appearance and outcome (Fakhry and Gillison, 2006). HPV positivity is associated with a good prognosis in patients with oropharyngeal tumours (D'Souza et al., 2007). HPV-related cancers are characterised by over expression of *CDKN2A* (Fakhry et al., 2008, Lassen et al., 2010, Lassen et al., 2009, Kumar et al., 2008). As the HPV E7 oncogene product prevents the action of retinoblastoma protein, *CDKN2A* is upregualted via the loss of the negative feedback from retinoblastoma protein. It has been suggested that *CDKN2A* expression can be used as a marker of tumours with oncogenetically relevant HPV infection (Smeets et al., 2007, Weinberger et al., 2006). As mentioned above, positive *CDKN2A* expression was significant for locoregional control and cancer specific survival in the subset of tonsil tumours, consistent with other published series (Kong et al., 2009, Klussmann et al., 2003, Lassen et al., 2009).

HPV positivity confers a favourable advantage for local contol and survival in patients treated with chemoradiotherapy, with this observed improvement in prognosis thought to be due to three main factors: lack of field cancerization, immune response to viral antigens and improved response to radiotherapy. First, HPV positive patients are less likely to have field cancerization due to the lack of exposure to tobacco and alcohol, therefore reducing their risk of recurrence and second primaries (Licitra et al., 2006). The results from this series support this finding, with *CDKN2A* expression significantly associated with low alcohol intake ($p=0.003$) and non smokers ($p=0.005$). Second, HPV infection may lead to improved immune surveillance (Gillison, 2006). Millen et al demonstrated that patients with a higher viral load have a better prognosis (Mellin et al., 2002), and Rajjoub et al showed that patients with increased numbers of CD3 positive tumour infiltrating lymphocytes had significantly lower levels of cervical node metastasis and imporved survival (Rajjoub et al., 2007). Third, HPV positivity appears to be associated with an improved response to radiotherapy. Viral integration leads to increased expression of the oncogenes E6 and E7, causing genomic instability, and it has been suggested that E6 and E7 play a role in enhancing the radiosensitivity of HPV positive tumours (Duensing and Munger, 2004).The tumour microenviroment also influences radiosensitivity with hypoxia causing radioresistance. Interestingly hypoxia modification has been reported to improve outcome in *CDKN2A* negative but not *CDKN2A* positive tumours. This finding suggests that hypoxic radioresistance may not be clinically relevant in HPV positive patients (Lassen et al., 2010). The finding, therefore, of an association between high expression of HIF-1α

and *CDKN2A* (p=0.010) is contrary to what might be expected and further research is required to confirm whether the relationship is sound. Despite this finding HIF-1α and *CDKN2A* expression had independent prognostic significance in multivariate analysis.

The relationship between HPV and molecular markers in head and neck cancer clearly needs further investigation before any changes in management can be justified. Although HPV clearly offers a new and exciting factor in the world of head and neck cancer, these results highlight that the long known fact that hypoxic tumours have a poor prognosis should not be forgotten. Further research into hypoxia in HNSSC is still warranted and necessary.

In conclusion, this study confirms previous studies showing the significance of HIF-1α and *CDKN2A* expression on prognosis in patients with oropharyngeal cancer. It highlights the need for further research into the relationship between HPV and hypoxia.

# 4 Expression of hypoxia markers in surgically managed HNSCC

## 4.1 Abstract

Purpose: Low tumour expression of the hypoxia associated markers hypoxia inducible factor 1-α (HIF-1α) and carbonic anhydrase 9 (CA9) has been widely linked with a good prognosis in head and neck cancer. For the potential biomarkers to be useful in the clinic there is a need to show low expression predicts benefit from chemoradiotherapy/radiotherapy rather than being associated with a poor prognosis irrespective of treatment modality. This study explored the prognostic significance of the markers in a heterogeneous series of patients who underwent surgery as the primary modality of treatment.

Material and Methods: An audit identified 100 consecutive patients with histologically proven squamous cell carcinoma of the head and neck. All patients had surgery as the primary modality of treatment. Tumour expression of HIF-1α and CA9 was examined using immunohistochemistry.

Results: Extracapsular spread was significanltly associated with poor cancer specific survival (p=0.022). No other patient variables were associated with outcome. HIF-1α expression was significantly associated with poor tumour grade (p=0.019) and the tumour having a cohesive front (p=0.026).

Conclusion: If confirmed, this finding suggests that hypoxic head and neck cancers should undergo primary surgery rather than chemoradiotherapy/radiotherapy.

## 4.2 Introduction

Head and neck cancer is a relatively common malignancy, accounting for 5% of all new cancers diagnosed internationally (Parkin et al., 2005). Around 40% of patients that present with head and neck cancer have advanced disease at presentation, commonly involving regional lymph nodes (Ries, 2006). At present the management of HNSCC is based upon patient factors, including age and comorbidities, and tumour factors including site, stage and rersectability. Both surgery and radiotherapy play key roles in the management of HNSCC with the addition of chemotherapy in recent years. Management decisions are possibly more relavent now as there have been significant advances in this area recently. Progress in surgical techniques and reconstruction has pushed the bounderies of what is now regarded as operable disease. The imaging of tumours and the delivery of radiotherapy has evolved with the introduction of PET and IMRT. The role of systemic therapy has advanced from use only in palliative patients to now being a key component in HNSCC management. Concurrent chemoradiotherapy now offers an organ preserving treatment for advanced malignancy. Historically, patients with advanced disease (stage III/IV, accounting for up to 60% of patients) were treated with surgery followed by post-operative radiotherapy. More recently there has been a change in management, with these patients now commonly receiving organ sparing chemoradiotherapy. However, despite this change in practice, only 50% of patients respond well to chemoradiotherapy raising the question of whether some of patients would benefit from surgery rather than chemoradiotherapy as the primary treatment modality.

The difficulty is predicting which patients are likely to respond to a particular treatment, which would allow treatment to be tailored to the individual patient. Biomarkers that allow prediction of efficacy are therefore required. Tumour hypoxia is a well documented feature of many solid tumours, including HNSCC, with a large multi-centre analysis demonstrating an association between low tumour $pO_2$ and poor prognosis (Nordsmark et al., 2005). Hypoxia has an unfavourable effect on malignant progression (Semenza, 2010a). There are many ways to assess tumour hypoxia including the use of endogenous hypoxia-associated markers HIF-1α and CA9 (Bache et al., 2008). HIF-1 regulates a number of biological pathways including angiogenesis, apoptosis, cell migration, metastasis and proliferation via the transactivation of around 60 genes (Semenza, 2003). Under hypoxic conditions, the oxygen dependent degredation of HIF-1α is halted, causing accumulation if HIF-1α in hypoxic cells. One of the genes that HIF-1 regulates is carbonic anhydrase 9 (CA9). CA9 is a member of the carbonic anhydrase family, and it regulates the pH of cells via the reversible hydration of carbon dioxide to carbonic acid. CA9 is induced in increasing levels as $pO_2$ levels fall (Hoogsteen et al., 2007a). Although not a universal finding (Kappler et

al., 2008, Eriksen and Overgaard, 2007), many studies have shown overexpression of HIF-1α and CA9 to be associated with poor prognosis and treatment failure in HNSCC (Silva et al., 2008, Koukourakis et al., 2008, Winter et al., 2006). In this study we performd immunohistochemistry for HIf-1α and CA9 on a series of surgically managed HNSCC patients to investigate the relationship between markers of tumour hypoxia and outcome in a surgically managed cohort of patients.

## 4.3 Materials and Methods

### 4.3.1 Patients

Ethical approval was obtained from the local research ethics committee (Reference Number: 03/TG/076), appendix IV. All patients had histologically confirmed squamous cell carcinoma of the head and neck, diagnosed between 1999 and 2005. The demographic, clinico-pathologic and outcome patient data were collected retrospectively from the case notes and the Christie Head and Neck assessment form, see appendix V. Tumour blocks were requested from the referring hospitals in which the diagnostic biopsies were performed. Inclusion criteria for the study were patients with a histologically confirmed squamous cell carcinoma of the head and neck, patients treated at the Christie Hospital between January 1999 and December 2005, patients over the age of 16 years, patients who had surgery as their primary treatments All patients had surgery as the primary modality of treatment. Twenty-six had surgery only, 64 had surgery with post operative radiotherapy and 10 had surgery with post operative chemoradiotherapy. Radiotherapy was delivered using a 4-6 MeV photons linear accelerator. All patients received 52.5 Gy in 16 daily fractions over three weeks. Exclusion criteria were patients treated palliatively; patients that had unresectable tumours.

### 4.3.2 Immunohistochemistry

Immunohistochemical detection of HIF-1α was performed using the Tyramide Signal Amplification System (NEN Life Sciences, Boston), which is based on a streptavidin-biotin-horseradish peroxidase complex formation. Sections 4 μm thick were deparaffinised and antigen retrieval carried out by microwaving in 10 mmol/L citrate buffer for 20 min. The sections were rinsed, soaked in tris buffered saline (TBS) for 5 min, and immersed in TBS and 30% hydrogen peroxide. The sections were washed, soaked in TBS again, and TNB (Tris NaCl Blocking) reagent applied for 30 min. The mouse monoclonal IgG1 (immunoglobulins isotype 1) antibody (BD Biosciences 610958) in TNB was applied to the sections, with mouse IgG1 (Dako X0931) used as a

negative control sections. Sections were incubated overnight at 4$^{o}$C. The secondary antibody, a biotinylated rabbit anti-mouse immunoglobulin (RAMBO) (Dako E0413) in TCP (Tryptone Casein Peptone), was applied for 30 min followed by SA-HRP (Streptavidin-Horse Radish Peroxidase) from the TSA (tyramide signal amplification) Biotin Kit in TCP for 30 min. Biotinyl tyramide amplification reagent was applied to the sections for 8 min, followed by SA-HRP in TNB for 30 min. TNT was used to wash the sections in between applications. DAB (3,3' diaminobenzidine tetrahydrochloride) chromogen was then applied to the sections for 5 min. Gill's haematoxylin was used as a counterstain, prior to dehydration and cover slipping (Kim et al., 2003). Batch-to-batch variation was assessed by choosing two sections showing high and low HIF-1α expression and running additional sections from these biopsies with each batch. (Aebersold et al., 2001).

Immunohistochemical detection of CA9 was performed using the Envision kit system. Sections 4 μm thick were deparaffinised and then washed in TBS for 5 min. A 30% hydrogen peroxide block was applied for 15 min followed by a 10% casein block for 15 min. The primary antibody, a mouse monoclonal anti-human antibody (M75) raised to the external domain of CA9, was a gift from Profs S. Pastorekova and J. Pastorek, Slovak Academy of Sciences, Bratislava, Slovak Republic. The CA9 antibody was applied at a 1:50 dilution for 30 min and mouse IgG 2B (immunoglobulins isotype 2B) (Dako 0944) was used as the negative control. A secondary antibody was applied to the sections for 30 min and after rinsing, DAB+ (3,3' diaminobenzidine tetrahydrochloride) was applied to each section for 5 min. Both the secondary antibody and the DAB+ were from the Envision kit. Gill's haematoxylin was used to counterstain the sections, which were then rinsed, dehydrated and coverslipped.

### 4.3.3 Scoring method

The presence of SCC was confirmed by a senior pathologist (Dr Rachel Hall, Consultant Pathologist). For CA9 the scoring system was as follows: 0, less than 1% plasma membrane staining; 1, 1-9% plasma membrane staining; 2, 10-30% plasma membrane staining; 3, greater than 30% plasma membrane scoring. For analysis the scoring was further sub divided into low expression ( 0-9%) and high expression (10->30%) (see Fig 4.1). These are similar scoring systems used by previous authors (Loncaster et al., 2001) . The scoring system for HIF 1-α was as follows: 0, no nuclear staining; 1, less than 10% nuclear staining; 2, 10% to 29% nuclear staining; 3, 30% or greater nuclear staining (see Fig 4.2). This is a similar scoring to previous authors (Condon et al., 2002).

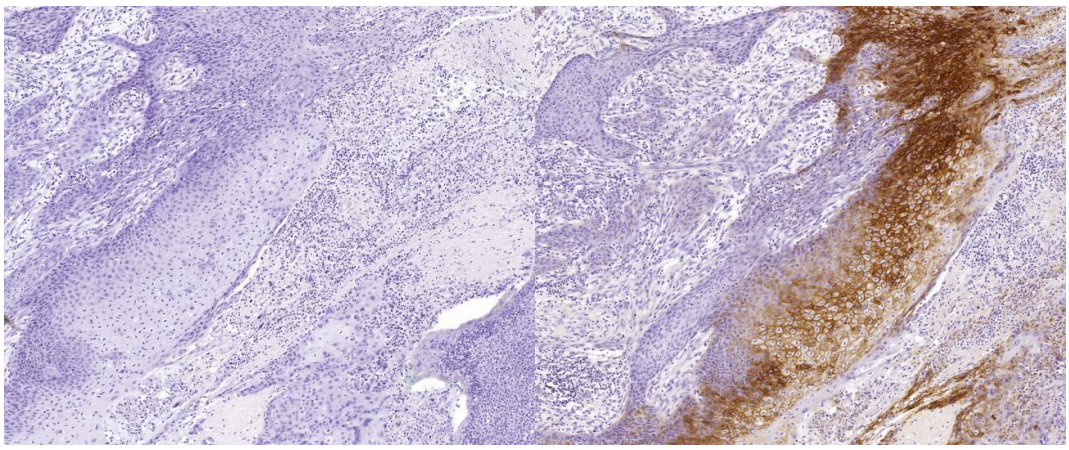


Figure 4-1Surgical series biopsy of negative control for CA9 expression (left) and high CA9 expression (right)

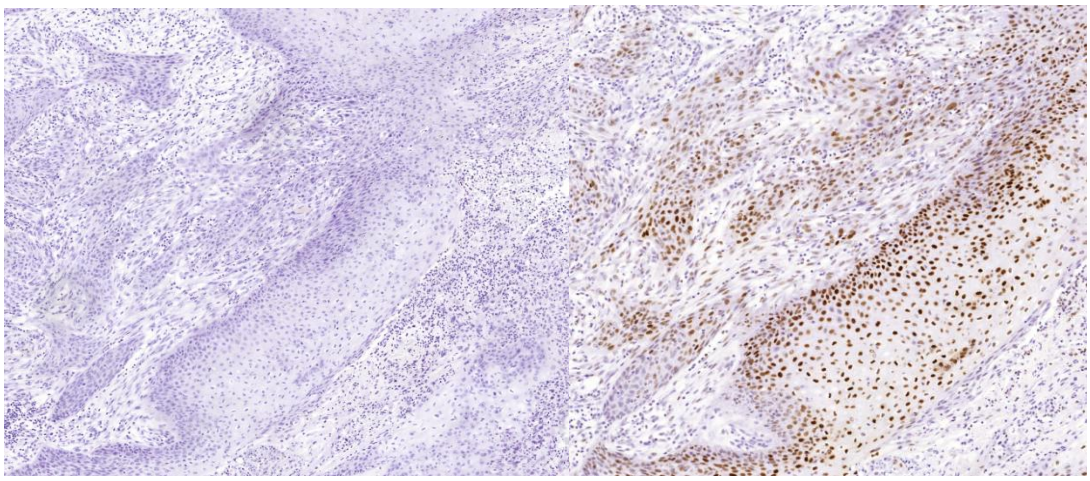


Figure 4-2 Surgical series biopsy of negative control for HIF-1α expression (left) and high HIF-1α expression (right)

### 4.3.4 Statistical analysis

Actuarial estimations of locoregional control and cancer specific survival were obtained using the Kaplain-Meier method. The survival curves were compared in univariate analysis using the logrank method. Analysis was carried out on all patients, and for tonsil and tongue subgroups. The variables analysed for prognostic significance were: smoking history, alcohol history, site, gender, age, pre-treatment Hb level, stage, tumour HIF-1α expression, tumour CA9 expression. The proportion of patients with high HIF-1α and CA9 expression was compared with various clinical factors using chi-squared test.

## 4.4 Results

### 4.4.1 Patient characteristics

The characteristics of the 100 patients are summarised in Table 5.1, see appendix VIII. The median duration of follow up was 3.6 (range 0.25-18) years.

### 4.4.2 Univariate and Multivariate analysis

The 5-year locoregional control, cancer specific survival and overall survival were 52%, 57% and 55% respectively. On univariate analysis the only significant factor associated with poor cancer specific survival was extracapsular spread (p=0.022) (Figure 5.1), which remained significant on multivariate anslysis (p=0.022). No other patient variables were significant for outcome (Table 5.2).

Table 4-1 Patient characteristics of surgical series

| **Feature** | | **No. of patients (%)** |
|---|---|---|
| Gender | Male | 64 (64) |
| | Female | 36 (36) |
| Age | Median (Range) | 60 (31-83) |
| Pre treatment Hb | Median (Range) | 13.8 (11.1-17.3) |
| Smoking History | Never | 8 (9) |
| | Current | 52 (59) |
| | Ex | 28 (32) |
| Alcohol History | Never/low | 27 (34) |
| | Mod | 18 (22) |
| | Heavy/Prev Heavy | 35 (44) |
| Site | Oral Cavity | 54 (54) |
| | Larynx | 25 (25) |
| | Oropharynx | 12 (12) |
| | Pharynx | 9 (9) |
| Stage | I-II | 23 (23) |
| | III-IV | 77 (77) |
| T Stage | T1 | 12 (12) |
| | T2 | 32 (32) |
| | T3 | 13 (13) |
| | T4 | 42 (43) |
| N Stage | N0 | 45 (45) |
| | N1 | 20 (20) |
| | N2 | 29 (32) |
| | N3 | 3 (3) |
| Grade | Well | 7 (11) |
| | Moderate | 41 (68) |
| | Poor | 13 (21) |
| Lymphovascular invasion | No | 44 (80) |
| | Yes | 11 (20) |
| Cohesive front | No | 33 (92) |
| | Yes | 3 (8) |
| Margins | Clear | 42 (42) |
| | Close | 43 (43) |
| | Involed | 15 (15) |
| Extracapsular spread | No | 76 (76) |
| | Yes | 24 (24) |
| HIF-1α Expression | None | 9 (14) |
| | Low | 37 (58) |
| | Moderate | 17 (27) |
| | High | 1 (1) |
| CA9 | None | 6 (9) |
| | Low | 32 (49) |
| | Moderate | 20 (30) |
| | High | 8 (12) |

Table 4-2 Treatment outcome analysis for surgical patients

| **Feature** | | **LRC HR (95% CI)** | **p** | **CSS HR (95%CI)** | **P** |
|---|---|---|---|---|---|
| Gender | Male | 1 | | 1 | |
| | Female | 0.88 (0.45-1.71) | 0.701 | 0.74 (0.36-1.55) | 0.430 |
| Age | 70+ | 1 | | 1 | |
| | <50 | 0.68 (0.30-1.54) | | 0.59 (0.24-1.43) | |
| | 51-70 | 1.28 (0.49-3.35) | 0.251 | 1.34 (0.49-3.64) | 0.118 |
| Pre treatment Hb | <13.8 | 1 | | 1 | |
| | ≥ 13.8 | 0.53 (0.13-2.22) | 0.385 | 0.70 (0.16-3.14) | 0.643 |
| Smoking History | Never | 1 | | 1 | |
| | Current | 1.58 (0.35-7.07) | | 1.28 (0.28-5.83) | |
| | Ex | 1.53 (0.35-6.56) | 0.832 | 1.07 (0.24-4.69) | 0.884 |
| Alcohol History | Never/low | 1 | | 1 | |
| | Mod | 1.18 (0.48-2.88) | | 1.23 (0.43-3.50) | |
| | Heavy/Prev Heavy | 0.64 (0.28-1.46) | 0.380 | 0.86 (0.35-2.13) | 0.791 |
| Site | Oral cavity | 1 | | 1 | |
| | Larynx | 0.89 (0.39-2.02) | | 0.93 (0.36-2.37) | |
| | Oropharynx | 1.27 (0.51-3.18) | | 1.18 (0.45-3.08) | |
| | Pharynx | 0.73 (0.22-2.45) | 0.868 | 1.01 (0.29-3.48) | 0.984 |
| T Stage | T1 | 1 | | 1 | |
| | T2 | 1.14 (0.42-3.12) | | 0.85 (0.30-2.41) | |
| | T3 | 0.47 (0.11-1.96) | | 0.46 (0.11-1.95) | |
| | T4 | 0.88 (0.32-2.43) | 0.543 | 0.72 (0.25-2.06) | 0.73 |
| N Stage | N0 | 1 | | 1 | |
| | N1 | 0.98 (0.43-2.28) | | 1.05 (0.41-2.69) | |
| | N2 | 0.95 (0.44-2.08) | | 1.56 (0.67-3.63) | |
| | N3 | 1.46 (0.33-6.32) | 0.959 | 2.44 (0.55-10.94) | 0.555 |
| Grade | Well | 1 | | 1 | |
| | Moderate | 10.8 (0.31-3.72) | | 1.41 (0.39-5.10) | |
| | Poor | 0.54 (0.11-2.72) | 0.550 | 0.61 (0.09-3.89) | 0.498 |
| Lymphovascular invasion | No | 1 | | 1 | |
| | Yes | 0.81 (0.23-2.84) | 0.747 | 1.10 (0.31-3.96) | 0.883 |
| Cohesive front | No | 1 | | 1 | |
| | Yes | 0.03 (0.0-112.7) | 0.422 | 0.03 (0.0-258.1) | 0.465 |
| Margins | Clear | 1 | | 1 | |
| | Close | 1.42 (0.71-2.86) | | 1.62 (0.75-3.50) | |
| | Involed | 1.91 (0.76-4.81) | 0.351 | 1.90 (0.69-5.22) | 0.354 |
| Extracapsular spread | No | 1 | | 1 | |
| | Yes | 1.54 (0.76-3.12) | 0.229 | 2.42 (1.34-5.16) | 0.022 |
| HIF-1α Expression | None | 1 | | 1 | |
| | Low | 2.17 (0.49-9.46) | | 2.38 (0.53-10.7) | |
| | Moderate | 1.95 (0.39-9.72) | | 2.94 (0.56-15.3) | |
| | High | 0.00 | 0.786 | 0.000 | 0.646 |
| CA9 | None | 1 | | 1 | |
| | Low | 0.32 (0.10-1.03) | | 0.41 (0.13-1.34) | |
| | Moderate | 0.42 (0.13-1.42) | | 0.38 (0.11-1.41) | |
| | High | 0.24 (0.04-1.32) | 0.233 | 0.35 (0.06-1.98) | 0.438 |

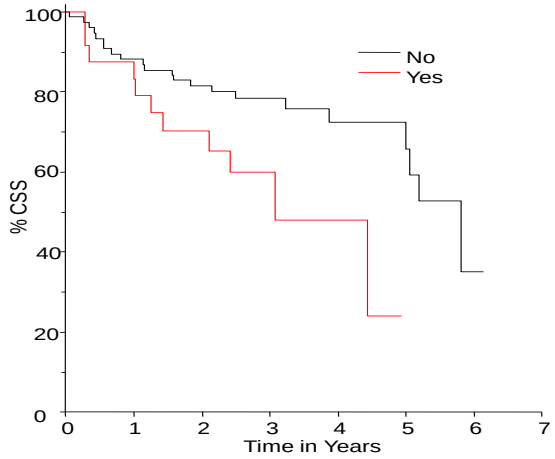



Figure 4-3 Kaplan-Meier survival curve showing the relationship between extracapsular spread and cancer-specific survival (no-upper curve; yes-lower curve).

### 4.4.3 HIF-1α and CA9 expression

Tissue sections were suitable for HIF-1α analysis in 64 patients. They were suitable for CA9 analysis in 66 of the 100 patients. This discrepancy represents logistical difficulties obtaining the original surgical specimens for all patients. There was a significant association between HIF-1α expression and poorlt differentiated tumour grade (p=0.019) and cohesive front (p=0.026). There was a significant association between HIF-1α expression and CA9 expression (p=0.021), and a significant correlation between HIF-1α expression and CA9 expression (Spearman's rho =0.351, p=0.004). There was no association between any patient characteristics and HIF-1α and CA9 expression (see Table 5.3).

Table 4-3 Patient characteristics/histology in relation to HIF-1α and CA9 expression

| **Feature** | **HIF-1α P value** | **CA9 P value** |
|---|---|---|
| Gender | 0.404 | 0.775 |
| Pre treatment Hb | 0.766 | 0.267 |
| Smoking History | 0.946 | 0.829 |
| Alcohol History | 0.120 | 0.149 |
| Site | 0.177 | 0.214 |
| T Stage | 0.954 | 0.679 |
| N Stage | 0.977 | 0.558 |
| Grade | 0.019 | 0.720 |
| Lymphovascular invasion | 0.789 | 0.270 |
| Cohesive front | 0.026 | 0.695 |
| Margins | 0.914 | 0.379 |
| Extracapsular spread | 0.247 | 0.121 |
| HIF-1α expression | | 0.021 |
| CA9 | 0.021 | |

## 4.5 Discussion

A major problem in the management of HNSCC is the unpredictable response of the cancer to treatment, being able to accurately predict responders and non-responders would therefore be invaluable. At present, tumour stage, morphology and grade are the main prognostic factors in HNSCC, yet frequently this information does not give reliable predictive information. There is good evidence to show that HNSCC are hypoxic and more resistant to radiotherapy (Nordsmark et al., 2005). Overgaard demonstrated that the addition of the hypoxia radiosensitiser nimorazole to a series of supraglottic larynx and pharynx patients treated with radiotherapy significantly improved outcome (Overgaard et al., 1998). Targeting hypoxia has been of clinical interest for a number of years, however the clinical curiosity has been hampered by the lack of ability to acurately ascertain which patients would benefit from hypoxia targeted therapy. It is well established that HIF-1α and CA9 are strongly inducible by hypoxia (Harris, 2002). Several studies have shown that HIF-1α overexpression is linked to poor outcome in patients treated with radiotherapy (Koukourakis et al., 2002, Aebersold et al., 2001, Silva et al., 2008). However, we do not know if HIF-1α is a genuine marker of poor response to radiotherapy or whether it just reflects a more aggressive tumour that is associated with a poor outcome irrespective of treatment modality.

This study investigated the immunohistochemical expression of HIF-1α and CA9 in a series of patients who were treated by a modality that was not dependent on hypoxia, i.e., surgery. Although the sample size was small, there was no indication for any trend that HIF-1α and CA9 expression would be associated with a poor outcome. We did not find an association between HIF-1α and CA9 expression and outcome in this series of patients, consistent with other authors who have found similar results (Kappler et al., 2008, Kyzas et al., 2005b, Eriksen and Overgaard, 2007). It has also been reported that overexpression of HIF-1α is associated with a significantly imporved outcome in surgically managed patients. Beasley et al looked at a series of 79 patients treated surgically, demonstrating that high HIF-1α expression was associated with improved disease free survival (Beasley et al., 2002). Fillies also reported similar results; showing that overexpression of HIF-1α was significantly associated with improved 5 year survival rates (Fillies et al., 2005).

It has long been recognised that, in comparison with well differentiated tumours, histologically high grade poorly differentiated tumours are more aggressive and have a poorer outcome. We found that there was a significant positive association between HIF-1α expression and grade ($p=0.019$) and cohesive front ($p=0.026$). Cells respond to decreasing oxygen levels via hypoxia-inducible transcription factor 1 (HIF-1). HIF-1 is a hetrodimer composed of HIF-1α and HIF-1β. If oxygen is not present, HIF-1 binds

to hypoxia response elements (HREs) activating a series of hypoxia response genes. These hypoxia regulated genes regulate several key biological processes in tumour development: cell proliferation, angiogensis, cell migration, immortilization and gene instability, and apoptosis (Harris, 2002). These factors convey a more aggressive phenotype in hypoxic tumours, which may manifest clinically as a poorly differentiated tumour, which may benefit from surgical management or hypoxic radiosensitisers to improve outcome.

No association was found between hypoxia-associated marker expression and outcome. However, a weak but statistically significant positive correlation was found between HIF-1α and CA9 expression (Spearman's $r$ =0.35, p=0.004), consistent with the laryngeal series described in Chapter 3 (Spearman's $r$ = 0.27, p=<0.0001). The degredation of HIF-1α and CA9 are very different, with the half life of HIF-1α being minutes compared to days for CA9 (Turner et al., 2002). CA9 is also known to be a downstream gene of HIF-1 pathway. This may explain why, although there is an association between the two markers, the correlation coefficient was low, a finding also reported by Winter et al (Winter et al., 2006).

Another important finding in this chapter was that patients with extracapsular spread (ECS) had significantly worse cancer specific survival (p=0.022), in agreement with many previously published series (Imre et al., 2008, Puri et al., 2003). Nodal metastasis is the most significant prognostic factor in HNSCC. Nodal histopathological features such as the number of positive nodes and the presence of ECS have prognostic significance with regards to cervical recurrence, distant metastasis and survival (De Zinis et al., 2006). There is evidence that ECS is indicative of an aggressive tumour type, associated with increased rates of locoregional and distant failure, with one series reporting a 40% decrease in tumour recurrence rate and 45% decrease in survival for patients with compared to those without ECS (Prim et al., 1999). Furthermore, ECS can often be present in the clinically negative neck, with one series observing that 49% of patients had ECS in a clinically N0 neck (Alvi and Johnson, 1996).

In summary, this paper highlights that HIF-1α and CA9 may be useful markers for helping to guide management decisions regarding the use of radiotherapy versus surgery. The work suggests that the markers should be explored further surgically treated series of patients with HNSCC. This paper also highlights that clinic-pathological information is still a very useful to help guide management decisions and this information should not be forgotten in the race to find the perfect biomarker.

# 5 Overall Conclusion

Cree recently pointed out that a cancer is as individual as its host, possibly even more so, with the heterogeneity being influenced by genotypic and phenotypic differences between patients (Cree, 2009). Despite the obvious heterogeneity of HNSCC, the management of these patients remains largely pragmatic, on the basis of randomised clinical trials that treat all patients as if they were identical. However, as has been discussed in the thesis, two patients with apparently similar tumours and comorbidities may respond in very different ways despite receiving exactly the same treatment. Unfortunately, predictive information on the likely response of a given tumour before starting treatment is not currently available routinely. The tailoring of treatment based on an individual patient's tumour biology would represent a very significant step forward in head and neck cancer. Recent research in other fields of cancer has been encouraging. The K-ras mutations in colorectal cancer and the associated response to anti-EGFR antibodies like cetuximab are the beginning of individualised treatment in cancer management along with HER2 in breast cancer and herceptin (Allegra et al., 2009, Wolff et al., 2007) . However, so far there is no equivalent in head and neck cancer.

Currently, today's guidelines for the management of HNSCC are based on phase III trials and meta-analyses, with a wealth of radiotherapy and chemotherapy trials but unfortunately due to the nature of surgery, randomised trials are not often performed. Recently there has been a considerable increase in new therapeutic agents for HNSCC. Ironically, as the number of possible treatment options supported by completed randomised trials increases, the scientific literature becomes increasingly hazy for guiding doctors. Moreover, clinicians are confronted on almost a daily basis by decisions that have not been addressed by randomised trial evaluation. Indeed, a great many promising observations will never make it to prospective controlled evaluations because of scarce resources. Nevertheless, clinical decisions are made according to experience, new basic science insights, bias or personal preference, philosophical beliefs, and so on - in short, the so-called art of medicine.

Additional clinical research is needed for patients with intermediate disease. Currently research focuses on early disease and late disease, but there is a group of patients in the middle who are often enrolled in phase III trials, along with patients with advanced disease. However, it may not be necessary to subject these patients to such intense treatment and single modality treatment may be just as effective. The results presented in Chapter two show that there is a significant difference between T2a and T2b glottic cancer. T2 disease is often treated within the "early disease" category with single modality treatment. The current guidelines advise larynx preservation, and

chemotherapy is not currently recommended for early stage disease (Pfister et al., 2006). However, our results show that patients with T2b disease are likely to benefit from more intensive management compared to T2a disease for larynx preservation. The work reported in this thesis suggests that a trial randomising patients with T2b disease to radiotherapy versus chemoradiotherapy should be considered.

The role of endoscopic surgery in the management of early larynx cancer is well established. There appears to be clinical equipoise between the use of surgery and radiotherapy, although radiotherapy is used more commonly. This is an ideal situation where a biomarker to predict treatment response should be used. Identifying those patients that are likely to have a radioresistant tumour would mean that they would be offered conservative laryngeal surgery with larynx preservation, therefore preventing the need for more radical surgery and possibly total laryngectomy if they have a radioresidtant recurrence. There is good evidence to show that hypoxic tumours are more resistant to radiotherapy. The work described in Chapter 3 showed that tumour overexpression of CA9 in combination with low Hb was significantly associated with worse locoregional control. The significance of CA9 expression and outcome in a series of glottis cancer patients has been reported previously, suggesting that CA9 may be a useful marker for helping to predict radioresistant tumours. The combined prognostic role of CA9 and Hb has not been previously reported. This work obviously needs validating in further series of glottis cancer patients. If the finding is confirmed then CA9+Hb might have potential as a biomarker to stratify patients to radiotherapy or surgery.

The results in Chapter 4 show that high HIF-1α expression is highly significant for outcome in a series of oropharyngeal patients treated with radiotherapy. This finding is consistent with other published series, suggesting that high expression of HIF-1α indicates a more radioresistant tumour. This is supported by the finding in Chapter 5 in which HIF-1α expression was not related to outcome in a series of surgically managed patients. It has been consistently demonstrated that the presence of HPV in oropharyngeal tumours is prognostic for outcome. It is increasingly apparent that HPV positive tumours exhibit a different molecular pathogenesis from tobacco and alcohol associated tumours, as confirmed by the results in Chapter 4. We demonstrated that tumour *CDKN2A* expression is a significant independent adverse prognostic factor for locoregional control and cancer specific survival in a subgroup of tonsil patients, but not tongue base patients. There was also a significant association between *CDKN2A* expression and tobacco and alcohol use. At present, HPV status is not routinely tested in HNSCC. However, there are several clinical indications for HPV testing, which are: to indicate prognosis (treatment selection and possible de-escalation); to classify neck nodes with an unknown primary; for patient education and the use of/development of

future preventative or therapeutic vaccines. The discovery that HPV 16 causes a distinct, and rising, group of HNSCC offers the opportunity for prevention and treatment with vaccines designed to induce immune response. The improved survival of HPV positive patients may be due to these tumours being more radiosensitive. There was a mild association between high HIF-1α expression and *CDKN2A* expression suggesting that there may be a link between the two. However, a recent study by Lassen et al did not show any improvement in survival with the addition of hypoxic radiosensitizers to *CDKN2A* positive patients suggesting that hypoxia may not be clinically relevant in these tumours (Lassen et al., 2010).

Despite the promising results reported in this thesis, an important question still remains: can any of the proposed biomarkers be developed so that they would be suitable for use in individualising patient treatment selection? So far, no biological predictive marker among the numerous investigated has entered clinical practice. At present the predictive values of individual or combined molecular markers is insufficient for use in routine clinical practice. In theory, a panel of markers would seem more robust than a single marker, but at present there are no clinical data to support this. Despite significant advances in the field of both hypoxia and HPV, we still need more research into head and neck cancer before individualised patient treatment becomes routine. In particularly, collaborative multi-centre studies are required so that validated biomarkers can be developed.

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# Appendix I - Randomised phase III trials of chemoradiotherapy in head and neck cancer patients*

| Site | Pts | Control Arm | Treatment | Primary endpoint | Outcome | Reference |
|---|---|---|---|---|---|---|
| **Chemoradiotherapy vs conventional radiotherapy alone as postoperative treatment** | | | | | | |
| Oral cavity, oropharynx, hypopharynx, larynx | 334 | Radiotherapy | Radiotherapy + Cs (100mg/m$^2$) d1,22,43 | Progression free survival | 47%vs 36% at 5 yrs, p=0.04. Overall survival 53% vs 40% at 5 years, p= 0.04 | (Bernier et al., 2004) |
| Oral cavity, oropharynx, hypopharynx, larynx | 444 | Radiotherapy | Radiotherapy + Cs (20mg/m$^2$ ci) + F(600 mg/m$^2$ ci) d1-5, 29-33) | Locoregional control | 88% vs 62% at 5 years, p=0.0006. Overall survival 58% vs 49%, p=0.11 | (Fietkau et al., 2006) |
| **Chemoradiotherapy vs radiotherapy alone as primary treatment** | | | | | | |
| oropharynx | 222 | Radiotherapy | Radiotherapy + Cb (70 mg/m$^2$, d1-4) + F (600mg/m$^2$ ci, d1-4) for 3 cycles | Overall survival | 51% vs 31% at 3 yrs, p=0.02. 22.4% vs 15.8% at 5 years, p=0.05 | (Denis et al., 2004) |
| Oral cavity, oropharynx, hypopharynx, larynx | 295 | Radiotherapy (C) | Radiotherapy + Cs (100mg/m$^2$) d1,22,43 (A) or Radiotherapy split course + Cs (75mg/m$^2$ d1) + F (1000mg/m$^2$ ci, d1-4 for 3 cycles (B) | Overall survival | (A) 84% vs (C) 37% (p=0.014) vs (B) 27% (p=NS) at 3 years | (Adelstein et al., 2003) |
| Larynx | 510 | Cs (100mg/m$^2$ d1) + F (1000 mg/m$^2$/d ci d1-5) for 3 cycles followed by radiotherapy alone in responders (C) | Radiotherapy + Cs (100 mg/m$^2$) d 1,22,43 (A) or radiotherapy alone (B) | Laryngeal preservation | (A) 84% vs (B) 66% (p=0.00017) vs (C) 70% (p=0.0029), at 5 years. | (Forastiere et al., 2003) |
| **Chemoradiotherapy vs altered fractionation radiotherapy alone as primary treatment** | | | | | | |
| Oropharynx, hypopharynx (unresectable) | 240 | Hfx Acc radiotherapy | Hfx Acc radiotherapy + Cb (70 mg/m$^2$ d1-4) + F (600mg/m$^2$ ci, d1-4) for 2 cycles | Locoregional control | 22.7% vs 12.6% at 5 yrs, p = 0.01 Overall survival 26.1% vs 13% at 5 yrs, p=0.008 | (Semrau et al., 2006) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Oral cavity, oropharynx, hypopharynx (unresectable) | 384 | Hfx Acc radiotherapy (77.6 Gy) | Hfx Acc radiotherapy (77.6 Gy) + F (600mg/$m^2$ ci, d1-5) + M (10 mg/$m^2$ bolus) d 1,36 | Locoregional control | 49.9% vs 37.4% at 5 years, p=0.001. Overall survival 28.6% vs 23.7% at 5 years, p=0.023 | (Budach et al., 2005) |
| Oral cavity, oropharynx, hypopharynx , larynx (78 unresectable) | 224 | Hfx Acc radiotherapy | Hfx Acc radiotherapy + Cs (20mg/$m^2$, d1-5) for 2 cycles | Time to treatment failure | 27% vs 37.4% at 5 years, p >0.05. Overall survival 46% vs 32% at 5 years, p=0.15 | (Huguenin et al., 2004) |
| Oropharynx (unresectable) | 192 | Conventional radiotherapy or Hfx Acc radiotherapy | Radiotherapy (66-70 Gy) + Cb (75 mg/$m^2$ d1-4) + F (1000mg/$m^2$ ci, d1-4) for 3 cycles | Overall survival | 51% vs 40% vs 37% at 2 years, p=0.129 | (Olmi et al., 2003) |

*Adapted from (Argiris et al., 2008); Acc=accelerated; Cs=cisplatin; Cb=carboplatin; ci=continuous infusion; d=day of treatment cycle; F=fluorouracil; Fo=folinate; Hfx=hyperfraqctionated; HR=hazard ratio; M=mitomycin; NS=not significant; HNSCC=head and neck squamous cell carcinoma.

# Appendix II - Prognostic significance of pre-treatment anaemia for radiotherapy outcome in patients with head and neck cancer

| T/TNM Stage | Pts | Treatment | Outcome | Reference |
|---|---|---|---|---|
| T1-T2 larynx | 735 | 50 Gy in 20 fractions over 4 weeks | ↓ pre-treatment Hb associated with risk of local relapse | **(Warde et al., 1998)** |
| T1 larynx | 139 | Median total dose 66 Gy (range 50–70.4 Gy). Dose per # 1.7–2.2 Gy once daily, over 47 days ( 29–63), 114 patients (72%) completing RT with no or only one day of interruption. | ↓ pre-treatment Hb level (≤13 gm/dL) associated with ↓ OS. No association with DSS | **(Canaday et al., 1999)** |
| T1N0M0 larynx | 235 | The individual total dose, dose per fraction, and overall treatment time (OTT) ranged from 51-70 Gy, 1.5-3.0 Gy, and 24-79 days, respectively | ↓ in Hb level of 1g/dL (13.8g/dL to 12.8g/dL) →6% reduction in TCP (if OTT was 45 days) | **(Skladowski et al., 1999)** |
| T1N0M0 larynx | 238 | 6- or 8 MV X-ray or $^{60}$Co radiation in parallel-opposed fields (median size: 22.5 cm(2)) over a median of 52 days to a median dose of 68 Gy. | ↓Hb level during treatment associated with reduced LC rate | **(Jin et al., 2002)** |
| Stage III or IV, M0 HNC | 159 | 70 to 72.5 Gy acc HFRT (1.25 Gy b.i.d.) and CCT | Five-year FFS was 75% for patients ≥ Hb 13 g/dL vs. 50% for patients with Hb <13 g/dL had a ($p < 0.01$). | **(Prosnitz et al., 2005)** |
| Consecutive HNSCC patients | 78 | Concurrent chemoradiation for SCCHN. Patients were treated with IMRT to 70 Gy in 35 daily fractions to the high-dose target volume and 56 Gy to the elective target volume. | Loco-regional failure occurred in 7 of 19 patients (37%) whose pretreatment Hb level was <12 g/dL compared with 8 of 59 patients (14%) with Hb levels ≥12 ($p = 0.042$). | **(McCloskey et al., 2009)** |
| Stage III or IV HNC | 64 | 30 Gy in 20 fractions over 2 wks + CCT, repeated after 2 wk break up to a cumulative dose of 60 Gy and followed by a boost up to 70 Gy (69-70.5 Gy). | Univariate analysis of survival showed a better outcome for patients with a hemoglobin nadir >10.5 g/dL | **(Stadler et al., 2006)** |
| Larynx and pharynx | 414 | 62-68 Gy for 6-7 wks | Hb <9 mmol/L men and <8 mmol/L women associated with ↓LRC p=0.02 | **(Overgaard et al., 1989)** |
| Stage II-IV HNSCC | 196 | 66–70 Gy with concomitant chemotherapy (cisplatin). Neck dissection was performed when residual disease was noted. | MVA, anaemia most significant predictor of LC (HR 0.37, $p = 0.009$) + OS (HR 0.47, $p = 0.007$). A dose-effect relationship found for LC ($p = 0.04$) + OS (0.04) when grouping by Hb concentration: <12, 12-14 & >14 g/dL. | **(Fortin et al., 2008)** |

Hb=haemoglobin; OS=overall survival; TCP=tumour control probability; OTT=overall treatment time; LC=locoregional control. HFRT-hyperfractionated radiotherapy; CCT – concomitant chemoradiotherapy; FFS-failure free survival; HNC – head and neck cancer

# Appendix III - HPV status and prognosis in HNSCC

| Study Author | N | Tumour Subsite | HPV Positivity (%) | HPV Types Detected | Method Used | Tissue Prep | Treatment | Endpoints Studied | Survival Advantage |
|---|---|---|---|---|---|---|---|---|---|
| All Site Survival Studies[*] | | | | | | | | | |
| (Brandwein et al., 1994) | 55 | Oral Cavity | 78 | — | PCR | FFPE | — | DSS | No |
| | 9 | Oropharynx | 56 | | | | | | |
| (Clayman et al., 1994) | 6 | Hypopharynx | 100 | — | PCR | FFPE | S/XRT | DSS | No[**] |
| | 59 | Larynx | 41 | | | | | LRC | No |
| (Chiba et al., 1996) | 38 | Oral Cavity | 21 | 16 | PCR | FF | — | DFS | Yes |
| (Haraf et al., 1996) | 14 | Oral Cavity | 0 | 16/33 | PCR | FFPE | Mixed | OS | No |
| | 26 | Oropharynx | 38 | | | | | | |
| | 7 | Hypopharynx | 14 | | | | | | |
| | 19 | Larynx | 5 | | | | | | |
| (Snijders et al., 1996) | 63 | All sites | 21 | 16 | PCR | FFPE | — | OS | No |
| (Riethdorf et al., 1997) | 78 | Oral Cavity | 42 | 6/11/16/18 | PCR | FF | Mixed | OS | No |
| | 9 | Oropharynx | 44 | | | | | | |
| | 3 | Larynx | 67 | | | | | | |
| (Paz et al., 1997) | 15 | Tonsil | 60 | 16/6 | PCR, SB | FF | — | OS | No |
| | 152 | Other site | 11 | | | | | DSS | No |
| (Koch et al., 1999) | 211 | All sites | 18 | 16/33 | PCR | FF | — | OS | No |
| (Pintos et al., 1999) | 29 | Oral Cavity | 10 | — | PCR, SB | FFPE | — | OS | No |
| | 20 | Pharynx | 30 | | | | | DFS | No |
| | 52 | Larynx | 15 | | | | | | |
| (Shima et al., 2000) | 46 | Oral Cavity | 74 | 16/18 | PCR, SB | FF | — | OS | No |

| Study Author | N | Tumour Subsite | HPV Positivity (%) | HPV Types Detected | Method Used | Tissue Prep | Treatment | Endpoints Studied | Survival Advantage |
|---|---|---|---|---|---|---|---|---|---|
| (Schwartz et al., 2001) | 254 | Oral Cavity | 16 | 16+ | PCR | FFPE | Mixed | OS | Yes‡‡ |
| | 41 | Oral cavity | 5 | 16 | PCR | FF | — | OS | Yes |
| | 29 | Oropharynx | 52 | | | | | DSS | Yes |
| (Ringstrom et al., 2002) | 4 | Hypopharynx | 0 | | | | | | |
| | 10 | Larynx | 10 | | | | | | |
| | 5 | Other site | 0 | | | | | | |
| (Sisk et al., 2002) | 32 | Mixed sites | 47 | 16/18 | PCR | FF | — | OS | Yes |
| (Dahlgren et al., 2003) | 25 | Tonsil | 60 | 16/118/33 | PCR | FF | S/XRT | DSS | Yes |
| | 5 | Tonsil | 100 | 6/16/33/51/52 | PCR, ISH | FF | — | OS | No |
| | 15 | Tongue | 73 | | | | | | |
| (Koskinen et al., 2003) | 13 | Oral Cavity | 54 | | | | | | |
| | 10 | Hypopharynx | 50 | | | | | | |
| | 18 | Larynx | 50 | | | | | | |
| (Ritchie et al., 2003) | 94 | Oral Cavity | 11 | 16/18/33 | PCR | FFPE | Mixed | OS | Yes |
| | 45 | Oropharynx | 42 | | | | | | |
| (Azzimonti et al., 2004) | 25 | Larynx | 56 | 16/18 | PCR | FFPE | — | OS | No |
| | 9 | Tonsil | 56 | | | | | | |
| (Baez et al., 2004) | 36 | Oral Cavity | 36 | 16 | PCR | FF | — | OS | No |
| | 16 | Oropharynx | 63 | | | | | DFS | No |
| | 14 | Hypopharynx | 36 | | | | | | |
| | 52 | Larynx | 46 | | | | | | |
| (Dahlgren et al., 2004) | 85 | Oral Cavity | 2 | 16/18/33 | PCR | FFPE | S/XRT | DSS | Yes |

| Study Author | N | Tumour Subsite | HPV Positivity (%) | HPV Types Detected | Method Used | Tissue Prep | Treatment | Endpoints Studied | Survival Advantage |
|---|---|---|---|---|---|---|---|---|---|
| | 25 | Base of Tongue | 40 | | | | | | |
| | 20 | Tonsil | 55 | 16/33 | PCR, SB | FF | S/XRT | OS | No |
| | 4 | Oropharynx | 26 | | | | | DFS | No |
| (Hoffmann et al., 2005) | 6 | Oral Cavity | 67 | | | | | | |
| | 24 | Hypopharynx | 29 | | | | | | |
| | 19 | Larynx | 26 | | | | | | |
| (Kozomara et al., 2005) | 50 | Oral Cavity | 64 | 6/16/18/31 | PCR | FF/FFPE | S/XRT | OS | No** |
| (Vlachtsis et al., 2005) | 90 | Larynx | 40 | 16/18 | PCR | FF | S/XRT | OS | No |
| | 60 | Oral Cavity | 13 | 16/33/35/58 | PCR | FFPE | — | OS | Yes |
| | 2 | Nasal Cavity | 100 | | | | | DFS | No |
| (Badaracco et al., 2007) | 10 | Oropharynx | 0 | | | | | | |
| | 8 | Tonsil | 75 | | | | | | |
| | 5 | Hypopharynx | 20 | | | | | | |
| | 30 | Larynx | 13 | | | | | | |
| | 266 | Oral cavity | 14 | 16 | PCR | FF/FFPE | — | OS | Yes |
| (Furniss et al., 2007) | 78 | Oropharynx | 19 | | | | | | |
| | 33 | Hypopharynx | 18 | | | | | | |
| | 90 | Larynx | 16 | | | | | | |
| (Na et al., 2007) | 70 | Oral Cavity | 0 | 16 | PCR | FFPE | Mixed | OS | Yes |
| | 38 | Tonsil | 24 | | | | | | |
| (Sugiyama et al., | 66 | Oral Cavity | 36 | 16 | PCR | FFPE | Mixed | OS | No |

| Study Author | N | Tumour Subsite | HPV Positivity (%) | HPV Types Detected | Method Used | Tissue Prep | Treatment | Endpoints Studied | Survival Advantage |
|---|---|---|---|---|---|---|---|---|---|
| 2007) | | | | | | | | | |
| (Jo et al., 2009) | 14 | Oropharynx | 93 | 16 | PCR | FF/FFPE | C, S/XRT | OS | No |
| | 10 | Other site | 10 | | | | | PFS | No |
| | | | | Oropharynx Survival Studies‡ | | | | | |
| (Portugal et al., 1997) | 58 | Oral Cavity | 7 | — | PCR | FFPE | — | OS tonsil | Yes |
| | 42 | Tonsil | 19 | | | | | OS all sites | No |
| (Gillison et al., 2000) | 2 | Nasopharynx | 0 | 16/18/31/33 | PCR/ISH/SB | FF | Mixed | OS all sites | Yes |
| | 84 | Oral Cavity | 12 | | | | | DSS all sites | Yes |
| | 60 | Oropharynx | 57 | | | | | OS oropharynx | Yes |
| | 21 | Hypopharynx | 10 | | | | | DSS oropharynx | Yes |
| | 86 | Larynx | 19 | | | | | | |
| (Friesland et al., 2001) | 34 | Tonsil | 41 | 16 | PCR | FFPE | XRT | OS | Yes |
| | | | | | | | | DFS | No |
| (Lindel et al., 2001) | 99 | Oropharynx | 14 | 16/33/35/45 | PCR | FFPE | XRT/C | OS | No |
| (Mellin et al., 2002) | 22 | Tonsil | 55 | 16/33 | PCR | FF | — | OS | No |
| | | | | | | | | DSS | No |
| (Strome et al., 2002) | 52 | Tonsil | 46 | 16/59 | PCR | FFPE | — | OS | No |
| | | | | | | | | DFS | No |
| (Klussmann et al., 2003) | 34 | Tonsil | 53 | 16/33 | PCR | FFPE | S/C/XRT | OS | No |
| | | | | | | | | DFS | No |
| (Li et al., 2003) | 67 | Tonsil | 46 | 16+ | PCR | FFPE | Mixed | DSS | Yes |
| (Mellin et al., 2003) | 60 | Tonsil | 45 | 16/33 | PCR | FFPE | XRT/S | DSS | Yes |

| Study Author | N | Tumour Subsite | HPV Positivity (%) | HPV Types Detected | Method Used | Tissue Prep | Treatment | Endpoints Studied | Survival Advantage |
|---|---|---|---|---|---|---|---|---|---|
| (Wittekindt et al., 2005) | 34 | Tonsil | 53 | 16/18 | PCR | FF | — | OS | No |
| (De Petrini et al., 2006) | 23 | Oral Cavity | 39 | 16 | PCR | FFPE | — | DSS oropharynx | Yes |
| | 21 | Oropharynx | 52 | | | | | DSS oral cavity | no |
| (Licitra et al., 2006) | 90 | Oropharynx | 19 | 16 | PCR | FFPE | S/XRT | OS | Yes |
| (Weinberger et al., 2006) | 79 | Oropharynx | 61 | 16 | PCR | FFPE | Mixed | OS | Yes |
| | | | | | | | | DFS | Yes |
| (Reimers et al., 2007) | 106 | Oropharynx | 28 | 16/33 | PCR | FFPE | - | OS | No |
| | | | | | | | | DFS | No |
| (Fakhry et al., 2008) | 62 | Oropharynx | 61 | 16/33/35 | ISH | FFPE | IC, CRT/S | OS all sites | Yes |
| | 34 | Larynx | 0 | | | | | PFS all sites | Yes |
| | | | | | | | | OS oropharynx | Yes |
| | | | | | | | | PFS oropharynx | Yes |
| (Hafkamp et al., 2008) | 81 | Tonsil | 41 | 16 | PCR/ISH | FFPE | Mixed | OS | Yes |
| | | | | | | | | DSS | Yes |
| (Worden et al., 2008) | 26 | BOT | 62 | 16 | PCR | FFPE | IC, CRT/S | OS | Yes |
| | 16 | Tonsil | 69 | | | | | DSS | Yes |
| (Gillison, 2009) | 323 | Oropharynx | 64 | 16+ | ISH | - | CRT | OS | Yes |
| | | | | | | | | PFS | Yes |
| | | | | | | | | LRC | Yes |
| (Haughey, 2009) | 174 | Oropharynx | 72 | — | ISH | FFPE | S, C/XRT | OS | Yes |
| | | | | | | | | DFS | Yes |

| Study Author | N | Tumour Subsite | HPV Positivity (%) | HPV Types Detected | Method Used | Tissue Prep | Treatment | Endpoints Studied | Survival Advantage |
|---|---|---|---|---|---|---|---|---|---|
| | 25 | Oral Cavity | 36 | 16/6 | PCR | FFPE | S+ | OS oropharynx | Yes |
| (Ritta et al., 2009) | 22 | Oropharynx | 50 | | | | | OS other sites | No |
| | 12 | Larynx | 58 | | | | | | |
| | 49 | Oropharynx | 53 | 16 | ISH | FFPE | CRT | OS | Yes |
| (Sedaghat et al., 2009) | | | | | | | | DSS | Yes |
| | | | | | | | | RFS | Yes |
| | 28 | Oral cavity | 11 | 16 | PCR | FFPE | CRT | OS oropharynx | Yes‡‡ |
| (Settle et al., 2009) | 119 | Oropharynx | 50 | | | | | | |
| | 35 | Hypopharynx | 6 | | | | | | |
| | 55 | Larynx | 7 | | | | | | |
| (Shi et al., 2009) | 111 | Oropharynx | 66 | 16 | RT-PCR, ISH | FFPE | XRT, CRT | OS | Yes§§ |
| | | | | | | | | DFS | Yes |
| (Attner et al., 2010) | 87 | Oropharynx | 78 | 16 | PCR | FFPE | XRT,CRT | DFS | Yes |
| (Ang et al., 2010) | 323 | Oropharynx | 64 | 16 & 18 | ISH | FFPE | XRT | OS, RFS | Yes |
| (Rischin et al., 2010b) | 185 | Oropharynx | 57% | 16 & 18 | ISH & PCR | FFPE | CRT | OS, RFS | Yes |

Adapted from (Allen et al., 2010); * Studies that evaluate non oropharynx sites or studies that include oropharynx but do not correlate oropharynx site with survival seperately.
† Studies that correlate oropharynx site with survival. PCR = polymerase chain reaction; ISH = in-situ hybridization; SB = Southern blot; FFPE = formallin fixed paraffin embedded; FF = fresh frozen; S=surgery; XRT = external beam radiotherapy; C = chemotherapy; IC = induction chemoptherapy; CRT = concurrent chemoradiotherapy; DSS = disease-specific survival; LRC = local-regional control; DFS = disease-free survival; OS = overall survival; PFS = progression-free survival; RFS=recurrence free survival.
# Statistically significant ($p \leq 0.05$) for endpoint listed; ** statistically significant for HPV postivity as a negative prognostic variable; ††statistically significant for HPV type 16 only; ‡‡ Includes patients with tissue available for HPV analysis only; §§significant improvement in OS with RT-PCR only on multivariate analysis.

# Appendix IV - Ethical approval

MANCHESTER 1824

The University of Manchester

Secretary to Dr. T Stibbs
Room 2.005
John Owens Building
The University of Manchester
Oxford Road
Manchester M13 9PL

www.manchester.ac.uk

*ref: TPCS/CA/ethics/07285*

7th March 2008

Dr C West,
Cancer and Imaging Sciences,
Christie Hospital,
Wilmslow Road,
Withington,
Manchester

Dear Dr West,

**Committee on the Ethics of Research on Human Beings**

*07285 West, Silva, Homer, Slevin, Price: Markers of hypoxia and predictors of treatment response in head and neck carcinoma. (Tameside and Glossop ref: 03/TG/076)*

I write to confirm that at its meeting on 6th March 2008, the Committee received the report on the above project, which had been approved by a recognised ethics committee. That approval is therefore endorsed by the University Ethics Committee.

If you have cause to inform the REC of any unusual or unexpected results that raise questions about the safety of the research, you should also forward a copy to our office. We also ask that you provide us with details of any substantial amendments approved by the REC.

I am pleased to say that we now have a facility to accept and store electronic versions of documents, particularly copies of COREC application forms. From now on, therefore, electronic documents can be emailed to me at the address below. We are happy to accept documents in hard copy. Typically LREC approval letters will be in this form and we would prefer to have the ethics insurance form as a hard copy with a signature.

Yours sincerely

Catherine Atkinson
**Secretary to Dr T P C Stibbs**
Catherine Atkinson
Compliance and Risk Management
0161 275 2206
0161 275 5697
Catherine.atkinson-2@manchester.ac.uk

NHS
Central Office for Research Ethics Committees
(COREC)

## NOTICE OF SUBSTANTIAL AMENDMENT

*For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at http://eudract.emea.eu.int/document.html#guidance.*

*To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.*

*Further guidance is available at http://www.corec.org.uk/applicants/apply/amendments.htm.*

**Details of Chief Investigator:**


| | |
|---|---|
| *Name:* | Dr Catharine West |
| *Address:* | c/o Dr Jo Cresswell<br>Academic Department of Radiation Oncology<br>Christie Hospital NHS Trust<br>Wilmslow Road, Withington, Manchester M20 4BX |
| *Telephone:* | 0161 446 8268 |
| *E-mail:* | jo.cresswell@manchester.ac.uk |
| *Fax:* | 0161 446 8111 |


| | |
|---|---|
| **Full title of study:** | Markers of hypoxia and predictors of treatment response in head and neck carcinoma |
| **Name of main REC:** | Tameside & Glossop |
| **REC reference number:** | 03/TG/076 |
| **Date study commenced:** | October 2003 |
| **Protocol reference *(if applicable)*, current version and date:** | |
| **Amendment number and date:** | Amendment 3: 5th September 2006 |

**Type of amendment (indicate all that apply in bold)**

*(a) Amendment to information previously given on the REC application form*

*Yes* ■

*If yes, please refer to relevant sections of the REC application in the "summary of changes" below.*

*(b) Amendment to the protocol*

■ *No*

*If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text.*

*(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study*

■ *No*

*If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.*

**Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?**

■ *No*

**Summary of changes**

*Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study. In the case of a modified amendment, highlight the modifications that have been made.*

*If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.*

Based on the data so far obtained, we would like to extend this study to investigate a further 200-300 patients with the following disease types (ICD classifications are shown in brackets):

- Base of tongue (C01)
- Other tongue (C02)
- Floor of mouth (C04)
- Tonsillar (C09)
- Oropharynx (C10)
- Piriform fossa (C12)
- Hypopharynx (C13)
- Larynx (C32.9)

We will identify all patients between 1998 and 2004 who received surgery as the primary treatment for these tumours at either the Christie Hospital NHS Trust or Manchester Royal Infirmary. We will then investigate tissue samples from those patients with the aim of correlating expression of biological markers with outcome following surgery.

The research aims of the project remain the same – to identify biological markers which can be used to predict outcome following treatment for head and neck cancer. Primary head and neck cancer may be treated by either surgery or radiotherapy. Treatment decisions are based mainly on tumour size, stage and grade. Radiotherapy is often the preferred treatment for smaller tumours, due the lower risk of morbidity, however salvage surgery following failed radiotherapy is associated with a very poor prognosis. There is an aim therefore to identify those patients who will respond best to radiotherapy and those who will respond best to surgery, before the decision to treat is made.

From the original study we have a large amount of biological marker data relating to outcome following radiotherapy. This extension will allow us to investigate biological markers relating to outcome where surgery is the primary treatment. It is hoped that by comparing marker profiles for these two treatment modalities, profiles may emerge which can be used to provide patients with the most suitable treatment.

This amendment will involve auditing notes of all patients with the above listed cancer types treated with primary surgery at the Christie Hospital and Manchester Royal Infirmary between 1998 and 2004.

Retrospective tissue samples will be requested from the Pathology archives of the referring hospital, and samples prepared for immunohistochemical analysis. In the original design, immunohistochemical analysis was carried out on individual sections cut from the tissue blocks held with Pathology. Since the origination of this project, the group has gained access to tissue microarray (TMA) facilities. For TMAs, multiple small sections (cores) are removed from a single tissue block. This enables a greater number of sections to be taken from the same block, and a larger number of markers to be studies for each section. Immunohistochemical analysis is then carried out as normal.

We have the full support of a Consultant Surgeon (Mr Jarrod Homer), Consultant Pathologist (Professor Phil Sloan) and a Consultant Clinical Oncologist (Dr Nick Slevin)

**Any other relevant information**

*Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.*

With the exception of this change, the original study design will be observed in that slides will be cut from the pathology archive blocks and codes so as to be anonymous. As the study has similar outcome measures and laboratory techniques as outlined in our original ethics submission, we do not anticipate any ethical problems.

Stockport NHS
Primary Care Trust
**Tameside & Glossop Local Research Ethics Committee**
Room 181
Gateway House
Piccadilly South
Manchester
M60 7LP
0161 237 2336
carol.ebenezer@northwest.nhs.uk

27 September 2006

Dr Catherine West
c/o Jo Cresswell
Academic Department of Radiation Oncology
c/o Christie Hospital NHS Trust
Wilmslow Road
Withington
Manchester
M20 4BX

Dear Dr West,

**Full title of study:** **Markers of hypoxia and predictors of treatment response in head and neck carcinoma**
**REC reference number : 03/TG/076**
**Protocol number:**
**Amendment: 3, dated 5 September 2006**

The above amendment received on 11 September 2006, was reviewed by the sub-committee at the meeting held on 27 September 2006.

**Ethical opinion**

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form.

**Approved documents**

The documents reviewed and approved at the meeting were:

- Notice of Amendment form dated 5 September 2006
- Covering letter

**Membership of the Committee**

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

**Management approval**

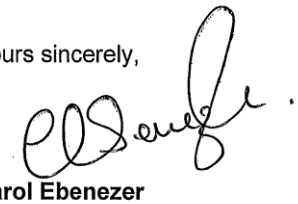
Before implementing the amendment, you should check with the host organisation whether it affects their approval of the research.

**Statement of compliance (from 1 May 2004)**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

| **REC reference number: 03/TG/076** | **Please quote this number on all correspondence** |
|---|---|

Yours sincerely,



**Carol Ebenezer**
**Committee Co-ordinator**

# Appendix V - Christie Head and Neck Assessment Form

**The Christie NHS Foundation Trust**

| | |
|---|---|
| **Name**<br>**CX Number**<br>**Date of Birth** | **HEAD AND NECK ASSESSMENT FORM** |

| **Complete ALL sections** | **Date** ___/___/___ | **Completed by** | | **Consultant** | |
|---|---|---|---|---|---|

**HISTORY** (additional to front sheet)

**PERSONAL HISTORY**

Lives with: _____ Occupation: _____

**Smoking**: 0 1 2 3 (see below)

Max ever smoked regularly _____ c p d

**Alcohol** (see below)
1. Never heavy
2. Previous heavy
3. Current heavy

**PMH:**

Other malignancies Family Hx cancer

**MEDICATION:**

**Allergies**:

| Height _____ cm | Weight ____kg | BSA ___ $m^2$ | Current diet:<br>1.Normal 2.Soft 3.Liquid 4.Tube | |
|---|---|---|---|---|

**EXAMINATION** (see diagrams over)

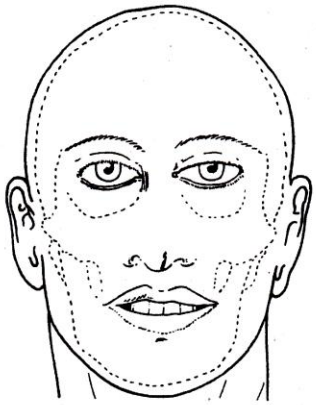
| **Primary site** | **Stage T N** | **WHO score** | |
|---|---|---|---|

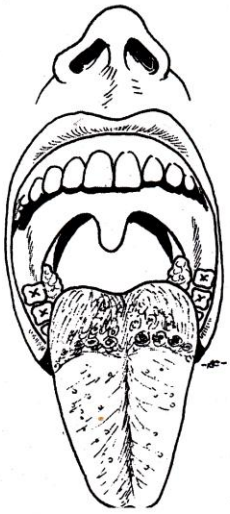
**Smoking:**
0 never smoked
1 ex-smoker < 1 year
2 ex-smoker > 1 year
3 current smoker

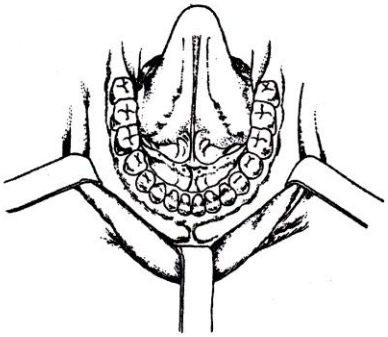
**Alcohol**
Heavy = male > 40 upw; female > 30 upw

**WHO score**
0 – Able to carry out all activity without restriction
1 – Restricted in physically strenuous activity but ambulatory and able to carry out light work
2 – Ambulatory and capable of self-care but unable to carry out any work; up and about more than 50% of waking hours
3 – Capable of only limited self-care; confined to bed or chair more than 50% of waking hours
4 – Completely disabled; cannot carry out any self-care; totally confined to bed or chair

AP1235





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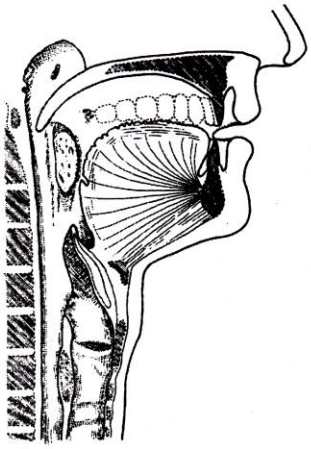
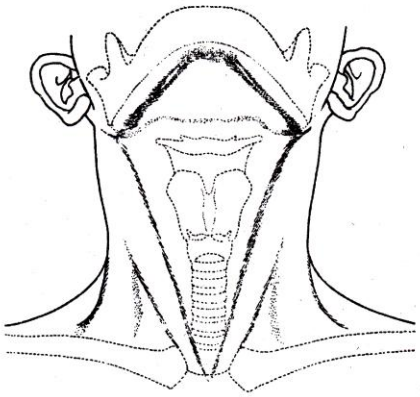
L





## The Christie NHS Foundation Trust

**MOULD ROOM INSTRUCTIONS – please tick all sections**

**Diagnosis**_____ **Side R L**

**PRIMARY**

| Parallel pair | Wedge pair | Three field | Single phase | 2 phase | Bite block | Tongue depressor |
|---|---|---|---|---|---|---|
| | | | | | | |

**NECK**

| Anterior | Bilateral | R | L | Electrons |
|---|---|---|---|---|
| | | | | |

**NECK POSITION**

| Straight | Hyper-extended |
|---|---|
| | |

**LOCAL-ISATION**

| Gold Seed | Ba paste | Mark scar | Mark node | RTP scan / other |
|---|---|---|---|---|
| | | | | |

2

**SET UP**

| 100 SSD | Isocentric |
|---|---|
| | |

**SHELL**

| Thermoplastic | Vivak |
|---|---|
| | |

**The Christie NHS Foundation Trust**

| | | | |
|---|---|---|---|
| **ALL PATIENTS SHOULD HAVE:** | | Date of test | Sign when results known |
| | FBC and biochemical profile | | |
| | Recent (< 6 months) CXR | | |
| | Dental assessment (as appropriate) | | |
| **CLINICAL TRIALS** (Please indicate which trial and date information sheet given) | | | |

**MANAGEMENT PLAN**

Treatment intent: Radical / Palliative

Date of proposed XRT ____ / ____ / ____

Proposed dose _____ cGy in _____ fractions

Chemotherapy: N Y:

Other eg plan for tube feeding, referrals etc

**PRE-RADIOTHERAPY ANNOTATIONS**

| Date | | Signature |
|---|---|---|
| | | |

# Appendix VI – Glottic Series

| Age | Gender | Smoker | Alcohol | T stage | Overall stage | Pre trt HB | Recurrence | 1o Failure | Node Failure | Distant Failure | Date Last Seen or of Death | Status | CA9 % staining | HIF-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 81 | M | E>1 | L | 2a | 2 | 14.7 | No | No | No | No | 16-Oct-2007 | Alive | <1 | |
| 72 | M | E<1 | L | 2a | 2 | 15.9 | No | No | No | No | 10-Aug-2007 | Alive | 10 | 11<=30 |
| 72 | M | E>1 | L | 1a | 1 | 13.3 | No | No | No | No | 07-Nov-2007 | Alive | 30 | |
| 53 | F | Ct | L | is | 0 | 15.2 | No | No | No | No | 19-Oct-2007 | Alive | 3 | |
| 81 | M | Ct | NK | 2a | 2 | 11.8 | Yes | Yes | Yes | No | 08-Nov-2001 | DdthisC | 1 | 11<=30 |
| 73 | M | No | N | 2a | 2 | 15.0 | No | No | No | No | 25-Feb-2001 | ICT | 15 | 1<=10 |
| 74 | M | No | L | 1a | 1 | 15.6 | No | No | No | No | 29-Oct-2007 | Alive | 35 | 1<=10 |
| 83 | M | E>1 | PH | 2a | 2 | 13.7 | No | No | No | No | 08-Mar-2003 | ICT | 15 | 11<=30 |
| 69 | M | E>1 | L | 2a | 2 | 12.3 | No | No | No | No | 16-Mar-2001 | DotherC | <1 | |
| 54 | F | Ct | L | 3 | 2 | 99.9 | No | No | No | No | 27-Jun-2007 | Alive | 5 | 1<=10 |
| 56 | F | E<1 | L | 3 | 2 | 99.9 | No | No | No | No | 01-Oct-2007 | Alive | 15 | 11<=30 |
| 58 | F | Ct | NK | is | 0 | 99.9 | No | No | No | No | 21-Jan-2004 | LFU | <1 | 11<=30 |
| 47 | F | No | NK | 2a | 2 | 12.8 | Yes | No | Yes | No | 30-Aug-2007 | Alive | 10 | >30 |
| 52 | M | Ct | H | 1a | 1 | 99.9 | No | No | No | No | 14-Dec-2006 | LFU | 3 | |
| 54 | M | E>1 | L | 1a | 1 | 13.8 | No | No | No | No | 01-Oct-2007 | Alive | <1 | 1<=10 |
| 54 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 07-Jan-2007 | Alive | 1 | 1<=10 |
| 72 | M | E>1 | H | 1a | 1 | 99.9 | No | No | No | No | 13-Sep-2007 | Alive | 5 | 11<=30 |
| 78 | M | E>1 | L | 2a | 2 | 99.9 | No | No | No | No | 27-Sep-2007 | Alive | 8 | <1 |
| 74 | F | No | NK | 1a | 1 | 99.9 | No | No | No | No | 25-Jan-2003 | ICT | 5 | <1 |
| 70 | M | E>1 | L | 1a | 1 | 99.9 | Yes | Yes | No | No | 02-Oct-2007 | Alive | 20 | 1<=10 |
| 71 | F | E>1 | NK | 2b | 2 | 12.9 | Yes | Yes | No | No | 20-Apr-2002 | DotherC | 4 | >30 |
| 39 | M | E>1 | NK | 1a | 1 | 99.9 | No | No | No | No | 10-May-2000 | LFU | 5 | 11<=30 |
| 59 | M | Ct | H | 1a | 1 | 12.2 | Yes | Yes | No | No | 07-Jun-2007 | Alive | 3 | 1<=10 |
| 70 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 16-Nov-2004 | ICT | 30 | |
| 86 | M | Ct | NK | 1a | 1 | 14.2 | No | No | No | No | 11-Feb-2006 | ICT | <1 | 1<=10 |
| 49 | M | Ct | H | 1a | 1 | 14.6 | No | No | No | No | 15-Jun-2007 | Alive | <1 | >30 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 75 | M | E<1 | L | 2b | 2 | 13.0 | No | No | No | No | 24-Jul-2002 | ICT | 3 | |
| 73 | M | E>1 | L | 2a | 2 | 15.3 | No | No | No | No | 18-Feb-2007 | ICT | <1 | 1<=10 |
| 69 | M | E<1 | L | is | 0 | 14.3 | No | No | No | No | 01-Oct-2007 | Alive | 3 | 1<=10 |
| 55 | M | E<1 | L | 2a | 4 | 14.5 | Yes | Yes | Yes | No | 24-Feb-2003 | DdthisC | <1 | |
| 35 | F | Ct | NK | 3 | 2 | 12.6 | Yes | Yes | No | No | 14-Aug-2006 | Alive | 3 | 11<=30 |
| 62 | M | Ct | L | 3 | 2 | 15.7 | No | No | No | No | 17-Jun-2004 | DdthisC | <1 | 11<=30 |
| 55 | M | Ct | H | 2a | 2 | 13.9 | No | No | No | No | 16-May-2007 | Alive | 10 | 11<=30 |
| 72 | M | E>1 | L | 3 | 2 | 14.0 | No | No | No | No | 12-Mar-2005 | ICT | 5 | 11<=30 |
| 67 | M | Ct | L | 1b | 1 | 16.8 | No | No | No | No | 01-Sep-2003 | DotherC | 3 | |
| 78 | M | E>1 | NK | 3 | 2 | 99.9 | No | No | No | No | 09-Feb-2007 | ICT | 5 | |
| 59 | M | E<1 | NK | 2b | 2 | 12.0 | No | No | No | No | 24-Oct-2004 | DotherC | 10 | |
| 62 | M | NK | NK | 1a | 1 | 14.3 | No | No | No | No | 07-Dec-2007 | Alive | 15 | 11<=30 |
| 57 | M | E>1 | L | 1a | 1 | 14.9 | No | No | No | No | 03-Sep-2007 | Alive | 3 | |
| 53 | M | NK | NK | 1a | 1 | 15.0 | No | No | No | No | 01-Oct-2007 | Alive | 40 | |
| 51 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 05-Nov-2007 | Alive | 3 | <1 |
| 72 | M | E>1 | H | 1a | 1 | 99.9 | No | No | No | No | 26-Nov-2007 | Alive | 10 | |
| 76 | F | Ct | L | 2b | 2 | 13.9 | No | No | No | No | 10-Jun-2002 | ICT | 20 | |
| 64 | M | E>1 | H | 1a | 1 | 15.2 | No | No | No | No | 18-Oct-2007 | Alive | <1 | |
| 53 | M | E<1 | H | 1a | 1 | 99.9 | Yes | Yes | No | No | 07-Nov-2007 | Alive | 30 | |
| 46 | M | E>1 | L | 1a | 1 | 14.1 | No | No | No | No | 12-Oct-2007 | Alive | <1 | 11<=30 |
| 74 | M | E>1 | L | 1a | 1 | 11.4 | No | No | No | No | 05-Mar-2006 | DotherC | 3 | |
| 63 | M | E>1 | L | 2a | 4 | 14.9 | Yes | No | Yes | Yes | 25-Jul-2002 | DdthisC | 3 | >30 |
| 62 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 09-Jul-2007 | Alive | <1 | |
| 54 | M | No | L | 1a | 1 | 99.9 | No | No | No | No | 24-Sep-2003 | Alive | 20 | |
| 79 | M | E>1 | H | is | 0 | 15.6 | No | No | No | No | 15-Oct-2007 | Alive | 1 | 1<=10 |
| 68 | M | Ct | L | 2a | 2 | 99.9 | No | No | No | No | 20-May-2004 | ICT | 8 | 11<=30 |
| 45 | M | Ct | H | 2a | 2 | 16.4 | No | No | No | No | 13-Sep-2007 | Alive | 20 | 1<=10 |
| 30 | F | E>1 | N | 1b | 1 | 12.4 | No | No | No | No | 09-Oct-2003 | Alive | 30 | 11<=30 |
| 79 | M | NK | NK | 2a | 2 | 14.7 | No | No | No | No | 30-Sep-2002 | DotherC | 30 | >30 |
| 57 | M | No | NK | 1a | 1 | 14.8 | No | No | No | No | 12-Mar-2002 | LFU | 10 | 11<=30 |
| 51 | M | No | L | is | 0 | 15.3 | No | No | No | No | 15-Nov-2004 | LFU | 40 | |
| 70 | M | Ct | L | 2a | 2 | 13.7 | No | No | No | No | 15-Oct-2007 | Alive | 8 | 11<=30 |
| 63 | M | E<1 | H | 2a | 2 | 15.2 | No | No | No | No | 11-Feb-2004 | DotherC | <1 | 11<=30 |
| 75 | M | E<1 | L | 2b | 2 | 99.9 | No | No | No | No | 06-Nov-2006 | ICT | 30 | 11<=30 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | M | E>1 | L | 1a | 1 | 14.6 | No | No | No | No | 08-Nov-2006 | Alive | 3 | 1<=10 |
| 68 | M | Ct | H | 1a | 1 | 12.5 | No | No | No | No | 02-Dec-2001 | ICT | 1 | 1<=10 |
| 71 | M | No | N | 1a | 1 | 15.4 | No | No | No | No | 02-Aug-2005 | ICT | 25 | 11<=30 |
| 67 | M | E>1 | H | 1b | 1 | 15.2 | No | No | No | No | 26-Jul-2007 | Alive | 20 | |
| 75 | M | No | N | 1a | 1 | 99.9 | No | No | No | No | 18-Sep-2002 | ICT | 8 | 1<=10 |
| 72 | M | E<1 | L | 1b | 1 | 13.5 | No | No | No | No | 05-Aug-2002 | DotherC | <1 | 11<=30 |
| 77 | F | E>1 | L | 1a | 1 | 14.5 | No | No | No | No | 04-Oct-2007 | Alive | 20 | |
| 73 | F | Ct | N | 1a | 1 | 12.8 | No | No | No | No | 29-Oct-2007 | Alive | <1 | 1<=10 |
| 85 | M | Ct | H | 1a | 1 | 13.7 | No | No | No | No | 17-Dec-2004 | ICT | 5 | 1<=10 |
| 67 | M | Ct | H | 1a | 1 | 13.9 | No | No | No | No | 12-Jan-1999 | LFU | 2 | 11<=30 |
| 54 | M | E>1 | H | 1b | 1 | 15.5 | No | No | No | No | 30-Oct-2006 | Alive | 3 | |
| 60 | M | E>1 | H | 2a | 2 | 14.2 | No | No | No | No | 04-Dec-2004 | DotherC | 15 | 1<=10 |
| 56 | M | NK | NK | 1a | 1 | 99.9 | No | No | No | No | 09-Nov-2007 | Alive | 20 | |
| 72 | F | E>1 | L | 2a | 2 | 13.8 | No | No | No | No | 22-Apr-1999 | LFU | <1 | |
| 44 | M | NK | NK | 1a | 1 | 99.9 | No | No | No | No | 11-Oct-2007 | Alive | 20 | |
| 62 | M | E<1 | L | 1a | 1 | 99.9 | No | No | No | No | 28-Sep-2007 | Alive | <1 | 1<=10 |
| 69 | M | E>1 | NK | 2b | 2 | 14.9 | No | No | No | No | 14-Sep-2007 | ICT | 30 | 1<=10 |
| 64 | M | Ct | L | 1a | 1 | 13.3 | No | No | No | No | 22-Oct-2003 | LFU | 1 | 11<=30 |
| 62 | M | NK | L | 1a | 1 | 14.2 | No | No | No | No | 07-Sep-2007 | Alive | 3 | |
| 65 | M | Ct | H | 1a | 1 | 13.6 | No | No | No | No | 12-Apr-2000 | LFU | <1 | 11<=30 |
| 54 | M | Ct | L | 1a | 1 | 14.5 | No | No | No | No | 12-May-2000 | LFU | 1 | 11<=30 |
| 52 | M | E<1 | L | 2a | 2 | 99.9 | No | No | No | No | 11-Apr-2007 | Alive | 3 | 1<=10 |
| 75 | M | E>1 | NK | 1b | 1 | 14.7 | No | No | No | No | 06-Mar-2006 | ICT | 35 | 1<=10 |
| 57 | M | E>1 | N | 1a | 1 | 12.5 | No | No | No | Yes | 01-Apr-2000 | DdthisC | 3 | <1 |
| 62 | M | Ct | NK | 1a | 1 | 14.3 | No | No | No | No | 18-Jul-2007 | Alive | 70 | 1<=10 |
| 48 | M | E>1 | L | 1a | 1 | 14.1 | No | No | No | No | 19-Sep-2007 | Alive | 8 | |
| 50 | M | Ct | H | is | 0 | 13.2 | No | No | No | No | 01-Oct-2007 | Alive | 8 | >30 |
| 79 | F | Ct | L | 2a | 2 | 12.4 | No | No | No | No | 28-May-2005 | ICT | 25 | |
| 51 | M | E>1 | L | 1a | 1 | 14.1 | No | No | No | No | 13-Mar-2006 | Alive | 10 | >30 |
| 45 | M | Ct | H | 1b | 1 | 15.4 | Yes | Yes | No | No | 15-Jul-2007 | Alive | 5 | |
| 62 | M | E>1 | NK | 1a | 1 | 13.6 | No | No | No | No | 15-Mar-2005 | Alive | 8 | >30 |
| 53 | M | E>1 | H | 1a | 1 | 99.9 | No | No | No | No | 04-Jun-2007 | Alive | 8 | 1<=10 |
| 53 | M | E>1 | NK | 1a | 1 | 14.7 | Yes | Yes | No | Yes | 20-Jul-2001 | DdthisC | 3 | 11<=30 |
| 73 | M | Ct | NK | 2a | 2 | 16.3 | No | No | No | No | 06-Jan-2004 | LFU | 8 | <1 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 74 | F | No | N | 1a | 1 | 14.7 | No | No | No | No | 20-Aug-2000 | ICT | <1 | 1<=10 |
| 76 | M | E>1 | NK | 2a | 2 | 11.9 | No | No | No | No | 18-Sep-2007 | Alive | <1 | |
| 56 | M | Ct | L | 1a | 1 | 15.3 | No | No | No | No | 06-Oct-2006 | Alive | <1 | 11<=30 |
| 64 | M | Ct | L | 2a | 2 | 13.1 | No | No | No | No | 16-Oct-2007 | Alive | 30 | 11<=30 |
| 68 | M | Ct | H | 1a | 1 | 15.8 | No | No | No | No | 20-Oct-2003 | LFU | 40 | 11<=30 |
| 50 | M | Ct | H | 2b | 2 | 14.5 | Yes | Yes | No | No | 14-Feb-2000 | ICT | 15 | |
| 72 | M | E<1 | L | 1b | 1 | 14.7 | No | No | No | No | 13-Aug-2007 | Alive | 3 | |
| 56 | M | Ct | NK | 1a | 1 | 99.9 | No | No | No | No | 07-Nov-2005 | Alive | <1 | |
| 50 | M | E<1 | L | 2a | 2 | 16.5 | No | No | No | No | 17-Aug-2004 | LFU | <1 | <1 |
| 57 | M | Ct | NK | 1b | 1 | 14.2 | Yes | No | Yes | Yes | 18-Oct-2007 | Alive | 15 | 11<=30 |
| 39 | M | E<1 | H | 1b | 1 | 14.7 | No | No | No | No | 04-May-2007 | Alive | 20 | >30 |
| 87 | M | E>1 | NK | 1a | 1 | 11.0 | No | No | No | No | 30-Jun-1999 | DdthisC | 5 | <1 |
| 56 | M | Ct | H | 1b | 1 | 14.8 | No | No | No | No | 02-Feb-2005 | LFU | <1 | 11<=30 |
| 49 | M | E<1 | L | 3 | 2 | 15.2 | No | No | No | No | 31-Aug-2004 | DdthisC | 30 | 11<=30 |
| 60 | M | E>1 | L | 1a | 1 | 15.1 | No | No | No | No | 28-Aug-2007 | Alive | 8 | 11<=30 |
| 72 | M | E<1 | NK | 1a | 1 | 13.4 | No | No | No | No | 28-May-2007 | ICT | <1 | 11<=30 |
| 60 | M | E<1 | L | 1a | 1 | 99.9 | No | No | No | No | 31-Oct-2007 | Alive | <1 | 1<=10 |
| 67 | M | Ct | NK | 3 | 2 | 99.9 | No | No | No | No | 29-Oct-2002 | ICT | 30 | >30 |
| 74 | M | Ct | L | 2a | 2 | 14.3 | No | Yes | No | Yes | 26-Jun-2006 | DdthisC | 8 | 1<=10 |
| 71 | M | No | L | 1a | 1 | 13.5 | No | No | No | No | 22-Oct-2007 | Alive | 25 | |
| 70 | M | E>1 | H | 1a | 1 | 13.9 | No | No | No | No | 25-Oct-2007 | Alive | <1 | >30 |
| 64 | M | Ct | NK | 3 | 2 | 13.9 | Yes | No | Yes | No | 07-Jul-2000 | DdthisC | 20 | >30 |
| 79 | M | E>1 | L | 2a | 2 | 12.8 | No | No | No | No | 10-Oct-2007 | Alive | 5 | <1 |
| 49 | M | Ct | L | 1a | 1 | 14.9 | No | No | No | No | 19-Sep-2005 | DdthisC | 30 | 1<=10 |
| 66 | M | Ct | L | 1a | 1 | 14.2 | No | No | No | No | 05-Nov-2007 | Alive | 8 | 11<=30 |
| 70 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 24-Feb-2000 | LFU | 10 | |
| 65 | M | E>1 | L | 2a | 4 | 15.5 | Yes | Yes | Yes | No | 29-Apr-2002 | DdthisC | 30 | 11<=30 |
| 51 | M | E>1 | L | 1a | 1 | 14.1 | No | No | No | No | 03-Sep-2007 | Alive | 30 | 11<=30 |
| 78 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 01-Jan-2000 | DotherC | <1 | 11<=30 |
| 53 | M | E>1 | H | 1b | 1 | 14.6 | No | No | No | No | 20-Feb-2007 | Alive | 30 | >30 |
| 85 | M | E>1 | NK | 1a | 1 | 14.9 | No | No | No | No | 04-Apr-2003 | ICT | 30 | |
| 71 | M | E>1 | NK | 2b | 2 | 14.1 | No | No | No | No | 06-Oct-2006 | DdthisC | 10 | >30 |
| 66 | M | E<1 | H | 1b | 1 | 13.5 | No | No | No | No | 14-Nov-2007 | Alive | 1 | >30 |
| 81 | M | E>1 | N | 2a | 2 | 13.0 | No | No | No | No | 21-Oct-2004 | ICT | 40 | >30 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 75 | M | NK | NK | 2b | 2 | 15.3 | No | No | No | No | 13-Aug-2007 | Alive | 20 | 1<=10 |
| 44 | M | E<1 | H | 1a | 1 | 15.0 | No | No | No | No | 16-Sep-2007 | Alive | 3 | <1 |
| 68 | F | E<1 | L | Is | 0 | 13.1 | No | No | No | No | 12-Apr-2001 | DotherC | 10 | |
| 37 | M | Ct | L | 2a | 2 | 99.9 | No | No | No | No | 01-May-2007 | Alive | <1 | 11<=30 |
| 69 | M | E>1 | L | 2a | 2 | 13.9 | No | No | No | No | 12-Oct-2007 | Alive | 8 | 1<=10 |
| 76 | M | E>1 | N | 3 | 2 | 16.4 | No | No | No | No | 10-Jul-2005 | ICT | 3 | 11<=30 |
| 69 | M | NK | NK | 1a | 1 | 13.1 | No | No | No | No | 02-Nov-2007 | Alive | 3 | 11<=30 |
| 77 | F | Ct | L | 2a | 2 | 12.0 | No | No | No | No | 04-Feb-2000 | DdthisC | 1 | 11<=30 |
| 67 | M | NK | L | 1a | 1 | 13.8 | No | No | No | No | 11-Oct-2007 | Alive | 40 | >30 |
| 68 | M | E<1 | L | 1a | 1 | 99.9 | No | No | No | No | 07-Sep-2007 | Alive | 20 | |
| 69 | M | E<1 | NK | 2a | 2 | 13.9 | No | No | No | No | 15-Oct-2003 | DdthisC | 5 | 1<=10 |
| 52 | M | E<1 | L | 1b | 1 | 14.3 | No | No | No | No | 02-Nov-2005 | Alive | 3 | 11<=30 |
| 54 | M | Ct | H | 1a | 1 | 14.0 | No | No | No | No | 08-Feb-2005 | LFU | 10 | |
| 45 | M | Ct | L | 1a | 1 | 15.5 | No | No | No | No | 02-Apr-2007 | Alive | <1 | 11<=30 |
| 72 | M | No | H | 1b | 1 | 12.9 | No | No | No | No | 25-Sep-2000 | DdthisC | 5 | <1 |
| 80 | M | E>1 | L | 2b | 2 | 13.9 | No | No | No | No | 02-May-2002 | ICT | 30 | 1<=10 |
| 54 | M | E<1 | H | 2b | 2 | 14.5 | No | No | No | No | 06-May-2005 | Alive | 5 | |
| 72 | M | Ct | L | 2b | 2 | 13.5 | No | No | No | No | 09-Nov-2000 | DdthisC | 3 | 11<=30 |
| 63 | M | E<1 | NK | 1b | 1 | 13.8 | No | No | No | No | 11-May-2005 | DotherC | <1 | |
| 80 | M | E>1 | PH | 1b | 1 | 10.8 | No | No | No | No | 21-Nov-2002 | ICT | <1 | |
| 62 | M | NK | L | 1a | 1 | 13.3 | No | No | No | No | 25-Oct-2006 | ICT | 15 | 1<=10 |
| 57 | M | E<1 | H | 1a | 1 | 14.3 | No | No | No | No | 24-Apr-2005 | Alive | 3 | <1 |
| 73 | M | Ct | L | 1a | 1 | 14.5 | No | No | No | No | 21-Apr-2006 | Alive | 50 | 11<=30 |
| 45 | M | Ct | N | 1a | 1 | 13.8 | No | No | No | No | 31-Jul-2007 | Alive | <1 | |
| 65 | M | NK | H | 1a | 1 | 14.4 | No | No | No | No | 20-Sep-2007 | Alive | <1 | |
| 67 | M | No | H | 1a | 1 | 14.3 | No | No | No | No | 02-Dec-2004 | LFU | 5 | <1 |
| 70 | M | E>1 | L | 2b | 2 | 14.3 | Yes | No | Yes | No | 21-Feb-2002 | DdthisC | 1 | 1<=10 |
| 50 | M | E<1 | NK | 2b | 2 | 14.8 | Yes | Yes | No | No | 14-Feb-2003 | LFU | 20 | |
| 66 | F | Ct | L | 1a | 1 | 99.9 | No | No | No | No | 07-Mar-2006 | ICT | 1 | |
| 59 | M | E>1 | NK | 1a | 1 | 15.8 | No | No | No | No | 18-Dec-2006 | Alive | <1 | 1<=10 |
| 74 | M | E>1 | NK | 1a | 1 | 13.9 | No | No | No | No | 18-Sep-2007 | Alive | <1 | <1 |
| 70 | M | E>1 | NK | 2b | 2 | 15.2 | No | No | No | No | 07-Nov-2007 | Alive | 1 | |
| 51 | M | Ct | L | 1a | 1 | 15.0 | No | No | No | No | 24-Aug-2004 | LFU | 30 | |
| 70 | M | Ct | L | 2b | 2 | 15.1 | No | No | No | No | 02-Jan-2005 | DotherC | 5 | >30 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 68 | M | E>1 | N | 1a | 1 | 14.9 | No | No | No | No | 10-Oct-2007 | Alive | 1 | 1<=10 |
| 68 | M | E>1 | NK | 1a | 1 | 13.4 | No | No | No | No | 23-Aug-2007 | Alive | 20 | 1<=10 |
| 66 | M | E>1 | L | 2b | 2 | 15.4 | No | No | No | No | 13-May-2005 | ICT | 1 | |
| 39 | M | Ct | PH | is | 0 | 15.6 | No | No | No | No | 19-Feb-2006 | Alive | 5 | |
| 80 | M | E>1 | L | 2b | 2 | 12.1 | No | No | No | No | 11-Jun-2007 | Alive | 5 | 11<=30 |
| 70 | F | E>1 | N | 1a | 1 | 13.4 | No | No | No | No | 20-Nov-2007 | Alive | 3 | 11<=30 |
| 53 | M | E>1 | L | 1a | 1 | 13.8 | No | No | No | No | 01-Apr-2005 | Alive | 50 | >30 |
| 79 | M | No | L | 1a | 1 | 15.3 | No | No | No | No | 06-Aug-2007 | Alive | <1 | 1<=10 |
| 65 | M | E<1 | L | 2a | 2 | 14.3 | No | No | No | No | 22-Jul-2003 | DotherC | <1 | >30 |
| 73 | M | E>1 | L | is | 0 | 15.3 | Yes | Yes | No | No | 18-Sep-2007 | Alive | <1 | |
| 61 | M | E<1 | NK | 1a | 1 | 13.4 | No | No | No | No | 27-Jun-2007 | Alive | <1 | 1<=10 |
| 69 | M | Ct | H | 2a | 2 | 13.4 | No | No | No | No | 28-Dec-2006 | Alive | <1 | |
| 69 | M | E>1 | H | 1a | 1 | 14.1 | No | No | No | No | 07-Aug-2007 | Alive | 10 | 11<=30 |
| 41 | M | E<1 | L | 2a | 2 | 14.7 | Yes | Yes | Yes | No | 03-Mar-2001 | DdthisC | 30 | 11<=30 |
| 50 | M | E<1 | H | 2a | 2 | 13.2 | No | No | No | No | 18-Oct-2007 | Alive | 1 | |
| 78 | M | Ct | L | is | 0 | 15.3 | No | No | No | No | 26-Jan-2003 | ICT | 35 | 1<=10 |
| 80 | M | E>1 | H | is | 0 | 13.6 | No | No | No | No | 25-Oct-2006 | ICT | 5 | |
| 79 | M | No | L | 2b | 2 | 99.9 | Yes | Yes | No | No | 25-Oct-2006 | DotherC | <1 | >30 |
| 70 | F | Ct | L | 2a | 2 | 13.2 | No | No | No | No | 18-Jan-2006 | Alive | 1 | 11<=30 |
| 74 | F | E<1 | L | 2a | 2 | 99.9 | No | No | No | No | 15-Dec-2004 | LFU | <1 | |
| 76 | M | E<1 | L | 1a | 1 | 11.5 | No | No | No | No | 17-Apr-2001 | ICT | <1 | >30 |
| 67 | M | E>1 | L | 3 | 2 | 13.0 | No | No | No | No | 06-Sep-2007 | Alive | <1 | 1<=10 |
| 48 | M | Ct | H | 2a | 2 | 15.0 | No | No | No | No | 16-Oct-2007 | Alive | 3 | |
| 61 | F | E<1 | L | 1a | 1 | 99.9 | No | No | No | No | 05-Sep-2000 | LFU | 5 | 1<=10 |
| 68 | M | E<1 | Med | 1b | 1 | 12.6 | No | No | No | No | 23-Nov-2000 | LFU | 10 | 11<=30 |
| 86 | M | No | N | 2a | 2 | 12.5 | No | No | No | No | 27-Jan-2007 | ICT | 15 | 11<=30 |
| 74 | M | E<1 | L | 1a | 1 | 10.3 | No | No | No | No | 25-Sep-2007 | Alive | 5 | 11<=30 |
| 77 | M | Ct | H | 2a | 2 | 13.5 | No | No | No | No | 17-Sep-2007 | Alive | 3 | >30 |
| 63 | M | E>1 | NK | 2a | 2 | 12.6 | No | No | No | No | 26-Jun-2005 | ICT | 50 | |
| 69 | M | E>1 | L | is | 0 | 14.6 | Yes | Yes | No | No | 20-Feb-2002 | DdthisC | 10 | 11<=30 |
| 57 | M | Ct | H | 3 | 2 | 13.7 | Yes | No | Yes | No | 26-Dec-2000 | DdthisC | 5 | 11<=30 |
| 51 | F | Ct | N | 1a | 1 | 15.1 | No | No | No | No | 15-Oct-2007 | Alive | <1 | |
| 60 | M | Ct | H | 1a | 1 | 15.0 | No | No | No | No | 26-Sep-2007 | Alive | 30 | 11<=30 |
| 70 | M | E<1 | L | 1a | 1 | 15.4 | Yes | Yes | No | No | 05-Nov-2005 | ICT | 10 | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 69 | F | E<1 | NK | 2a | 2 | 13.2 | No | No | No | No | 15-Nov-2007 | Alive | 15 | |
| 54 | M | E>1 | H | 1a | 1 | 99.9 | No | No | No | No | 16-Jun-2006 | Alive | 55 | 1<=10 |
| 56 | M | E<1 | H | 1a | 1 | 15.8 | No | No | No | No | 18-Feb-2007 | DdthisC | <1 | |
| 63 | F | Ct | N | 3 | 2 | 13.9 | No | No | No | No | 03-Oct-2007 | Alive | 15 | 1<=10 |
| 47 | M | E<1 | H | 2b | 2 | 15.4 | Yes | Yes | No | No | 28-Apr-2003 | Alive | 40 | 1<=10 |
| 78 | M | E>1 | NK | 1a | 1 | 11.6 | No | No | No | No | 03-Feb-2003 | DdthisC | <1 | 11<=30 |
| 61 | F | E<1 | L | 1a | 1 | 14.8 | No | No | No | No | 17-Oct-2007 | Alive | <1 | 11<=30 |
| 56 | M | E>1 | PH | 2a | 2 | 14.2 | No | No | No | No | 17-Apr-2007 | Alive | 20 | 1<=10 |
| 68 | M | E<1 | N | 2a | 2 | 14.1 | No | No | No | No | 04-Jul-2007 | Alive | 8 | 1<=10 |
| 43 | M | Ct | H | 2a | 2 | 13.8 | No | No | No | No | 01-Oct-2007 | Alive | 5 | 1<=10 |
| 63 | M | E>1 | L | 2a | 2 | 14.2 | Yes | Yes | No | No | 12-Apr-2002 | DdthisC | 20 | 11<=30 |
| 51 | M | E<1 | H | 1a | 1 | 15.0 | No | No | No | No | 16-Aug-2007 | Alive | 5 | |
| 70 | M | E>1 | N | 1a | 1 | 13.6 | No | No | No | No | 28-Sep-2000 | LFU | 50 | |
| 69 | M | E>1 | PH | 1a | 1 | 12.7 | No | No | No | No | 04-Dec-2007 | Alive | 5 | |
| 59 | M | Ct | H | 3 | 2 | 14.4 | No | No | No | No | 22-Nov-2007 | Alive | 30 | 1<=10 |
| 55 | M | E>1 | H | 2b | 2 | 14.8 | No | No | No | No | 02-Apr-2007 | ICT | 4 | 1<=10 |
| 57 | M | E>1 | L | 1b | 1 | 15.2 | No | No | No | No | 15-Nov-2000 | LFU | 5 | 1<=10 |
| 72 | M | E>1 | H | is | 0 | 14.8 | No | No | No | No | 02-Aug-2007 | Alive | <1 | 11<=30 |
| 60 | M | E>1 | H | 1a | 1 | 13.4 | No | No | No | No | 26-Mar-2007 | Alive | 17 | 1<=10 |
| 61 | M | Ct | PH | 1a | 1 | 14.5 | No | No | No | No | 12-Oct-2007 | Alive | <1 | 1<=10 |
| 59 | M | E<1 | H | 2b | 2 | 14.7 | No | No | No | No | 17-Apr-2006 | ICT | 10 | 11<=30 |
| 81 | M | E>1 | L | 1a | 1 | 13.4 | No | No | No | No | 02-Feb-2006 | Alive | 50 | 11<=30 |
| 79 | M | E<1 | N | 2a | 2 | 14.2 | Yes | No | No | Yes | 14-Aug-2003 | DdthisC | 8 | |
| 65 | F | Ct | L | 2a | 2 | 12.9 | No | No | No | No | 28-Dec-2004 | ICT | 8 | |
| 79 | M | E>1 | NK | 1a | 1 | 13.5 | No | No | No | No | 15-Dec-2005 | Alive | 10 | 1<=10 |
| 82 | M | E>1 | H | 2a | 2 | 15.2 | No | No | No | No | 28-Aug-2007 | Alive | <1 | >30 |
| 74 | M | E>1 | NK | 2b | 2 | 15.9 | No | No | No | No | 15-Feb-2005 | DdthisC | <1 | |
| 63 | M | E<1 | N | 1a | 1 | 15.5 | Yes | No | Yes | No | 01-May-2004 | DdthisC | <1 | |
| 74 | M | E<1 | L | is | 0 | 13.4 | No | No | No | No | 29-Jun-2004 | DotherC | 5 | |
| 81 | M | E>1 | L | 1a | 1 | 15.2 | No | No | No | No | 17-Oct-2007 | Alive | 5 | 1<=10 |
| 49 | F | E<1 | NK | 3 | 2 | 13.6 | No | No | No | No | 15-Jan-2007 | ICT | 8 | <1 |
| 82 | M | Ct | L | 2a | 2 | 15.7 | No | No | No | No | 30-Nov-2005 | DotherC | <1 | |
| 56 | M | Ct | L | 2a | 2 | 14.4 | No | No | No | No | 27-Sep-2007 | Alive | 5 | |
| 63 | M | Ct | H | 1a | 1 | 17.0 | No | No | No | No | 09-Apr-2004 | DotherC | 25 | 11<=30 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 63 | M | E<1 | L | 2b | 2 | 15.4 | No | No | No | No | 05-Oct-2007 | Alive | 3 | 11<=30 |
| 51 | M | Ct | NK | 1b | 1 | 15.2 | No | No | No | No | 02-Jul-2007 | Alive | <1 | |
| 53 | M | E<1 | L | 2a | 2 | 14.5 | No | No | No | No | 25-Apr-2007 | Alive | 8 | 1<=10 |
| 48 | M | E<1 | L | is | 0 | 15.1 | No | No | No | No | 18-May-2007 | Alive | 3 | |
| 56 | M | Ct | H | 2a | 2 | 14.3 | No | No | No | No | 22-Sep-2007 | Alive | 8 | 11<=30 |
| 77 | M | E>1 | L | 2a | 2 | 14.9 | No | No | No | No | 27-Sep-2007 | Alive | <1 | |
| 45 | M | Ct | H | is | 0 | 99.9 | No | No | No | No | 14-Mar-2001 | LFU | <1 | |
| 55 | M | E>1 | L | 3 | 2 | 15.3 | Yes | No | Yes | No | 06-Jun-2002 | DdthisC | <1 | |
| 54 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 19-Apr-2004 | LFU | 15 | <1 |
| 56 | M | E>1 | H | 1a | 1 | 14.9 | No | No | No | No | 12-Jan-2007 | Alive | 15 | >30 |
| 75 | M | E>1 | L | 1a | 1 | 14.9 | No | No | No | No | 20-Mar-2005 | ICT | <1 | 1<=10 |
| 55 | F | Ct | H | 2a | 2 | 13.8 | Yes | Yes | No | No | 09-Oct-2002 | DdthisC | <1 | 1<=10 |
| 61 | M | E<1 | L | 2a | 2 | 16.3 | No | No | No | No | 01-Dec-2002 | ICT | 4 | |
| 41 | M | Ct | H | 1b | 1 | 14.8 | No | No | No | No | 05-Jul-2007 | Alive | 20 | |
| 72 | M | E>1 | L | 1a | 1 | 15.0 | Yes | Yes | Yes | Yes | 11-Nov-2001 | DdthisC | 3 | |
| 81 | M | Ct | L | 1a | 1 | 16.5 | No | No | No | No | 22-Apr-2004 | DotherC | <1 | 1<=10 |
| 64 | M | E>1 | L | 1a | 1 | 13.0 | No | No | No | No | 20-Dec-2001 | ICT | 30 | >30 |
| 81 | M | E>1 | PH | 2a | 2 | 14.0 | No | No | No | No | 18-Mar-2004 | ICT | <1 | 1<=10 |
| 60 | M | E<1 | H | 1a | 1 | 15.4 | No | No | No | No | 14-Mar-2007 | Alive | 30 | 1<=10 |
| 77 | M | E<1 | PH | 1a | 1 | 12.4 | No | No | No | No | 18-Nov-2006 | DotherC | <1 | 11<=30 |
| 55 | F | E>1 | L | is | 0 | 13.5 | No | No | No | No | 05-Sep-2007 | Alive | 3 | <1 |
| 70 | M | No | L | is | 0 | 14.4 | No | No | No | No | 10-Oct-2007 | Alive | 3 | |
| 63 | M | E<1 | H | 2a | 2 | 14.5 | No | No | No | No | 16-Oct-2001 | ICT | 15 | |
| 65 | F | Ct | L | is | 0 | 15.3 | No | No | No | No | 20-Aug-2007 | Alive | 8 | 11<=30 |
| 81 | F | E>1 | N | 1a | 1 | 13.2 | No | No | No | No | 19-Oct-2005 | LFU | <1 | <1 |
| 59 | M | No | L | 2a | 2 | 15.0 | No | No | No | No | 16-Jan-2006 | LFU | <1 | |
| 78 | M | E<1 | H | 1a | 1 | 17.0 | No | No | No | No | 04-Jul-2006 | LFU | <1 | 1<=10 |
| 58 | M | E<1 | H | 1a | 1 | 15.6 | No | No | No | No | 17-Oct-2007 | Alive | <1 | 1<=10 |
| 62 | M | NK | L | 1a | 1 | 13.6 | No | No | No | No | 07-Nov-2007 | Alive | 8 | |
| 59 | M | E>1 | L | 1a | 1 | 14.0 | No | No | No | No | 29-Oct-2007 | Alive | 40 | >30 |
| 62 | M | No | L | 2b | 2 | 15.1 | Yes | Yes | No | No | 21-Sep-2007 | Alive | <1 | |
| 68 | M | E<1 | L | 1a | 1 | 13.7 | No | No | No | No | 19-Feb-2003 | DotherC | 5 | 1<=10 |
| 76 | M | E>1 | L | 1a | 1 | 15.6 | No | No | No | No | 24-Nov-2005 | ICT | 30 | <1 |
| 52 | M | Ct | PH | 1b | 1 | 13.3 | No | No | No | No | 12-Oct-2007 | Alive | 8 | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 71 | F | E<1 | NK | 3 | 2 | 15.5 | No | No | No | No | 01-Jan-2002 | ICT | <1 | 1<=10 |
| 51 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 20-Aug-2007 | Alive | <1 | |
| 65 | M | E<1 | L | 1a | 1 | 15.3 | No | No | No | No | 03-Oct-2007 | Alive | 25 | >30 |
| 51 | M | E<1 | L | 1a | 1 | 14.1 | No | No | No | No | 05-Feb-2007 | Alive | 30 | <1 |
| 59 | F | E>1 | H | 1a | 1 | 14.0 | No | No | No | No | 13-Jul-2007 | Alive | 2 | 11<=30 |
| 82 | M | E>1 | L | 3 | 2 | 11.9 | No | No | No | No | 18-Sep-2004 | LFU | 5 | 11<=30 |
| 79 | M | E>1 | L | 1a | 1 | 12.8 | No | No | No | No | 21-Mar-2004 | DotherC | 1 | |
| 61 | F | Ct | L | is | 0 | 13.4 | No | No | No | No | 03-Jul-2007 | Alive | <1 | |
| 70 | M | Ct | H | 2b | 2 | 13.2 | No | No | No | No | 01-Apr-2002 | ICT | <1 | >30 |
| 73 | M | Ct | PH | 1b | 1 | 99.9 | No | No | No | No | 01-Aug-2007 | Alive | <1 | |
| 89 | F | E>1 | NK | 1a | 1 | 99.9 | No | No | No | No | 16-Aug-2006 | Alive | 15 | |
| 55 | F | NK | NK | 2b | 2 | 12.9 | Yes | No | Yes | No | 23-Oct-2007 | Alive | 1 | |
| 59 | M | Ct | H | 2a | 2 | 13.7 | No | No | No | No | 22-Nov-2007 | Alive | <1 | 11<=30 |
| 77 | M | E>1 | L | 1a | 1 | 14.2 | Yes | Yes | No | No | 04-Aug-2006 | Alive | 40 | 1<=10 |
| 53 | M | E>1 | L | 1a | 1 | 14.0 | No | No | No | No | 19-Jun-2007 | Alive | 3 | 1<=10 |
| 80 | M | Ct | NK | 1a | 1 | 11.9 | No | No | No | No | 15-Mar-2005 | DdthisC | <1 | |
| 56 | M | E>1 | L | 1a | 1 | 15.6 | Yes | Yes | No | No | 14-Jun-2007 | Alive | 40 | 1<=10 |
| 54 | M | E<1 | H | 3 | 2 | 14.4 | No | No | No | No | 17-Sep-2007 | Alive | <1 | |
| 77 | M | E>1 | L | is | 0 | 16.0 | No | No | No | No | 12-Oct-2007 | Alive | <1 | <1 |
| 80 | F | E>1 | L | 1b | 1 | 13.4 | No | No | No | No | 25-Dec-2002 | DdthisC | 70 | 11<=30 |
| 70 | M | E>1 | L | 1b | 1 | 14.6 | No | No | No | No | 05-Sep-2007 | Alive | 15 | 11<=30 |
| 76 | M | E>1 | L | 1b | 1 | 14.8 | No | No | No | No | 11-Sep-2007 | Alive | 3 | |
| 69 | M | No | L | 1a | 1 | 14.5 | No | No | No | No | 13-Jun-2007 | Alive | 4 | |
| 67 | M | E>1 | H | 1a | 1 | 14.1 | No | No | No | No | 25-Jul-2007 | Alive | 1 | >30 |
| 60 | M | E<1 | H | 3 | 2 | 14.3 | No | No | No | No | 11-Oct-2007 | Alive | 1 | 11<=30 |
| 56 | M | E<1 | H | 2b | 2 | 14.9 | No | No | No | No | 14-Jul-2005 | DotherC | <1 | 1<=10 |
| 62 | M | Ct | L | 2b | 2 | 14.0 | Yes | Yes | Yes | No | 04-Nov-2002 | DdthisC | 10 | 11<=30 |
| 77 | M | E>1 | L | 2b | 2 | 12.4 | No | No | No | No | 29-Dec-2001 | ICT | 3 | |
| 56 | M | E<1 | L | 2a | 2 | 14.7 | No | No | No | No | 08-May-2007 | Alive | 30 | <1 |
| 71 | F | E>1 | L | 1a | 1 | 12.3 | No | No | No | No | 25-Jun-2007 | Alive | <1 | >30 |
| 78 | M | E>1 | L | 1a | 1 | 11.9 | No | No | No | No | 25-May-2004 | DdthisC | <1 | 1<=10 |
| 88 | M | E>1 | L | 1a | 1 | 11.6 | Yes | Yes | No | No | 27-Nov-2005 | ICT | 20 | 1<=10 |
| 51 | M | E<1 | L | 1a | 1 | 13.3 | No | No | No | No | 27-Apr-2005 | DotherC | 30 | 11<=30 |
| 80 | M | No | N | 1a | 1 | 13.2 | No | No | No | No | 21-Oct-2009 | Alive | <1 | 11<=30 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 75 | M | E>1 | L | 1a | 1 | 13.2 | No | No | No | No | 24-Jul-2007 | ICT | <1 | |
| 50 | M | Ct | L | 2a | 2 | 13.3 | No | No | No | No | 19-Oct-2007 | Alive | 10 | |
| 74 | M | No | L | is | 0 | 99.9 | No | No | No | No | 31-Jan-2007 | Alive | 4 | 1<=10 |
| 66 | M | Ct | H | 1b | 1 | 99.9 | No | No | No | No | 13-Nov-2006 | ICT | 30 | <1 |
| 61 | M | E>1 | L | 1a | 1 | 15.1 | No | No | No | No | 23-Oct-2007 | Alive | 60 | 11<=30 |
| 48 | F | Ct | L | 1a | 1 | 12.9 | No | No | No | No | 02-Oct-2007 | Alive | 15 | |
| 75 | M | E>1 | L | 1a | 1 | 12.1 | No | No | No | No | 24-Feb-2005 | DotherC | <1 | 1<=10 |
| 49 | F | E>1 | L | 1a | 1 | 13.2 | No | No | No | No | 01-Nov-2007 | Alive | 12 | 11<=30 |
| 62 | M | E>1 | L | 1a | 1 | 14.0 | No | No | No | No | 12-Sep-2007 | Alive | 10 | |
| 63 | M | Ct | L | 1a | 1 | 15.9 | No | No | No | No | 15-May-2002 | DdthisC | 30 | <1 |
| 67 | M | No | L | 1b | 1 | 15.2 | Yes | No | Yes | No | 05-Jun-2003 | DdthisC | <1 | |
| 76 | M | E>1 | PH | 1a | 1 | 16.4 | No | No | No | No | 15-Oct-2007 | Alive | <1 | 11<=30 |
| 65 | M | Ct | PH | 1b | 1 | 14.4 | No | No | No | No | 17-Oct-2007 | Alive | 30 | 1<=10 |
| 61 | M | E<1 | H | 2a | 2 | 14.8 | No | No | No | No | 07-Nov-2006 | Alive | <1 | |
| 84 | M | E>1 | L | 3 | 2 | 16.0 | No | No | No | No | 18-Oct-2007 | Alive | 3 | 1<=10 |
| 74 | F | E>1 | L | 1a | 1 | 14.4 | No | No | No | No | 16-Oct-2007 | Alive | <1 | 11<=30 |
| 47 | M | No | L | 2a | 2 | 13.5 | No | No | No | No | 17-Sep-2007 | Alive | 5 | |
| 54 | M | Ct | L | 3 | 2 | 15.0 | No | No | No | No | 25-Jun-2007 | Alive | | <1 |
| 64 | M | E>1 | L | 1a | 1 | 12.7 | No | No | No | No | 01-Feb-2006 | LFU | | |
| 73 | M | No | L | is | 0 | 15.2 | No | No | No | No | 26-Jan-2007 | Alive | | |
| 71 | M | E<1 | L | 1b | 1 | 16.9 | No | No | No | No | 07-Feb-2007 | Alive | | 11<=30 |
| 82 | M | E>1 | L | 2b | 2 | 12.3 | No | No | No | No | 20-Apr-2002 | DotherC | | 11<=30 |
| 64 | M | Ct | L | 2a | 2 | 14.7 | No | No | No | No | 06-Aug-2007 | Alive | | |
| 64 | M | E>1 | H | 3 | 2 | 14.8 | Yes | Yes | No | No | 22-Aug-2007 | Alive | | <1 |
| 46 | M | E>1 | L | 1a | 1 | 15.8 | Yes | Yes | No | No | 07-May-2003 | LFU | | 11<=30 |
| 48 | M | E<1 | PH | 2a | 2 | 13.1 | No | No | No | No | 02-Jul-2007 | Alive | | 1<=10 |
| 67 | M | Ct | H | is | 0 | 99.9 | No | No | No | No | 07-Jan-2005 | ICT | | |
| 49 | M | E<1 | L | 1a | 1 | 14.2 | No | No | No | No | 15-Dec-2006 | Alive | | 1<=10 |
| 80 | M | E>1 | L | 3 | 2 | 13.2 | No | No | No | No | 16-Oct-2007 | Alive | | |
| 51 | M | E<1 | L | is | 0 | 13.0 | No | No | No | No | 22-Aug-2007 | Alive | | 11<=30 |
| 55 | M | E<1 | H | 1a | 2 | 13.5 | Yes | No | Yes | No | 31-May-2003 | DdthisC | | 11<=30 |
| 52 | M | Ct | L | 1a | 1 | 99.9 | No | No | No | No | 11-Oct-2007 | Alive | | 11<=30 |
| 78 | M | E>1 | L | 1a | 1 | 14.6 | No | No | No | No | 11-Oct-2007 | Alive | | 1<=10 |
| 68 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 15-Mar-2007 | Alive | | <1 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 71 | M | E>1 | L | 3 | 2 | 10.2 | Yes | No | No | Yes | 26-Jun-2002 | DdthisC | | 1<=10 |
| 54 | M | E>1 | PH | 1a | 1 | 99.9 | No | No | No | No | 05-Mar-2007 | Alive | | 11<=30 |
| 42 | M | E<1 | PH | 1a | 1 | 99.9 | No | No | No | No | 08-Mar-2007 | Alive | | 1<=10 |
| 65 | M | E<1 | H | 1a | 1 | 13.6 | No | No | No | No | 23-May-2007 | Alive | | 1<=10 |
| 53 | M | E<1 | L | 1a | 1 | 14.9 | No | No | No | No | 15-Feb-2007 | Alive | | 1<=10 |
| 62 | M | E<1 | L | 2b | 2 | 16.0 | No | No | No | No | 15-Oct-2007 | Alive | | 1<=10 |
| 62 | M | Ct | PH | 2a | 2 | 13.7 | No | No | No | No | 14-Nov-2007 | Alive | | 1<=10 |
| 83 | F | E<1 | L | 1a | 1 | 15.0 | No | No | No | No | 12-Apr-2006 | Alive | | 1<=10 |
| 63 | M | E>1 | NK | 1a | 1 | 99.9 | Yes | Yes | No | No | 19-Oct-2004 | LFU | | <1 |
| 68 | F | E<1 | L | 1a | 1 | 10.6 | No | No | No | No | 02-Nov-2007 | Alive | | 1<=10 |
| 89 | M | E>1 | L | 1a | 1 | 15.0 | No | No | No | No | 22-Oct-2006 | DdthisC | | 1<=10 |
| 67 | M | Ct | NK | 1a | 1 | 14.3 | No | No | No | No | 01-Oct-2007 | Alive | | 11<=30 |
| 63 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 24-Oct-2007 | Alive | | 1<=10 |
| 77 | M | E>1 | PH | 1a | 1 | 14.2 | No | No | No | No | 14-Jul-2005 | LFU | | |
| 63 | M | E<1 | H | 1a | 1 | 14.5 | No | No | No | No | 06-Mar-2007 | Alive | | 1<=10 |
| 64 | M | E>1 | L | 1a | 1 | 15.4 | No | No | No | No | 02-Jul-2007 | Alive | | 1<=10 |
| 51 | M | E<1 | L | 1a | 1 | 14.5 | No | No | No | No | 07-Jun-2006 | Alive | | 1<=10 |
| 59 | M | E>1 | L | 1b | 1 | 14.4 | No | No | No | No | 26-Sep-2006 | Alive | | 1<=10 |
| 56 | M | E>1 | PH | 1a | 1 | 13.5 | No | No | No | No | 02-Nov-2007 | Alive | | |
| 79 | F | E>1 | L | 2a | 2 | 14.1 | No | No | No | No | 05-Sep-2007 | Alive | | 11<=30 |
| 84 | M | E>1 | L | 1a | 1 | 12.8 | No | No | No | No | 13-Sep-2005 | ICT | | 1<=10 |
| 54 | M | NK | H | 1a | 1 | 15.0 | No | No | No | No | 05-Oct-2006 | Alive | | <1 |
| 65 | M | E<1 | PH | 1a | 1 | 14.6 | No | No | No | No | 28-Aug-2007 | Alive | | <1 |
| 47 | M | E<1 | H | 1a | 1 | 14.5 | No | No | No | No | 07-Apr-2006 | Alive | | 1<=10 |
| 81 | M | No | L | 1a | 1 | 15.2 | No | No | No | No | 08-Mar-2007 | Alive | | 11<=30 |
| 70 | M | NK | L | is | 0 | 14.6 | No | No | No | No | 05-Nov-2007 | Alive | | |
| 62 | M | E<1 | PH | 1a | 1 | 13.8 | No | No | No | No | 31-Oct-2007 | Alive | | 1<=10 |
| 65 | M | E>1 | L | 2a | 2 | 14.3 | No | No | No | No | 19-Jun-2006 | LFU | | |
| 65 | M | E>1 | L | 2a | 2 | 12.2 | No | No | No | No | 26-May-2006 | Alive | | 1<=10 |
| 59 | M | Ct | H | 1b | 1 | 14.5 | No | No | No | No | 04-Oct-2007 | Alive | | 11<=30 |
| 58 | M | Ct | L | 1a | 1 | 17.8 | No | No | No | No | 02-Oct-2007 | Alive | | |
| 63 | M | E>1 | L | 1a | 1 | 11.7 | No | No | No | No | 22-Oct-2007 | Alive | | 1<=10 |
| 76 | M | E<1 | L | 1a | 1 | 14.5 | No | No | No | No | 07-Aug-2006 | DotherC | | 1<=10 |
| 60 | M | E<1 | H | 1a | 1 | 14.0 | No | No | No | No | 27-Sep-2007 | Alive | | 1<=10 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 70 | M | E>1 | PH | 1a | 1 | 13.8 | No | No | No | No | 12-Sep-2007 | Alive | | 11<=30 |
| 65 | M | E>1 | L | 1a | 1 | 13.6 | No | No | No | No | 02-Aug-2006 | LFU | | 1<=10 |
| 65 | M | Ct | H | 1a | 1 | 12.1 | No | No | No | No | 24-May-2003 | DdthisC | | >30 |
| 55 | M | E>1 | L | 2a | 2 | 15.4 | No | No | No | No | 21-Oct-2009 | Alive | | <1 |
| 59 | M | E>1 | L | 1b | 1 | 13.8 | No | No | No | No | 01-Mar-2007 | Alive | | 1<=10 |
| 68 | M | E>1 | L | 1a | 1 | 14.1 | No | No | No | No | 11-Jul-2007 | Alive | | 1<=10 |
| 62 | F | Ct | L | 1a | 1 | 99.9 | No | No | No | No | 05-Oct-2007 | Alive | | |
| 71 | M | E>1 | H | 1a | 1 | 99.9 | No | No | No | No | 08-Aug-2007 | Alive | | <1 |
| 66 | M | Ct | PH | 2b | 2 | 12.3 | Yes | Yes | No | No | 26-Sep-2003 | ICT | | 1<=10 |
| 80 | M | E>1 | L | 1b | 1 | 12.6 | No | No | No | No | 30-Nov-2005 | LFU | | >30 |
| 58 | M | Ct | PH | 1a | 1 | 15.0 | No | No | No | No | 18-Sep-2007 | Alive | | |
| 73 | M | E<1 | L | 3 | 2 | 13.4 | No | Yes | No | No | 29-Jan-2003 | DdthisC | | 1<=10 |
| 69 | M | E<1 | L | 1a | 1 | 99.9 | No | No | No | No | 16-Oct-2007 | Alive | | 11<=30 |
| 65 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 09-Oct-2007 | Alive | | |
| 71 | M | E<1 | L | 1b | 1 | 15.6 | No | No | No | No | 03-Oct-2007 | Alive | | <1 |
| 56 | M | E<1 | L | 2a | 2 | 15.3 | No | No | No | No | 12-Jun-2007 | Alive | | 11<=30 |
| 78 | M | No | L | 2a | 2 | 13.4 | No | No | No | No | 10-Oct-2007 | Alive | | 1<=10 |
| 59 | M | E<1 | H | 1a | 1 | 14.1 | No | No | No | No | 26-Jul-2007 | Alive | | |
| 87 | M | E>1 | PH | 2b | 2 | 11.4 | No | No | No | No | 19-Aug-2003 | ICT | | >30 |
| 71 | M | E>1 | L | 1a | 1 | 99.9 | No | No | No | No | 31-May-2007 | Alive | | 1<=10 |
| 54 | M | E<1 | L | 2b | 2 | 99.9 | No | No | No | No | 10-May-2007 | Alive | | 11<=30 |
| 43 | F | NK | NK | 1a | 1 | 99.9 | No | No | No | No | 23-Oct-2007 | Alive | | |
| 56 | M | E<1 | H | 1a | 1 | 14.3 | No | No | No | No | 02-Feb-2007 | Alive | | |
| 51 | M | Ct | L | 1a | 1 | 16.5 | No | No | No | No | 25-Jun-2007 | Alive | | 11<=30 |
| 47 | M | Ct | N | 2a | 2 | 99.9 | Yes | Yes | No | No | 18-Feb-2003 | LFU | | 1<=10 |
| 65 | M | E<1 | L | 2a | 2 | 99.9 | Yes | Yes | No | No | 13-Oct-2007 | Alive | | 11<=30 |
| 64 | F | Ct | L | 1a | 1 | 14.1 | No | No | No | No | 01-Oct-2007 | Alive | | |
| 54 | M | Ct | L | 1a | 1 | 99.9 | No | No | No | No | 05-Apr-2007 | Alive | | |
| 74 | M | E>1 | H | 1a | 1 | 14.1 | No | No | No | No | 20-Sep-2007 | Alive | | 1<=10 |
| 48 | M | NK | L | 1a | 1 | 14.0 | No | No | No | No | 07-Sep-2007 | Alive | | <1 |
| 72 | M | E<1 | L | 1a | 1 | 12.6 | No | No | No | No | 04-Oct-2007 | Alive | | |
| 72 | F | E<1 | L | 3 | 2 | 12.5 | No | No | No | No | 11-Jul-2007 | Alive | | 1<=10 |
| 56 | F | E>1 | L | 2a | 2 | 14.0 | No | No | No | No | 10-Apr-2007 | Alive | | |
| 63 | M | E>1 | L | 1a | 1 | 13.5 | No | No | No | No | 16-Oct-2007 | Alive | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 63 | M | E<1 | L | 1a | 1 | 15.7 | No | No | No | No | 20-Jul-2007 | Alive | | <1 |
| 59 | M | E>1 | H | 1a | 1 | 14.8 | No | No | No | No | 23-Mar-2007 | Alive | | |
| 75 | M | No | L | 1a | 1 | 13.7 | No | No | No | No | 30-Nov-2006 | LFU | | <1 |
| 79 | M | E>1 | L | 2b | 2 | 14.3 | No | No | No | No | 04-Jul-2007 | Alive | | 11<=30 |
| 67 | M | E<1 | PH | 1b | 1 | 13.6 | No | No | No | No | 19-May-2004 | ICT | | 11<=30 |
| 72 | M | E>1 | L | 1a | 1 | 14.2 | No | No | No | No | 20-Aug-2007 | Alive | | 1<=10 |
| 74 | M | E>1 | L | 2b | 2 | 15.9 | No | No | No | No | 17-Jul-2007 | Alive | | 1<=10 |
| 66 | M | E>1 | L | 2b | 2 | 14.6 | No | No | No | No | 05-Oct-2007 | Alive | | <1 |
| 57 | M | Ct | PH | 2b | 2 | 14.2 | No | No | No | Yes | 26-Nov-2004 | DotherC | | |
| 55 | M | No | L | 2b | 2 | 99.9 | Yes | Yes | No | No | 14-Sep-2005 | DotherC | | 1<=10 |
| 65 | M | Ct | H | 2b | 2 | 14.4 | No | No | No | No | 11-Jun-2007 | Alive | | >30 |
| 71 | M | E>1 | L | 1b | 1 | 15.3 | No | No | No | No | 19-Jul-2007 | Alive | | 1<=10 |
| 75 | M | Ct | H | 1a | 1 | 14.6 | Yes | Yes | No | No | 01-Oct-2007 | Alive | | 1<=10 |
| 80 | M | E>1 | L | 1a | 1 | 15.4 | No | No | No | No | 18-Jun-2005 | DotherC | | <1 |
| 82 | F | Ct | L | 1a | 1 | 12.5 | No | No | No | No | 13-Oct-2006 | ICT | | |
| 77 | M | E>1 | H | 1a | 1 | 13.2 | Yes | No | No | Yes | 08-Sep-2004 | DotherC | | 1<=10 |
| 59 | M | Ct | PH | 2a | 2 | 12.9 | No | No | No | No | 31-Oct-2007 | Alive | | 1<=10 |
| 88 | M | E>1 | L | 1a | 1 | 14.1 | No | No | No | No | 28-Sep-2005 | LFU | | 1<=10 |
| 50 | M | Ct | L | 1b | 1 | 14.1 | No | No | No | No | 11-Apr-2007 | Alive | | <1 |
| 56 | M | E>1 | L | 1a | 1 | 14.8 | Yes | Yes | No | No | 25-Sep-2007 | Alive | | |
| 76 | M | E>1 | L | 1a | 1 | 14.5 | No | No | No | No | 16-Oct-2007 | Alive | | 1<=10 |
| 60 | M | Ct | H | 2a | 2 | 13.0 | No | No | No | No | 26-Sep-2004 | DotherC | | |
| 78 | M | Ct | L | 2a | 2 | 12.9 | No | No | No | No | 19-Apr-2006 | LFU | | |
| 58 | M | E>1 | L | 1a | 1 | 13.1 | No | No | No | No | 16-Aug-2007 | Alive | | 1<=10 |
| 57 | M | E>1 | L | 2a | 2 | 14.0 | No | No | No | No | 31-May-2007 | Alive | | |
| 52 | F | Ct | H | 2a | 2 | 12.0 | No | No | No | No | 19-Jun-2007 | Alive | | 1<=10 |
| 88 | M | E<1 | L | 1a | 1 | 16.0 | No | No | No | No | 28-Mar-2005 | ICT | | 11<=30 |
| 62 | M | E<1 | H | 1a | 1 | 15.8 | No | No | No | No | 04-Sep-2007 | Alive | | <1 |
| 68 | M | E>1 | H | 1a | 1 | 14.4 | No | No | No | No | 01-Apr-2004 | ICT | | 1<=10 |

F=female, M=male, E>1=ex smoker > 1 year, Ct=current smoker, E<1=ex smoker < 1 year, L=low, H=high, PH=previously, N=none, Med=medium, NK= not known, ICT= intercurrent death, LFU=lost to follow up, DotherC=death other cancer, DthisC=death this cancer,HB=haemoglobin.

# Appendix VII – Oropharyngeal series

| Age | Gender | Tumour Site | Alcohol | Smoker | Pre trt HB | T stage | N stage | 1°Recurrence | Neck Recurrence | Distant Recurrence | Current Status | CA9 | HIF-1 | PCR | *CDKN2A* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 59 | M | TB | Md | Ct | 13.4 | 2 | 2c | No | Yes | No | DthisC | | | NK | |
| 42 | F | TB | Hv | NK | 8.5 | 4 | 1 | No | No | No | DthisC | 1 | 3 | NK | |
| 61 | F | TL | NK | NK | 12.1 | 4 | 1 | Yes | No | No | DthisC | 1 | 3 | NK | |
| 56 | F | TL | Lw | Ct | 12.9 | 2 | 1 | No | No | No | Al | 1 | 2 | NK | Pos |
| 46 | M | TL | Hv | Ct | 11.0 | 2 | 2b | No | No | No | ID | | | NK | |
| 56 | M | TL | Md | Ct | 13.5 | 2 | 0 | No | Yes | No | Al | | 3 | NK | |
| 72 | M | TL | NK | Ct | 14.8 | 1 | 0 | No | No | No | ID | | 2 | NK | |
| 61 | F | TL | Hv | Ct | 14.7 | 2 | 0 | No | No | No | ID | 1 | 2 | NK | |
| 48 | M | TL | Lw | Ct | 14.6 | 2 | 0 | No | No | No | Al | | 1 | NK | |
| 62 | M | TL | Lw | Ct | 14.1 | 2 | 2 | No | Yes | No | DthisC | | 2 | NK | |
| 64 | M | TB | Hv | Ct | 10.2 | 4 | 0 | No | No | No | DthisC | 2 | 2 | NK | |
| 53 | F | TL | NK | Ct | 14.1 | 2 | 1 | No | No | No | Al | 1 | 1 | NK | |
| 55 | M | TL | Hv | Ct | 11.2 | 2 | 3 | No | No | No | DthisC | | 2 | NK | |
| 51 | M | TL | Md | Ct | 14.8 | 2 | 1 | No | Yes | No | DthisC | | 2 | NK | |
| 57 | F | TL | Hv | Ct | 12.2 | 4 | 2b | No | No | No | DthisC | 3 | 3 | NK | |
| 92 | M | TL | NK | NK | 11.1 | 2 | 0 | No | No | No | DthisC | 0 | 2 | NK | |
| 50 | M | TL | Lw | Ct | 14.3 | 3 | 0 | No | No | No | Al | 0 | 1 | NK | Pos |
| 65 | F | TL | Nn | No | 14.2 | 2 | 1 | No | Yes | No | DthisC | 3 | 2 | NK | Pos |
| 64 | M | TL | Lw | Ct | 14.5 | 2 | 0 | No | Yes | No | DthisC | | 3 | NK | |
| 58 | M | TL | Hv | Ct | 12.4 | 3 | 2b | No | No | No | Al | 1 | 0 | NK | Neg |
| 53 | M | TL | Hv | Ct | 15.2 | 3 | 3 | Yes | No | No | DthisC | 3 | 2 | NK | Neg |
| 47 | M | TL | Lw | Ct | 14.5 | 2 | 2a | No | No | No | Al | 2 | 0 | NK | |
| 49 | F | TL | Lw | Ct | 13.0 | 1 | 2b | No | No | No | Al | | | NK | |
| 41 | M | TL | Md | No | 13.0 | 4 | 3 | Yes | No | No | Al | 1 | 3 | NK | |
| 49 | M | TL | NK | NK | 13.8 | 2 | 2b | No | No | Yes | DthisC | | | NK | |
| 51 | M | TL | Hv | Ct | 14.5 | 2 | 0 | Yes | No | Yes | DthisC | | | NK | |
| 39 | M | TL | Md | No | 14.3 | 1 | 1 | No | No | Yes | DotherC | | | NK | |
| 56 | M | TL | Lw | Ct | 13.2 | 2 | 0 | No | No | No | ID | | | NK | |
| 62 | M | TL | Md | Ct | 15.3 | 1 | 2b | No | No | No | Al | 2 | 1 | NK | Neg |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 68 | M | TL | Lw | Ct | 14.5 | 3 | 0 | No | No | No | ID | 2 | 1 | NK | |
| 58 | F | TL | Md | Ct | 10.1 | 2 | 0 | No | No | No | DthisC | 1 | 2 | NK | |
| 63 | F | TB | Lw | Ct | 99.9 | 3 | 2a | No | No | No | DthisC | | | NK | |
| 48 | M | TL | Hv | Ct | 13.0 | 4 | 0 | Yes | Yes | No | DthisC | | 2 | NK | |
| 66 | M | TL | NK | NK | 8.5 | 4 | 2b | No | No | No | DthisC | 0 | 3 | NK | |
| 61 | M | TL | Lw | Ct | 14.1 | 2 | 2a | No | No | No | Al | 0 | 1 | NK | Pos |
| 59 | M | TL | Lw | Ct | 13.1 | 4 | 2b | No | No | No | ID | | 1 | NK | Neg |
| 47 | M | TL | Nn | No | 13.0 | 1 | 1 | No | No | No | Al | 2 | 0 | NK | Pos |
| 57 | M | TL | Nn | No | 13.4 | 1 | 0 | Yes | No | No | Al | | | NK | |
| 69 | M | TL | Lw | No | 10.6 | 4 | 0 | No | No | No | ID | | | NK | |
| 53 | M | TL | Hv | Ct | 9.3 | 4 | 2b | No | No | No | DthisC | | 3 | NK | |
| 58 | M | TL | Hv | Ct | 14.5 | 2 | 2b | Yes | No | No | DthisC | 1 | 0 | NK | |
| 45 | F | TL | NK | NK | 13.1 | 2 | 1 | Yes | No | No | DthisC | | | NK | |
| 52 | M | TL | Hv | Ct | 13.3 | 4 | 2 | No | No | No | ID | 1 | 2 | NK | |
| 77 | M | TL | Md | Ct | 9.2 | 4 | 0 | No | Yes | No | DthisC | | | NK | |
| 62 | M | TL | Lw | Ct | 14.6 | 2 | 0 | No | No | No | Al | | | NK | |
| 53 | M | TL | Md | Ct | 10.2 | 4 | 2c | Yes | No | No | DthisC | | | NK | |
| 80 | M | TL | NK | Ct | 10.3 | 4 | 2 | No | No | No | DthisC | 1 | 3 | NK | |
| 46 | M | TL | NK | NK | 11.0 | 4 | 0 | No | No | Yes | DthisC | 3 | 3 | NK | |
| 52 | M | TL | NK | NK | 7.8 | 4 | 2b | Yes | No | No | DthisC | 2 | 2 | NK | Pos |
| 48 | M | TL | NK | NK | 9.5 | 3 | 1 | No | No | No | ID | 3 | 2 | NK | Pos |
| 54 | M | TB | Hv | Ct | 12.5 | 4 | 1 | No | No | No | ID | | | NK | |
| 60 | M | TL | Md | Ct | 99.9 | 2 | 0 | No | No | Yes | DthisC | | 1 | NK | |
| 82 | M | TL | Lw | Ct | 9.9 | 4 | 3 | No | No | No | DthisC | | 3 | NK | Neg |
| 40 | M | TL | Hv | Ct | 11.9 | 3 | 1 | No | No | Yes | ID | 1 | 1 | NK | |
| 49 | F | TL | Md | Ct | 13.8 | 2 | 0 | No | No | No | Al | 0 | 1 | NK | |
| 53 | M | TL | Lw | Ct | 14.3 | 2 | 2c | No | No | No | Al | | 1 | NK | |
| 57 | M | TL | Lw | Ct | 15.2 | 2 | 2b | No | Yes | No | DthisC | | | NK | |
| 69 | F | TL | Lw | No | 12.4 | 1 | 2a | No | No | No | Al | | | NK | |
| 57 | M | TL | Lw | Ct | 11.8 | 2 | 1 | No | No | No | Al | | | NK | |
| 68 | M | TL | NK | Ct | 13.2 | 1 | 0 | No | No | No | Al | | | NK | |
| 57 | M | TB | Md | Ct | 16.0 | 1 | 0 | Yes | No | No | DthisC | 3 | 2 | NK | Neg |
| 63 | M | TL | Lw | No | 99.9 | 2 | 0 | No | No | Yes | DthisC | | | NK | |
| 48 | M | TL | Lw | No | 14.1 | 4 | 1 | No | No | No | Al | | | NK | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 62 | M | TL | Nn | No | 16.0 | 1 | 2a | No | No | No | Al | | | NK | |
| 67 | M | TB | Md | Ct | 10.9 | 4 | 0 | Yes | No | No | DthisC | 2 | 3 | NK | |
| 64 | M | TB | Nn | Ct | 8.2 | 4 | 2c | Yes | No | No | DthisC | | 3 | NK | |
| 51 | M | TB | Hv | Ct | 13.5 | 4 | 1 | No | Yes | No | DthisC | | 1 | NK | |
| 58 | F | TL | Lw | Ex | 10.3 | 2 | 2 | No | No | No | Al | | | NK | |
| 68 | M | TB | Ph | Ct | 11.6 | 4 | 2 | No | Yes | No | DthisC | 1 | 1 | Ng | Neg |
| 49 | M | TB | Lw | Ex | 14.0 | 2 | 2 | No | No | No | Al | | | NK | |
| 63 | F | TB | Lw | Ex | 12.8 | 2 | 2 | No | No | No | Al | | | NK | |
| 91 | M | TL | NK | Ex | 11.9 | 4 | 0 | Yes | No | No | DthisC | 1 | 1 | Ng | Neg |
| 62 | M | TB | Hv | Ct | 15.6 | 1 | 0 | No | No | No | ID | | | NK | |
| 53 | M | TB | Hv | Ct | 13.7 | 2 | 0 | No | No | No | Al | | 1 | NK | |
| 62 | M | TL | Lw | Ct | 13.4 | 2 | 3 | No | No | No | Al | | 1 | NK | |
| 59 | M | TB | Hv | Ct | 13.3 | 3 | 0 | No | Yes | No | DthisC | | | NK | |
| 79 | M | TB | Nn | Ex | 13.2 | 3 | 1 | No | No | No | DthisC | | | NK | |
| 51 | F | TL | Lw | Ex | 11.8 | 4 | 2 | No | No | No | DthisC | | | NK | |
| 42 | M | TL | Lw | No | 14.4 | 3 | 2 | Yes | No | No | DthisC | | | NK | |
| 47 | M | TB | Nn | No | 15.2 | 2 | 0 | No | No | No | Al | 1 | 1 | Pos | |
| 47 | M | TB | Lw | Ct | 15.3 | 2 | 2 | No | No | No | Al | 2 | 1 | Pos | Pos |
| 45 | M | TL | Lw | No | 15.7 | 1 | 1 | No | No | No | Al | 1 | | Pos | Pos |
| 72 | M | TL | Lw | Ct | 12.6 | 2 | 0 | No | No | No | Al | | | NK | |
| 45 | M | TL | Lw | No | 13.7 | 2 | 2 | No | No | No | Al | | | NK | |
| 56 | M | TL | Ph | Ex | 99.9 | 1 | 0 | No | No | No | Al | 2 | 1 | Pos | Pos |
| 82 | M | TL | Lw | Ex | 12.3 | 2 | 3 | No | No | No | DthisC | 2 | 2 | NK | Neg |
| 56 | M | TL | Hv | Ct | 13.3 | 1 | 2 | No | No | No | Al | | | NK | |
| 53 | M | TL | Hv | Ct | 13.2 | 2 | 2 | No | No | No | ID | 1 | 0 | Ng | Pos |
| 84 | M | TL | Ph | Ct | 12.1 | 2 | 0 | No | No | No | ID | 0 | | Ng | Neg |
| 56 | M | TL | Lw | Ex | 14.6 | 1 | 2 | No | No | No | Al | 1 | 1 | Pos | Pos |
| 42 | M | TL | Hv | No | 15.1 | 4 | 2 | No | No | No | Al | 1 | 0 | Pos | Pos |
| 70 | M | TB | Lw | Ct | 12.6 | 4 | 1 | No | No | No | DthisC | | | NK | |
| 56 | M | TL | Md | Ct | 14.3 | 2 | 1 | No | No | No | Al | 3 | 2 | NK | Pos |
| 63 | F | TL | Ph | Ct | 14.8 | 2 | 0 | No | Yes | No | Al | 1 | 2 | Ng | Neg |
| 61 | F | TL | Nn | No | 10.9 | 2 | 2 | No | Yes | Yes | DthisC | 2 | 1 | NK | Pos |
| 73 | M | TL | Lw | Ex | 14.3 | 3 | 1 | No | No | No | DthisC | 1 | 0 | Pos | Neg |
| 81 | M | TL | Lw | Ex | 15.9 | 2 | 2 | No | No | No | DthisC | | | NK | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 56 | M | TL | Nn | No | 15.2 | 2 | 0 | No | No | No | Al | 3 | 1 | Pos | Pos |
| 64 | M | TL | Ph | Ct | 13.5 | 4 | 1 | No | No | No | ID | | | NK | |
| 60 | M | TB | Lw | No | 12.8 | 4 | 1 | No | No | Yes | DthisC | 2 | 1 | NK | Pos |
| 73 | F | TL | Nn | Ct | 10.6 | 3 | 2 | No | No | No | DthisC | | | NK | |
| 85 | M | TB | Lw | Ex | 12.7 | 2 | 1 | No | No | No | Al | 1 | 2 | NK | |
| 64 | M | TL | Lw | Ct | 11.7 | 1 | 3 | No | No | No | Al | | | NK | |
| 58 | M | TL | Hv | Ex | 12.8 | 2 | 0 | No | No | Yes | DthisC | | | NK | |
| 65 | M | TL | NK | NK | 12.4 | 2 | 2 | No | No | No | Al | 1 | 2 | Pos | Pos |
| 48 | M | TL | Hv | Ex | 14.0 | 2 | 2 | Yes | Yes | No | DthisC | 2 | 1 | Pos | Pos |
| 62 | M | TB | Lw | Ex | 99.9 | 2 | 2 | No | No | No | Al | 1 | 2 | Pos | Pos |
| 70 | M | TB | Lw | No | 12.6 | 3 | 2 | No | No | Yes | DthisC | 2 | 1 | NK | Pos |
| 60 | M | TB | Hv | Ct | 11.2 | 4 | 0 | No | No | No | Al | 3 | 2 | NK | Neg |
| 38 | M | TL | Lw | Ex | 16.0 | 1 | 2 | No | No | No | Al | | | NK | |
| 54 | M | TL | Md | Ct | 16.1 | 2 | 1 | Yes | No | No | Al | 2 | 1 | Pos | |
| 66 | M | TL | Hv | Ex | 14.2 | 2 | 1 | No | No | No | Al | 2 | 1 | Pos | Pos |
| 49 | M | TL | Ph | Ct | 99.9 | 1 | 2 | No | No | No | Al | 2 | 0 | Pos | Pos |
| 70 | M | TL | Lw | No | 13.6 | 2 | 0 | No | No | No | Al | | 1 | Pos | Pos |
| 75 | M | TL | Ph | Ct | 12.8 | 2 | 0 | No | No | No | Al | 1 | 2 | Ng | Pos |
| 44 | F | TL | Lw | Ex | 12.1 | 1 | 2 | No | No | No | Al | 0 | 0 | NK | Pos |
| 57 | M | TL | Lw | No | 12.9 | 2 | 2 | No | No | No | Al | 1 | 1 | Pos | Pos |
| 43 | M | TB | Lw | No | 13.3 | 2 | 2 | No | No | No | Al | 0 | 1 | Pos | |
| 64 | F | TL | Nn | Ex | 13.0 | 2 | 2 | No | No | No | Al | 1 | 1 | Pos | |
| 55 | M | TL | Hv | No | 13.0 | 2 | 2 | No | No | No | Al | 2 | 2 | Pos | Pos |
| 48 | M | TL | Lw | Ct | 14.0 | 1 | 1 | No | No | No | Al | 1 | 1 | Pos | Pos |
| 50 | M | TB | Lw | Ex | 12.4 | 2 | 2 | No | No | No | Al | 1 | 0 | Pos | Pos |
| 52 | M | TL | Ph | Ex | 14.2 | 3 | 0 | No | No | No | Al | 3 | 1 | Ng | Neg |
| 51 | M | TL | Ph | Ex | 99.9 | 2 | 1 | No | No | No | Al | | | NK | |
| 51 | M | TL | Hv | Ex | 13.2 | 1 | 0 | No | No | No | Al | 0 | | NK | Neg |
| 43 | M | TL | Md | Ex | 14.2 | 2 | 2 | No | No | Yes | DthisC | 2 | 0 | Pos | Pos |
| 92 | F | TL | Nn | No | 10.1 | 2 | 0 | Yes | No | No | DthisC | 2 | 2 | Ng | Pos |
| 43 | F | TB | Nn | Ct | 13.8 | 3 | 0 | No | No | No | Al | | | NK | |
| 56 | M | TB | Lw | Ct | 13.4 | 2 | 2 | No | No | No | ID | 1 | 0 | Pos | Neg |
| 58 | M | TL | Hv | Ex | 16.3 | 1 | 1 | No | Yes | No | Al | 1 | 0 | Pos | Pos |
| 64 | M | TL | Lw | No | 13.4 | 1 | 2 | No | No | No | Al | | | NK | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 62 | M | TL | Hv | Ex | 13.3 | 2 | 1 | No | No | No | Al | | | NK | |
| 56 | M | TL | Hv | Ex | 14.7 | 2 | 0 | No | No | No | Al | 0 | 0 | Ng | Neg |
| 64 | F | TL | Lw | Ex | 13.8 | 1 | 1 | No | No | No | Al | 2 | 0 | Pos | Pos |
| 54 | F | TL | Md | Ex | 13.5 | 2 | 1 | No | No | No | Al | | | NK | |
| 72 | M | TL | Hv | Ex | 16.0 | 2 | 3 | No | No | No | ID | 2 | 3 | Ng | Neg |
| 46 | M | TB | Hv | Ct | 13.9 | 4 | 1 | No | No | No | Al | 2 | 1 | Ng | |
| 53 | M | TB | Hv | Ct | 12.8 | 4 | 2 | Yes | No | No | DthisC | 1 | 1 | NK | Neg |
| 63 | M | TL | Hv | Ct | 15.2 | 2 | 0 | No | No | No | DotherC | | | NK | |
| 63 | M | TL | Lw | Ex | 15.0 | 1 | 2 | No | No | No | Al | 1 | 1 | Pos | Pos |
| 58 | M | TL | Lw | Ct | 13.5 | 2 | 2 | No | No | No | Al | | | NK | |
| 56 | M | TL | Lw | Ex | 13.2 | 2 | 2 | No | No | No | Al | | | NK | |
| 58 | M | TL | Lw | Ex | 15.0 | 1 | 2 | No | No | No | Al | | | NK | |
| 55 | M | TB | Lw | Ex | 13.8 | 4 | 2 | Yes | No | No | DthisC | | | NK | Pos |
| 52 | M | TL | Hv | Ex | 14.3 | 1 | 1 | No | No | No | Al | 2 | 1 | Pos | Pos |
| 52 | M | TL | Hv | Ex | 13.6 | 3 | 2 | No | No | No | Al | 2 | 0 | NK | |
| 51 | M | TL | Hv | Ct | 13.9 | 1 | 2 | Yes | No | No | DthisC | 2 | 0 | Ng | Neg |
| 66 | F | TL | Lw | Ex | 12.4 | 1 | 0 | No | No | No | Al | 2 | 1 | Ng | Neg |
| 47 | M | TB | Md | Ct | 14.4 | 4 | 0 | Yes | No | No | DthisC | | | NK | |
| 59 | F | TL | NK | Ct | 11.2 | 2 | 2 | No | No | No | Al | 2 | 1 | Ng | Pos |
| 59 | F | TB | Lw | Ex | 11.9 | 3 | 0 | No | No | No | Al | 0 | 2 | Pos | Pos |
| 58 | M | TL | Lw | Ex | 99.9 | 2 | 2 | No | No | No | Al | 1 | | Ng | Neg |
| 45 | F | TL | Lw | No | 13.3 | 2 | 0 | No | No | No | Al | 1 | 2 | NK | Pos |
| 58 | M | TB | Lw | Ex | 13.5 | 3 | 2 | Yes | No | No | DthisC | 3 | 1 | Pos | |
| 49 | F | TL | Hv | Ct | 10.9 | 2 | 0 | No | No | No | Al | 1 | | Pos | Neg |
| 37 | F | TL | Hv | Ex | 13.2 | 2 | 2 | No | No | No | Al | | | NK | |
| 63 | M | TB | Lw | No | 14.9 | 2 | 1 | No | No | No | Al | 1 | 0 | Pos | Neg |
| 79 | M | TL | Hv | Ex | 15.7 | 1 | 1 | No | Yes | No | DthisC | | | NK | |
| 56 | M | TB | Hv | Ct | 14.0 | 3 | 2b | No | No | Yes | DthisC | 1 | 1 | Pos | Pos |
| 48 | F | TL | Lw | Ex | 12.7 | 2 | 0 | No | Yes | No | Al | | | NK | |
| 68 | M | TL | Md | Ex | 11.1 | 2 | 2b | No | No | No | Al | | | NK | |
| 47 | F | TL | Lw | Ex | 12.4 | 2 | 1 | No | No | No | Al | | | NK | |
| 60 | M | TB | Hv | Ct | 14.1 | 3 | 2 | No | No | No | DthisC | 3 | 1 | Ng | |
| 42 | F | TL | Hv | No | 13.2 | 2 | 1 | No | No | No | Al | 1 | 1 | Pos | Pos |
| 58 | M | TL | Hv | Ct | 13.8 | 2 | 0 | No | Yes | No | DthisC | 3 | | Ng | Neg |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 74 | M | TL | Lw | Ct | 10.3 | 4 | 2b | No | No | No | Al | 1 | 1 | Ng | Neg |
| 68 | M | TB | Hv | Ex | 14.5 | 4 | 0 | No | No | No | ID | 2 | 2 | NK | Pos |
| 55 | M | TL | Hv | Ct | 11.1 | 3 | 1 | No | No | No | DthisC | 2 | 1 | Pos | |
| 60 | M | TL | Lw | Ex | 12.7 | 2 | 2 | No | No | No | Al | 2 | 0 | Pos | Pos |
| 63 | M | TL | Md | Ct | 14.4 | 3 | 1 | No | No | No | Al | 2 | 1 | Pos | Pos |
| 61 | M | TL | Lw | Ex | 13.8 | 2 | 0 | No | No | No | Al | 1 | 2 | NK | Pos |
| 58 | F | TL | Lw | Ex | 12.5 | 2 | 0 | No | No | No | Al | 3 | 1 | Pos | Pos |
| 55 | M | TL | Lw | Ex | 16.4 | 2 | 0 | No | No | No | Al | | | NK | |
| 54 | M | TL | Hv | Ct | 11.9 | 2 | 0 | No | No | No | Al | | | NK | |
| 62 | M | TL | Hv | Ct | 13.1 | 2 | 0 | Yes | No | No | Al | 0 | 2 | NK | |
| 46 | M | TB | Hv | Ct | 10.0 | 4 | 2b | No | No | Yes | DthisC | | | Pos | |
| 54 | M | TL | Hv | Ex | 14.3 | 4 | 2 | Yes | No | No | DthisC | 1 | 1 | Pos | Pos |
| 72 | F | TL | Nn | No | 11.8 | 2 | 2a | No | No | No | Al | 1 | | Pos | Pos |
| 78 | F | TL | Lw | Ex | 13.2 | 2 | 2 | No | No | No | Al | 1 | 1 | Pos | Pos |
| 65 | M | TL | Lw | Ex | 15.6 | 2 | 2 | No | No | Yes | DthisC | 1 | 1 | Pos | Neg |
| 75 | F | TL | Lw | Ct | 14.9 | 3 | 1 | No | No | No | Al | | | NK | |
| 59 | M | TL | Hv | Ct | 14.4 | 2 | 0 | No | No | No | Al | 1 | 0 | Pos | Pos |
| 57 | M | TL | Md | Ct | 99.9 | 3 | 3 | No | No | No | DthisC | 1 | 1 | Ng | |
| 61 | M | TL | Ph | Ex | 10.8 | 4 | 2c | No | No | No | DthisC | 3 | 3 | Ng | Neg |
| 52 | F | TL | Hv | Ct | 12.2 | 2 | 2 | No | No | No | Al | 1 | 2 | Ng | Neg |
| 52 | M | TL | Hv | Ex | 11.6 | 1 | 0 | No | No | No | Al | 1 | 0 | Ng | Pos |
| 53 | M | TB | Hv | Ct | 12.9 | 4 | 2 | No | Yes | No | DthisC | | | Ng | Neg |
| 84 | M | TL | Ph | Ct | 14.1 | 4 | 1 | No | No | No | ID | 1 | 2 | Ng | Neg |
| 51 | F | TL | Hv | Ct | 14.2 | 1 | 0 | No | No | No | ID | 1 | 1 | Ng | Neg |
| 62 | M | TL | Hv | Ex | 12.9 | 3 | 2b | No | No | No | Al | 2 | 2 | Pos | Pos |
| 60 | M | TL | Hv | Ex | 13.4 | 2 | 0 | No | No | No | Al | 2 | 2 | Ng | Neg |
| 64 | M | TB | Hv | No | 13.4 | 2 | 2 | No | No | No | Al | 2 | 2 | Pos | Pos |
| 59 | M | TL | Hv | Ex | 11.9 | 3 | 2 | No | No | Yes | DthisC | 2 | 0 | Pos | |
| 81 | M | TB | Ph | Ct | 13.9 | 3 | 0 | Yes | No | No | DthisC | 0 | | Pos | Pos |
| 64 | F | TL | Lw | Ct | 14.1 | 2 | 0 | No | No | No | Al | 2 | 2 | Ng | Neg |
| 80 | F | TL | Nn | Ex | 12.5 | 3 | 2 | No | No | No | Al | | | NK | |
| 68 | M | TL | Lw | Ex | 13.2 | 2 | 2 | No | No | Yes | DthisC | 2 | 0 | NK | Pos |
| 54 | M | TL | Nn | Ct | 11.0 | 2 | 2 | Yes | No | Yes | DthisC | | | Pos | Pos |
| 58 | F | TL | Ph | Ex | 13.2 | 1 | 2 | No | No | No | DthisC | 1 | 2 | Ng | Neg |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 47 | M | TL | Lw | No | 12.7 | 1 | 2 | No | No | No | Al | | 0 | Pos | Pos |
| 56 | M | TL | Lw | No | 15.0 | 3 | 2c | No | No | No | Al | | | NK | |
| 63 | M | TL | Hv | Ex | 12.2 | 3 | 0 | No | No | Yes | DthisC | | | NK | |
| 56 | M | TB | Ph | Ex | 12.4 | 1 | 2 | No | No | No | Al | | | NK | |
| 84 | F | TL | NK | NK | 99.9 | 1 | 2b | No | No | No | Al | | | NK | |
| 79 | M | TB | Ph | Ex | 14.3 | 2 | 2c | No | No | No | Al | 1 | | Ng | Pos |
| 64 | M | TL | Nn | No | 14.7 | 2 | 3 | Yes | Yes | No | Al | | | NK | |
| 61 | M | TB | Ph | Ex | 15.2 | 4 | 2c | No | No | No | Al | | | NK | |
| 37 | F | TL | NK | NK | 99.9 | 1 | 2b | No | No | No | Al | 0 | 0 | Pos | Pos |
| 51 | M | TB | Lw | Ex | 15.9 | 2 | 2 | No | No | No | Al | 1 | 1 | Ng | Neg |
| 84 | F | TL | Lw | Ex | 10.5 | 2 | 3 | No | Yes | Yes | DthisC | | | NK | |
| 61 | M | TL | Ph | Ct | 11.8 | 4 | 0 | No | No | No | DthisC | 2 | 3 | Ng | Neg |
| 53 | M | TL | Hv | Ct | 99.9 | 3 | 3 | No | No | No | DthisC | 2 | 3 | Pos | Neg |
| 78 | M | TL | Ph | Ex | 13.8 | 4 | 2 | No | No | No | DthisC | 2 | 1 | Ng | |
| 51 | M | TL | Hv | Ct | 99.9 | 2 | 0 | No | No | No | Al | 2 | | Pos | Pos |
| 52 | M | TL | Md | Ct | 15.5 | 2 | 0 | No | Yes | No | Al | | | NK | |
| 60 | M | TB | Lw | Ex | 14.7 | 1 | 2 | No | No | No | Al | 2 | 0 | Pos | Pos |
| 60 | M | TL | Lw | Ct | 13.2 | 1 | 2 | No | No | No | Al | | | NK | |
| 76 | M | TB | Lw | Ct | 12.5 | 2 | 0 | No | No | No | DthisC | 2 | 1 | NK | |
| 55 | F | TB | Hv | Ct | 14.9 | 4 | 3 | No | No | Yes | DthisC | 2 | 3 | NK | Neg |
| 77 | M | TB | Lw | Ct | 14.5 | 3 | 2b | Yes | No | No | DthisC | 0 | 1 | NK | Pos |
| 42 | M | TB | Md | Ct | 13.4 | 1 | 2b | No | No | No | Al | 3 | 1 | NK | |
| 78 | M | TB | Lw | No | 12.7 | 2 | 1 | No | No | Yes | DthisC | 3 | 2 | NK | Neg |
| 66 | M | TB | Lw | Ct | 13.2 | 1 | 2b | No | No | No | Al | 1 | 1 | NK | Pos |
| 70 | F | TB | Nn | No | 11.6 | 3 | 0 | No | No | No | Al | | 1 | NK | |
| 51 | F | TB | Nn | Ct | 12.3 | 1 | 2b | No | Yes | No | DthisC | | 1 | NK | |
| 70 | M | TB | Md | Ct | 12.6 | 4 | 2c | No | No | No | DthisC | | | NK | |
| 64 | F | TB | Lw | Ct | 12.7 | 4 | 0 | No | Yes | No | DthisC | | | NK | |
| 70 | M | TB | Lw | Ct | 14.3 | 1 | 1 | No | No | No | ID | 1 | 3 | NK | |
| 74 | F | TB | Hv | Ct | 13.5 | 3 | 0 | No | No | No | DthisC | 3 | 3 | NK | |
| 32 | F | TB | Lw | No | 15.2 | 1 | 0 | No | No | No | Al | | 0 | NK | Neg |
| 28 | F | TB | Lw | Ct | 13.5 | 2 | 1 | No | No | No | Al | | | NK | |
| 64 | M | TB | Hv | Ct | 14.2 | 3 | 0 | No | No | Yes | ID | | 1 | NK | |
| 59 | F | TB | Hv | Ct | 15.0 | 3 | 0 | No | Yes | No | DthisC | 3 | 2 | NK | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 61 | F | TB | Lw | Ct | 13.2 | 1 | 2b | No | No | No | ID | | | NK | |
| 45 | F | TB | Md | Ct | 12.7 | 4 | 1 | No | No | No | Al | | | NK | |
| 56 | M | TB | Lw | Ct | 14.5 | 3 | 2b | Yes | No | No | Al | | | NK | |
| 61 | M | TB | Hv | Ct | 13.9 | 2 | 2a | No | No | No | ID | | | NK | |
| 62 | M | TB | Nn | No | 10.5 | 3 | 0 | No | No | No | ID | | | NK | |
| 79 | F | TB | Lw | No | 10.2 | 2 | 2c | No | No | No | DthisC | | | NK | |
| 76 | M | TB | Md | Ct | 9.8 | 4 | 2a | No | No | No | DthisC | | 3 | NK | |
| 50 | M | TB | Hv | Ct | 14.3 | 1 | 1 | No | No | No | Al | | | NK | |
| 87 | M | TB | Hv | Ct | 10.3 | 4 | 0 | No | No | No | DthisC | | 1 | NK | |
| 53 | F | TB | NK | NK | 11.2 | 3 | 1 | Yes | No | No | DthisC | | 1 | NK | |
| 57 | M | TB | Lw | Ct | 14.7 | 3 | 1 | No | No | No | ID | | 2 | NK | |
| 56 | M | TB | Lw | Ct | 14.4 | 2 | 3 | No | No | No | DthisC | | | NK | |
| 48 | M | TB | Lw | Ct | 13.2 | 3 | 1 | Yes | No | No | DthisC | | 1 | NK | |
| 65 | M | TB | NK | Ct | 13.3 | 4 | 1 | No | No | No | ID | | 1 | NK | |
| 65 | M | TB | Lw | No | 13.1 | 1 | 2a | No | No | No | Al | | | NK | |
| 64 | M | TB | Lw | Ct | 14.2 | 2 | 2a | No | No | No | DthisC | | 1 | NK | |
| 68 | M | TB | NK | NK | 9.7 | 4 | 0 | No | No | No | ID | | | NK | |
| 52 | M | TB | NK | Ct | 8.1 | 4 | 1 | No | Yes | No | DthisC | | 0 | NK | |
| 66 | F | TB | Lw | Ct | 14.3 | 2 | 0 | No | No | No | Al | | 1 | NK | |
| 70 | M | TB | Md | Ct | 14.0 | 2 | 2b | No | Yes | No | DthisC | | 1 | NK | |
| 58 | F | TB | Hv | Ct | 12.4 | 3 | 2a | No | No | No | ID | 1 | 1 | NK | |
| 55 | M | TB | Lw | Ct | 14.5 | 1 | 3 | No | No | No | DthisC | | 3 | NK | |
| 64 | M | TB | Hv | Ct | 14.0 | 1 | 2a | No | Yes | No | DthisC | | | NK | |
| 73 | M | TB | Lw | Ct | 12.2 | 2 | 0 | No | No | No | Al | | | NK | |
| 80 | M | TB | Nn | No | 11.1 | 2 | 2a | Yes | No | No | DthisC | | | NK | |
| 52 | M | TB | Hv | Ct | 99.9 | 3 | 2c | No | No | No | DthisC | | | NK | |
| 63 | M | TB | NK | NK | 11.1 | 4 | 1 | No | No | No | DthisC | | 3 | NK | |
| 59 | M | TB | Md | Ct | 14.4 | 3 | 0 | No | Yes | No | ID | | 2 | NK | |
| 58 | M | TB | Md | Ct | 13.2 | 4 | 1 | Yes | No | No | DthisC | | | NK | |
| 62 | M | TB | Hv | Ct | 10.7 | 4 | 2 | No | No | No | ID | | 1 | NK | |
| 63 | M | TB | NK | Ct | 14.5 | 1 | 1 | No | No | No | DthisC | | | NK | |
| 46 | M | TB | Md | Ct | 12.5 | 1 | 2b | No | No | No | Al | | | NK | |
| 74 | M | TB | Md | Ct | 9.5 | 4 | 2c | No | No | No | DthisC | | | NK | |
| 60 | M | TB | NK | NK | 13.4 | 4 | 2c | No | No | No | DthisC | | | NK | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 71 | M | TB | NK | Ct | 7.5 | 4 | 0 | No | No | No | Al | | | NK | |
| 76 | M | TB | NK | Ct | 99.9 | 2 | 0 | No | No | No | ID | | 2 | NK | |
| 82 | M | TB | Hv | Ct | 13.0 | 2 | 2c | No | No | No | ID | | | NK | |
| 75 | F | TB | Lw | Ct | 13.1 | 2 | 0 | No | No | No | ID | 1 | 2 | NK | |
| 42 | M | TB | Hv | No | 14.9 | 1 | 0 | No | No | No | Al | | | NK | |
| 69 | F | TB | Nn | No | 8.7 | 4 | 3 | No | No | No | DthisC | | 2 | NK | |
| 59 | F | TB | Hv | Ct | 12.7 | 2 | 2a | No | No | No | ID | | | NK | |
| 55 | M | TB | Hv | Ct | 13.1 | 4 | 1 | No | No | No | ID | | 2 | NK | |
| 67 | F | TB | Lw | Ct | 12.9 | 1 | 3 | No | No | No | DthisC | | | NK | |
| 81 | M | TB | Hv | Ct | 12.7 | 4 | 2 | No | No | No | DthisC | | | NK | |
| 47 | M | TB | Hv | Ct | 15.6 | 1 | 3 | Yes | No | No | DthisC | | | NK | |
| 58 | M | TB | Hv | Ct | 15.6 | 2 | 2a | Yes | No | No | DthisC | | | NK | |

F=female, M=male, TB=tongue base, TL=tonsil, L=low, H=high, PH=previously, N=none, Med=medium, NK= not known, E>1=ex smoker > 1 year, Ct=current smoker, E<1=ex smoker < 1 year, Al=alive, ID= intercurrent death, LFU=lost to follow up, DotherC=death other cancer, DthisC=death this cancer,HB=haemoglobin.

# Appendix VIII – Surgical series

| Age | Sex | Smkr | Alcohol | Pre trt HB | Tumour Site 1 | T stage | N stage | Any Recurrence | Primary Recurrence | Neck Recurrence | Distant Recurrence | Current Status | HIF-1 | CA 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 59 | F | Nk | Nk | Nk | Cancer; Larynx; | 4 | 0 | No | No | No | No | Al | 9 | 9 |
| 70 | M | Nk | Nk | Nk | Cancer; Hypopharyngeal wall | 4 | 2 | Yes | No | No | Yes | Al | 9 | 9 |
| 84 | F | No | Nn | Nk | Cancer; Cheek mucosa | 4 | 0 | No | No | No | No | Al | 2 | 2 |
| 74 | M | Ex | Md | 15.2 | Cancer; Pifirom sinus | 4 | 1 | No | No | No | No | Al | 1 | 1 |
| 73 | M | Ex | Md | 13.5 | Cancer; Supraglottis | 4 | 0 | Yes | No | Yes | No | DotherC | 2 | 2 |
| 56 | F | Cur | Ph | 17.3 | Cancer; Tonsil; unspecified | 1 | 1 | No | No | No | No | Al | 9 | 9 |
| 68 | F | Cur | Hv | Nk | Cancer; Mouth; retromolar area | 4 | 1 | Yes | Yes | No | No | DCKN | 1 | 2 |
| 78 | F | No | Nk | Nk | Cancer; Oropharynx; unspecified | 4 | 1 | Yes | Yes | No | No | DthisC | 9 | 9 |
| 67 | M | Cur | Lw | 13.2 | Cancer; Tonsil; unspecified | 1 | 1 | No | No | No | No | DthisC | 9 | 1 |
| 72 | F | Ex | Lw | Nk | Cancer; Bone; face | 2 | 0 | Yes | No | Yes | Yes | DthisC | 0 | 1 |
| 55 | F | Ex | Nk | Nk | Cancer; Tongue; border | 1 | 3 | Yes | No | Yes | Yes | DthisC | 1 | 1 |
| 78 | F | Cur | Lw | Nk | Cancer; Mouth; floor of; unspecified | 2 | 0 | Yes | No | No | Yes | DCKN | 9 | 9 |
| 74 | F | Cur | Nk | Nk | Cancer; Tongue; dorsal surface | 2 | 0 | Yes | No | No | Yes | DthisC | 0 | 0 |
| 74 | M | Ex | Lw | Nk | Cancer; Tonsil; unspecified | 3 | 1 | Yes | No | No | Yes | Al | 9 | 9 |
| 48 | M | Nk | Nk | Nk | Cancer; Tongue; unspecified | 2 | 0 | Yes | No | Yes | Yes | DCKN | 0 | 1 |
| 74 | M | Cur | Md | Nk | Cancer; Tongue; border | 2 | 1 | No | No | No | No | DthisC | 1 | 1 |
| 44 | F | Cur | Md | Nk | Cancer; Tongue; border | 2 | 0 | No | No | No | No | Al | 1 | 1 |
| 51 | F | Cur | Lw | Nk | Cancer; Tongue; ventral surface | 2 | 0 | Yes | Yes | Yes | Yes | DthisC | 9 | 9 |
| 82 | F | Cur | Md | Nk | Cancer; Tongue; border | 4 | 9 | No | No | No | No | DCKN | 9 | 9 |
| 61 | M | Ex | Lw | Nk | Cancer; Gum; unspecified | 2 | 2 | Yes | No | Yes | No | DthisC | 9 | 9 |
| 63 | M | Ex | Ph | Nk | Cancer; Tongue; border | 2 | 1 | No | No | No | No | Al | 9 | 9 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 63 | M | Cur | Lw | Nk | Cancer; Mouth; floor of; unspecified | 4 | 0 | Yes | Yes | No | No | Al | 1 | 2 |
| 57 | M | Cur | Nk | Nk | Cancer; Mouth; floor of; unspecified | 1 | 1 | No | No | No | No | Al | 9 | 9 |
| 61 | M | No | Lw | Nk | Cancer; Tongue; border | 1 | 0 | No | No | No | No | Al | 9 | 9 |
| 62 | M | Cur | Hv | Nk | Cancer; Tongue; border | 2 | 1 | Yes | Yes | No | No | Al | 9 | 9 |
| 79 | M | Ex | Lw | Nk | Cancer; Cheek mucosa | 2 | 9 | Yes | Yes | Yes | No | DCKN | 1 | 1 |
| 65 | M | Cur | Lw | Nk | Cancer; Mouth; anterior floor | 2 | 9 | No | No | No | No | Al | 9 | 9 |
| 66 | M | Cur | Md | Nk | Cancer; Glottis | 4 | 0 | No | No | No | No | Al | 9 | 9 |
| 61 | F | Ex | Lw | Nk | Cancer; Mouth; floor of; unspecified | 2 | 0 | No | No | No | No | Al | 1 | 2 |
| 38 | M | No | Md | Nk | Cancer; Tongue; unspecified | 1 | 0 | Yes | Yes | Yes | No | DthisC | 1 | 1 |
| 37 | M | Cur | Hv | Nk | Cancer; Tongue; base | 4 | 0 | No | No | No | No | Al | 2 | 3 |
| 67 | F | Cur | Hv | 11.7 | Cancer; Pifirom sinus | 4 | 2 | No | No | No | No | Al | 9 | 9 |
| 56 | F | Cur | Lw | Nk | Cancer; Larynx; unspecified | 4 | 0 | Yes | Yes | No | No | Al | 9 | 9 |
| 57 | M | Cur | Hv | Nk | Cancer; Pifirom sinus | 4 | 2 | Yes | No | No | Yes | DthisC | 1 | 1 |
| 54 | F | Cur | Nn | Nk | Cancer; Palate; hard | 1 | 0 | Yes | Yes | No | No | DthisC | 1 | 1 |
| 39 | M | Ex | Hv | 14.3 | Cancer; Oropharynx; unspecified | 3 | 0 | Yes | No | No | Yes | DthisC | 9 | 9 |
| 55 | M | Ex | Hv | Nk | Cancer; Mouth; floor of; unspecified | 4 | 2 | No | No | No | No | Al | 9 | 9 |
| 70 | M | Ex | Nk | Nk | Cancer; Tongue; anterior two thirds | 4 | 2 | Yes | Yes | No | No | DthisC | 1 | 2 |
| 57 | M | Cur | Hv | Nk | Cancer; Mouth; anterior floor | 2 | 1 | No | No | No | No | ID | 1 | 2 |
| 59 | M | Nk | Nk | Nk | Cancer; Bone; lower jaw | 4 | 1 | No | No | No | No | DotherC | 0 | 1 |
| 50 | M | Cur | Hv | 15.4 | Cancer; Tongue; base | 4 | 2 | Yes | Yes | No | No | DthisC | 9 | 9 |
| 63 | F | Cur | Hv | Nk | Cancer; Glottis | 3 | 0 | Yes | Yes | Yes | No | DthisC | 9 | 9 |
| 57 | F | Cur | Lw | 13.7 | Cancer; Larynx; unspecified | 4 | 0 | No | No | No | No | Al | 2 | 0 |
| 52 | M | Ex | Md | Nk | Cancer; Tongue; border | 1 | 0 | No | No | No | No | Al | 2 | 2 |
| 73 | F | Nk | Nk | Nk | Cancer; Tongue; unspecified | 2 | 1 | No | No | No | No | Al | 2 | 1 |
| 65 | M | Cur | Md | Nk | Cancer; Tongue; border | 2 | 0 | No | No | No | No | Al | 2 | 1 |
| 79 | F | Cur | Lw | Nk | Cancer; Tongue; border | 2 | 2 | No | No | No | No | Al | 0 | 2 |
| 74 | M | Cur | Hv | 13.5 | Cancer; Larynx; unspecified | 4 | 0 | Yes | No | No | Yes | DthisC | 9 | 9 |
| 67 | M | Cur | Hv | 15.1 | Cancer; Pifirom sinus | 4 | 1 | Yes | Yes | No | No | DthisC | 1 | 2 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 67 | M | Cur | Hv | 16.0 | Cancer; Glottis | 3 | 0 | No | No | No | No | Al | 2 | 2 |
| 65 | M | Ex | Ph | Nk | Cancer; Pifirom sinus | 4 | 2 | No | No | No | No | Al | 9 | 9 |
| 52 | F | Cur | Hv | Nk | Cancer; Tongue; border | 3 | 2 | No | No | No | No | Al | 2 | 2 |
| 51 | M | Ex | Ph | Nk | Cancer; Mouth; floor of; unspecified | 2 | 0 | No | No | No | No | Al | 1 | 1 |
| 57 | F | Ex | Lw | Nk | Cancer; Tongue; unspecified | 2 | 0 | No | No | No | No | Al | 0 | 2 |
| 60 | M | Cur | Hv | 13.6 | Cancer; Glottis | 3 | 0 | No | No | No | No | Al | 1 | 3 |
| 44 | M | Ex | Lw | Nk | Cancer; Tongue; border | 2 | 0 | Yes | Yes | No | No | DCKN | 1 | 1 |
| 58 | M | Nk | Md | Nk | Cancer; Tonsil; unspecified | 4 | 2 | Yes | Yes | No | No | DthisC | 9 | 9 |
| 34 | F | Cur | Hv | Nk | Cancer; Tongue; border | 2 | 0 | No | No | No | No | Al | 1 | 1 |
| 61 | M | Cur | Hv | 17.1 | Cancer; Pifirom sinus | 4 | 3 | Yes | No | No | Yes | Al | 2 | 1 |
| 40 | F | Cur | Hv | Nk | Cancer; Tongue; ventral surface | 2 | 2 | No | No | No | No | NK | 1 | 1 |
| 75 | M | Cur | Lw | Nk | Cancer; Cheek mucosa | 4 | 1 | No | No | No | No | Al | 9 | 0 |
| 46 | M | No | Md | 15.0 | Cancer; Tonsil; unspecified | 1 | 2 | No | No | No | No | Al | 0 | 1 |
| 52 | M | Nk | Nk | Nk | Cancer; Tongue; border | 2 | 0 | Yes | No | No | Yes | DCKN | 1 | 0 |
| 53 | M | Cur | Hv | Nk | Cancer; Mouth; floor of; unspecified | 4 | 0 | No | No | No | No | Al | 1 | 1 |
| 52 | F | Ex | Md | Nk | Cancer; Mouth; anterior floor | 1 | 2 | Yes | No | No | No | DthisC | 2 | 3 |
| 63 | M | Cur | Ph | 11.1 | Cancer; Supraglottis | 2 | 0 | Yes | No | Yes | No | DthisC | 2 | 3 |
| 60 | F | Ex | Lw | Nk | Cancer; Supraglottis | 4 | 2 | No | No | No | No | Al | 9 | 9 |
| 80 | F | No | Lw | Nk | Cancer; Gum; unspecified | 9 | 0 | No | No | No | No | Al | 9 | 9 |
| 81 | M | Ex | Ph | Nk | Cancer; Tongue; dorsal surface | 2 | 2 | Yes | No | Yes | No | DCKN | 1 | 0 |
| 60 | F | Cur | Lw | Nk | Cancer; Pifirom sinus | 4 | 2 | No | No | No | No | Al | 0 | 1 |
| 62 | M | Ex | Md | Nk | Cancer; Cheek mucosa | 2 | 1 | Yes | No | No | No | DthisC | 2 | 2 |
| 31 | M | No | Nn | Nk | Cancer; Tongue; border | 2 | 2 | No | No | No | No | Al | 1 | 2 |
| 44 | M | Cur | Hv | Nk | Cancer; Tongue; border | 4 | 2 | No | No | No | No | Al | 1 | 2 |
| 59 | F | Cur | Md | Nk | Cancer; Tongue; ventral surface | 2 | 0 | Yes | Yes | No | No | Al | 1 | 2 |
| 63 | M | Cur | Nk | Nk | Cancer; Subglottis | 4 | 0 | No | No | No | No | Al | 2 | 3 |
| 50 | M | Cur | Hv | Nk | Cancer; Mouth; floor of; unspecified | 2 | 0 | No | No | No | No | Al | 2 | 3 |
| 70 | M | Cur | Md | 12.5 | Cancer; Supraglottis | 4 | 0 | No | No | No | No | Al | 0 | 1 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 45 | M | No | Ph | Nk | Cancer; Oropharynx; unspecified | 3 | 2 | No | No | No | No | Al | 1 | 2 |
| 51 | F | Nk | Nk | Nk | Cancer; Tongue; border | 1 | 9 | No | No | No | No | Al | 9 | 9 |
| 54 | F | Nk | Nk | Nk | Cancer; Larynx; unspecified | 4 | 0 | No | No | No | No | Al | 3 | 3 |
| 61 | M | Cur | Md | Nk | Cancer; Tongue; ventral surface | 2 | 0 | Yes | No | Yes | No | Al | 1 | 1 |
| 50 | M | Cur | Md | Nk | Cancer; Tongue; border | 1 | 0 | No | No | No | No | Al | 1 | 1 |
| 53 | M | No | Ph | Nk | Cancer; Tongue; base | 4 | 2 | No | No | No | No | Al | 1 | 1 |
| 59 | M | Cur | Ph | 13.9 | Cancer; Supraglottis | 3 | 2 | No | No | No | No | Al | 1 | 2 |
| 58 | M | Nk | Nk | Nk | Cancer; Oropharynx; unspecified | 3 | 2 | No | No | No | No | Al | 9 | 9 |
| 63 | M | Ex | Ph | Nk | Cancer; Supraglottis | 4 | 2 | No | No | No | No | Al | 1 | 1 |
| 48 | F | Cur | Nn | Nk | Cancer; Tongue; base | 2 | 2 | Yes | Yes | No | No | DCKN | 2 | 2 |
| 55 | M | Nk | Nk | Nk | Cancer; Tongue; border | 3 | 2 | No | No | No | No | Al | 1 | 3 |
| 64 | M | Ex | Hv | Nk | Cancer; Supraglottis | 4 | 0 | No | No | No | No | Al | 9 | 9 |
| 48 | F | Cur | Nk | Nk | Cancer; Mouth; floor of; unspecified | 1 | 0 | No | No | No | No | Al | 1 | 1 |
| 51 | F | Cur | Hv | Nk | Cancer; Oropharynx; unspecified | 2 | 2 | No | No | No | No | Al | 1 | 1 |
| 50 | M | Ex | Nk | Nk | Cancer; Hypopharyngeal wall | 3 | 3 | Yes | No | No | Yes | DthisC | 1 | 1 |
| 60 | M | Cur | Nk | Nk | Cancer; Pifirom sinus | 3 | 2 | No | No | No | No | NK | 2 | 1 |
| 79 | F | Ex | Lw | Nk | Cancer; Cheek mucosa | 4 | 2 | No | No | No | No | DthisC | 2 | 0 |
| 62 | F | Cur | Lw | Nk | Cancer; Supraglottis | 4 | 2 | No | No | No | No | Al | 9 | 9 |
| 35 | F | Ex | Md | Nk | Cancer; Tongue; border | 2 | 1 | No | No | No | No | Al | 1 | 1 |
| 72 | M | Cur | Md | 14.1 | Cancer; Supraglottis | 3 | 1 | No | No | No | No | Al | 0 | 1 |
| 41 | M | Ex | Lw | 13.5 | Cancer; Supraglottis | 4 | 0 | Yes | No | Yes | No | Al | 9 | 9 |
| 68 | M | Ex | Md | 13.1 | Cancer; Larynx; unspecified | 4 | 0 | No | No | No | No | Al | 1 | 1 |
| 55 | M | Cur | Hv | Nk | Cancer; Subglottis | 4 | 0 | No | No | No | No | Al | 9 | 9 |
| 54 | M | Cur | Hv | Nk | Cancer; Supraglottis | 4 | 2 | Yes | No | Yes | No | DthisC | 9 | 9 |
| 52 | M | Cur | Nk | 14.1 | Cancer; Glottis | 4 | 1 | Yes | Yes | No | Yes | DthisC | 9 | 9 |
| 60 | M | Nk | Nk | Nk | Cancer; Supraglottis | 4 | 2 | No | No | No | No | Al | 9 | 9 |
| 61 | F | Cur | Hv | 13.6 | Cancer; Oropharynx; unspecified | 3 | 1 | No | No | No | No | Al | 1 | 1 |

F=female, M=male, Ex=ex smoker, Cur=current  smoker, L=low, Hv=heavy, PH=previously heavy, N=none, Md=medium, NK= not known Al=alive, ID= intercurrent death, LFU=lost to follow up, DotherC=death other cancer, DthisC=death this cancer,DCNK= death cause nt known, HB=haemoglobin, CA9 0=<1%, 1=1-10%, 2=11-29%, 3=≥30%, HIF-1=0=<1%, 1=1-10%, 2=11-29%, 3=≥30%, NK=not known .

# Appendix IX - Publications and presentations arising from the thesis

**Publications**

- **CM Douglas**, R Swindell, A Sykes, J Hommer, N Slevin. Management of T2 glottic cancer – conventional radiotherapy unacceptable? Submitted to Oral Oncology
- **C M Douglas**, V Ormston, C West, R Swindell, R Hall, N Slevin, J Homer. Glottic cancer and markers of hypoxia and radioresponsiveness. Submitted to International Journal Radiation Oncology Biology Physics
- **C M Douglas**, V Ormston, C West, R Swindell, R Hall, N Slevin, J Homer. Glottic cancer and markers of hypoxia and radioresponsiveness. Clinical Otolaryngology. 2009; 34; S31
- **C M Douglas**, R Swindell, A Sykes, N Slevin, J Homer. Management of T2 glottic cancer – conventional radiotherapy unacceptable? Clinical Otolaryngology. 2009; 34; S75
- **C M Douglas**, P Silva, E Rowlinson, H Valentine, R Swindell, C West, N Slevin, R Hall, J Homer. The prognostic significance of CA9 for outcome following radiotherapy in oropharyngeal cancer. Clinical Otolaryngology. 2009; 34; S86

**Oral Presentation**

- **C M Douglas**, V Ormston, C West, R Swindell, R Hall, N Slevin, J Homer. Glottic cancer and markers of hypoxia and radioresponsiveness. British Academic Conference of Otolaryngology. July 2009
- **C M Douglas,** V. Ormston, C. West, R. Swindell, R. Hall, N. Slevin, J. Homer. Glottic Cancer - Carbonic Anhydrase-9 and Haemoglobin as Markers of Treatment Response. West of Scotland Laryngology and Head and Neck Surgery Collaborative Group. May 2009 ( First prize)

**Poster Presentation**

- **C M Douglas**, P Silva, E Rowlinson, H Valentine, R Swindell, C West, N Slevin, R Hall, J Homer. The prognostic significance of CA9 for outcome following radiotherapy in oropharyngeal cancer. British Academic Conference of Otolaryngology. July 2009
- **C M Douglas**, R Swindell, A Sykes, N Slevin, J Homer. Management of T2 glottic cancer – conventional radiotherapy unacceptable? British Academic Conference of Otolaryngology. July 2009

- A Merve, **CM Douglas**, A Shah, C West, JJ Homer, N Slevin. Retrospective study of Early Glottis Carcinoma post Radical Radiotherapy. BAHNO meeting April 2007.