UNIVERSITY OF SOUTHAMPTON

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Identifying Prognostic Factors in Oropharyngeal Carcinoma

by

Matthew JA Ward

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ABSTRACT

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IDENTIFYING PROGNOSTIC FACTORS IN OROPHARYNGEAL CARCINOMA

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Oropharyngeal squamous cell carcinoma (OPSCC) is increasing in incidence due to the emergence of human papillomavirus (HPV) as a causative agent. Patients with HPV-positive OPSCC generally have improved survival outcomes compared to those with HPV-negative tumours, seemingly regardless of treatment modality. This has lead to 'treatment de-escalation' trials, which aim to maintain positive outcomes whilst minimising treatment-related morbidity. However, there remains a small but significant minority of patients with HPV-positive tumours who have a very poor prognosis. Whilst there is some evidence that heavy smoking and advanced cervical lymphadenopathy can negatively affect prognosis, there are no globally agreed criteria by which to identify these high-risk patients. The work presented in this thesis aimed to attempt to identify factors which alter outcome in HPV-associated OPSCC.

A retrospective review of over 400 consecutive cases of OPSCC was carried out and a large clinicopathological tumour database created. Archival tumour tissue was retrieved where available (n=290) and tissue microarrays were created. Tumours were categorised as HPV-positive or negative using a combination of p16 immunohistochemistry (IHC) and HPV *in-situ* hybridisation, and also assessed for levels of a tumour-infiltrating lymphocytes (TILs). A number of other biomarkers were also assessed using IHC. The effect of clinicopathological features and biomarkers on survival was established using a combination of Kaplan Meier analysis and Cox Proportional Hazards Regression. A predictive model for 3-year survival was also created using Logistic Regression.

While 'traditional' risk factors for poor outcome, including TNM-stage, did not predict for survival in HPV-positive patients, TIL-levels were highly prognostic. The most important finding was of a group of HPV-positive but TIL^{low} patients (~15% of HPV-positive tumours) who had no difference in survival from those who were HPV-negative. Furthermore, a predictive model containing TIL-levels, smoking and T-stage was highly predictive for 3-year survival in HPV-positive patients.

The impact of TILs on survival is well established in other tumour types, especially colorectal carcinoma. This is one of the first large scale studies to identify TILs as a significant prognostic marker in HPV-positive OPSCC. The identification of a group of TIL^{low} patients with poor survival is important when considering enrollment in 'de-escalation' studies. At present it is unclear why the majority of HPV-positive tumours contain high TIL-levels, however recognition of the importance of the immune system in survival from HPV-positive OPSCC raises interesting questions as to whether immunotherapy might have a therapeutic role in the future.

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DECLARATION OF AUTHORSHIP

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and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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Date:…….

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Definitions and abbreviations

5-FU	5-flurouracil
95% CI	95% Confidence Interval
Ad	Adenovirus
AJCC	American Joint Committee on Cancer
APC	Antigen Presenting Cell
APP-BP1	Amyloid precursor protein binding potein 1
Bcl-2	B Cell Lymphoma 2
BLT	Barts and the London NHS Trust
CD	Cluster of Differentiation
CDK	Cyclin Dependent Kinase
CDKI	Cyclin Dependent Kinase Inhibitor
CGH	Comparitive genomic hybridisation
CIS	Carcinoma in situ
CSF-1	Colony Stimulating Factor 1
CTL	Cytotoxic T-Lymphocyte
DAB	Diaminobenzidine
DAHNO	Data for Head and Neck Oncology
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DR	Detection Rate
DSS	Disease Specific Survival
E6AP	E6 Associated Protein
ECOG	Eastern Cooperative Oncology Group
eDOCS	Electronic Document System
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-mesenchymal transition
EPR	Electronic Patient Records
eQUEST	Electronic Request System
FFPE	Formulin Fixed and Paraffin Embedded
FPR	False Positive Rate
FTD	Feeding Tube Dependency
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papillomavirus
HR	Hazard Ratio
HRP	Horse Radish Peroxidase
hTERT	Human Telomerase Reverse Transcriptase

IAP-2	Inhibitor of Apoptosis 2
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-10	Interleukin-10
IL-10	Interleukin-1
IL-6	Interleukin-6
ISH	*In-situ* hybridisation
LOH	Loss of Heterozygosity
MDT	Multidisciplinary team
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
NF-κB	Nuclear Factor Kappa B
OPSCC	Oropharyngeal Squamous Cell Carcinoma
OR	Odds Ratio
ORF	Open Reading Frame
ORN	Osteoradionecrosis
OS	Overall Survival
OSCC	Oral Squamous Cell Carcinoma
p53	Tumour Supressor Protein 53
PACS	Picture Archiving and Communication System
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDZ	PSD95/Dlg/ZO-1
PFT	Poole Hospital NHS Foundation Trust
PI3K	PI3 Kinase
PIP_2	Phosphatidylinositol bisphaosphate
PIP_3	Phosphatidylinositol triphosphate
pRb	Retinoblastoma tumour suppression protein
PTEN	Phosphate and Tensin Homolog
QOL	Quality of Life
RBL1	Retinoblastoma Like 1
RBL2	Retinoblastoma Like 2
RNA	Ribonucleic acid
RTOG	Radiation Therapy Oncology Group
SCC	Squamous Cell Carcinoma
SEER	Surveillance Epidemiology and End Results, a branch of the United States National Cancer Institute
SMG-1	Suppressor with morphogenetic effect on genetalia
SPT	Second Primary Tumour

SV40	Simian Vacuolating Virus 40
SWPHO	South West Public Health Observatory
T Ag	Large Tumour Antigen (of SV40)
TAP	Transporter Associated with Antigen Processing
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor β
Th	Helper T-Cell
TIL	Tumour Infiltrating Lymphocyte
TLM	Ataxia Telangectasia
TMA	Tissue Microarray
TNFR1	TNF Receptor 1
TNF-α	Tumour Necrosis Factor α
TNM	Tumour Node Metastasis
TOR	Transoral Resection
T_{reg}	Regulatory T-Cell
UHS	University Hospitals Southampton NHS Foundation Trust
UK	United Kingdom
URR	Upstream Regulatory Region
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Head and Neck Cancer

1.1.1 An introduction to head and neck cancer

Head and Neck Cancer includes a diverse group of tumours affecting a number of different anatomical sub-sites within the head and neck region. These include: the lip and oral cavity, the oropharynx, the larynx, the hypopharynx, the nasopharynx, the nasal cavity and the paranasal sinuses, and the salivary glands, both major and minor (1). These tumours are a highly heterogeneous group with differing pathological features and pathogenic pathways. Furthermore, they present in a number of different fashions and respond in different ways to a variety of treatments. Approximately 90% of head and neck cancers are squamous cell carcinomas (HNSCCs), and although other tumour types (such as salivary gland tumours and adenocarcinomas) are described, they will not be discussed in any further detail here (2). Examples of oropharyngeal tumours are shown in Figure 1. 1: Examples of Oropharyngeal Squamous Cell Carcinomas. A: A right sided tonsillar tumour. B: A right sided tongue base tumour. T = Tumour, E = Epiglottis.

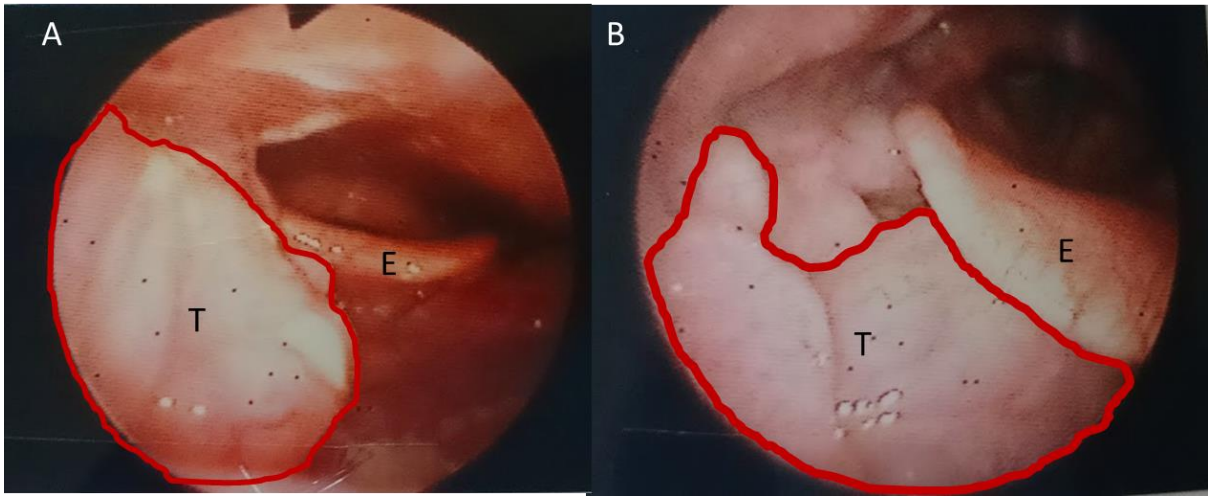


Figure 1. 1: Examples of Oropharyngeal Squamous Cell Carcinomas. A: A right sided tonsillar tumour. B: A right sided tongue base tumour. T = Tumour, E = Epiglottis

1.1.2 The epidemiology of head and neck squamous cell cancer

HNSCC is the 6th leading cause of cancer death worldwide, with approximately 650000 new cases diagnosed annually and 350000 deaths. Historically, approximately 50% of HNSCCs originate in the oral cavity, 30% in the larynx and 10% in the oropharynx (3-5). In the United States, HNSCC accounts for ~3.5% of all cancer cases and 1.5% of cancer

deaths (6). Although this represents a relatively small population, HNSCC often presents at a late stage and requires complex, multimodal treatment with severe and long-lasting side effects. This subsequently leads to extensive direct and indirect costs and the national economic burden of HNSCC in the United States, for example, is estimated to be in the region of $1 billion per year (7, 8).

HNSCC predominantly affects men (male:female ~ 3:1) and the median age of diagnosis is in the early 60s (4). The consumption of tobacco and alcohol has a multiplicative effect on the risk of HNSCC, and these risk factors account for approximately 75% of all cases (9-11). Alcohol is also an independent risk-factor, and never-smokers who consume at least three alcoholic drinks per day are at increased risk of HNSCC (12). The practise of chewing tobacco and Betel Nut is also associated with an increased risk of head and neck tumours, particularly those of the oral cavity (13, 14). There is some evidence for a protective role of fruit and vegetables, and increased consumption of these is associated with a reduced risk of HNSCC (15). Although the vast majority of head and neck cancers arise spontaneously, there is evidence of familial inheritance, and patients with cancer susceptibility syndromes (such as Li-Fraumeni syndrome or Fanconi's anaemia) are at an increased risk (16-18).

Somewhere in the region of 15-20% of cases of HNSCC occur in non-smokers and non-drinkers, and in recent years there has been increasing evidence of an association between human papillomavirus (HPV) and certain subsets of HNSCC, most notably oropharyngeal squamous cell carcinoma (OPSCC). HPV-positive tumours have different risk factors, pathogenesis and outcomes to HPV-negative tumours and are now considered a separate disease entity (19-24).

1.1.3 Changing trends in Head and Neck SCC

As mentioned above, alcohol and tobacco are traditionally the risk factors most associated with HNSCC. The most important of these has long been considered to be tobacco (25). Over the past 20-30 years, tobacco use in the United States and United Kingdom has declined. In 1965, 23% of adult Americans were considered heavy smokers (>20 cigarettes per day) compared with only 7% in 2007 (26, 27). In parallel with this, incidence rates for many subtypes of HNSCC have also declined (figure 1.2 A) (28, 29). The UK incidence of laryngeal cancer peaked in 1988 at 6.9 per 100000 population. By 2002 this had decreased to 5.3 per 100000 and has remained at this level since (30). In the United States, the incidence of oral cavity SCC (OSCC) was found to have decreased from 3.6 to 2.7 per 100000 between 1974 and 1999 in one study, and by 1.9% per year from 1973-2004 in another (29, 31).

In stark contrast to this, the incidence of OPSCC has increased over the past two decades (figure 1.2 B). Indeed, some studies have suggested that the incidence of OPSCC has increased by nearly 30% since the late 1980s (20, 32). Much of this increase has been observed in men under the age of 60, and in patients without a significant history of tobacco and alcohol exposure (5). For example, the incidence of tonsillar carcinoma in the United States has increased by between 0.6 and 3% per year since the mid-1970s, and similar patterns are seen in the United Kingdom, where the incidence of tonsillar cancer increased by 5.7% per year in men and 4.3% per year in women between 1985 and 2006 (29, 33, 34). Similar trends have been observed in a number of other western countries, including Canada and Australia (35-37). Furthermore, OPSCC has increased as a proportion of all HNSCC, with one study demonstrating a rise from 17.6% of all HNSCC in 1974-76 to 22.7% in 1998-99 (31).

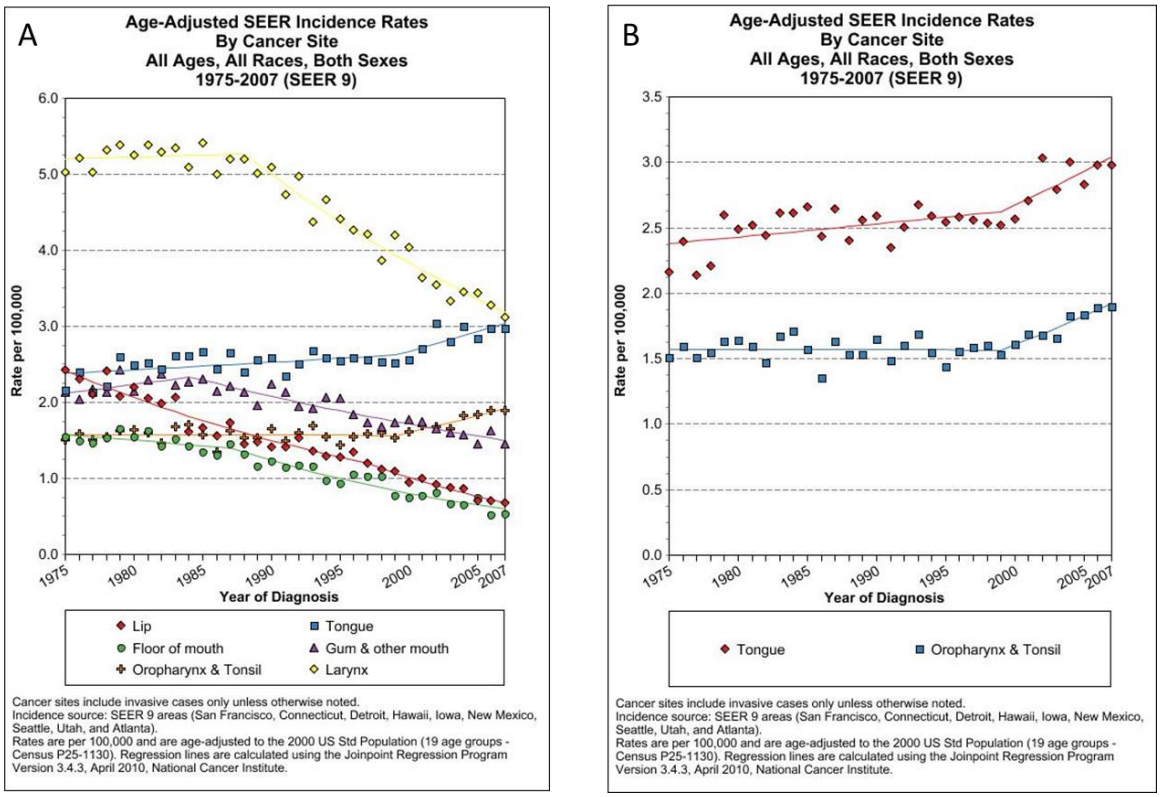


Figure 1. 2: Incidence rates for HNSCC since 1975. A: All subsites B: Tongue and Oropharynx. From seer.cancer.gov (accessed 1/11/2013)

The major contributing factor to the increasing incidence of OPSCC is thought to be HPV. When OPSCC is subcategorised by aetiology (i.e. HPV verses tobacco/alcohol) an interesting picture emerges. If HPV-negative OPSCC is considered as a separate entity from HPV-positive disease, then incidence rates are actually decreasing at a rate similar to other subtypes of HNSCC. In one recent cancer registry based study in the United States, Chaturvedi *et al* found that the population-level incidence of HPV-negative OPSCC decreased by 50% between 1988 and 2004 (20). Conversely the incidence of HPV-positive OPSCC has greatly increased over the past 20 to 30 years. In Chaturvedi *et al's* study, the population level incidence of HPV-positive OPSCC increased by over 200%, from 0.8/100 000 to 2.6/100 000 (20). Furthermore, the proportion of OPSCC related to HPV is also increasing. In Sweden, a retrospective study of archival tonsillar SCC specimens demonstrated that 23% of oropharyngeal tumours were HPV-positive in the 1970s compared to over 90% in 2006-2007(38). This is comparable to Chaturvedi *et al* who found that 16% of cases of OPSCC were HPV-positive in 1984-1989 compared to 73% between 2000 and 2004 (20).

Indeed, the incidence of HPV-positive OPSCC is climbing so rapidly that the total number of OPSCCs diagnosed annually in the United States has now surpassed the total number of cervical cancers. It is estimated that by 2020, there will be more HPV-positive OSCCs diagnosed on an annual basis than there will cervical cancers, making OPSCC the leading HPV-associated cancer. Furthermore, by 2030 it is likely that OPSCC will make up the majority of head and neck cancers, with estimates in the region of 45-50% (20). This pattern is replicated in other countries, including the United Kingdom (32).

1.1.4 Tumour Staging

Accurate tumour staging is generally considered to be the most important factor in terms of both prognosis and treatment planning (1). The most commonly used staging system is that proposed by the American Joint Committee of Cancer (AJCC), which is currently in its 7th edition (39). Head and neck tumours are staged according to the extent of the primary tumour (T1-T4), the presence, size and number of metastatic cervical lymph nodes (N0-N3), and the presence or absence of distant metastases (M0 or M1). The various combinations of TNM stage can be combined to give an overall disease stage from I to IV (39-41). The prognosis of any individual patient with HNSCC largely depends on the stage of their tumour at presentation and the subsite of their tumour. The 5-year survival for stage I lip cancer is over 90%, while that of stage IV hypopharyngeal cancer is less than 25% (42). Approximately two-thirds of HNSCCs present at an advanced stage (i.e. stage III or IV), usually due to involvement of regional lymph nodes, and the presence of cervical lymph node metastases reduces 5-

year survival by 50% (1, 43). Distant metastatic disease at presentation is rare, occurring in only 10% of cases, and is most commonly to the lungs (1).

1.1.5 Treatment of HNSCC

Individual patient treatment depends on a number of disease factors including tumour location, stage and grade and in addition, patient factors such as age, performance status and individual choice. Treatment includes surgery, radiotherapy and chemotherapy, either alone or, more commonly, in combination. Tumours of the head and neck are in a complicated anatomical location. Surgical resections may result in significant alteration of both cosmesis and function (including speech and swallow). Recent surgical advances, such as transoral laser or robotic surgery, may promote organ preservation and have reduced side effects, but are generally inappropriate for large primary tumours.

Radiotherapy and chemotherapy are not without their adverse effects, and most patients suffer to a varying degree with mucositis, dysphagia, xerostomia, loss of taste, laryngeal oedema and radiotherapy related skin irritation (44, 45). In addition to this, patients treated with chemotherapy are at risk of neutropenia and subsequent sepsis. Although quality of life generally improves in the first year following treatment, swallowing problems may be permanent and many patients require temporary (and sometimes permanent) nutritional support via nasogastric tube or percutaneous gastrostomy (46, 47). Xerostomia is a problem in the vast majority of patients following radiotherapy (48). Where the mandible is either within or adjacent to radiotherapy fields osteoradionecrosis (ORN) may occur, which can cause long term problems with chronic infection, pain and fistula formation (49). Other long term sequelae of treatment include dental caries, trismus, hypothyroidism, sensorineural hearing loss and subcutaneous fibrosis (1).

1.1.5.1 Treatment of early stage disease

Patients with stage I or II HNSCC (i.e. T1/2, N0) are considered to have early stage disease, and constitute approximately 33% of cases. Treatment is typically given with curative intent (possible in 90% of stage I and 70% of stage II tumours) and is usually either surgical or primary radiotherapy. Oral cavity tumours are most often treated surgically, and one argument for this is to avoid ORN (1). Radiotherapy alone is associated with high cure rates for early stage laryngeal, oropharyngeal and hypopharyngeal tumours, and is usually preferred over open surgical treatment due to improved organ preservation (1, 50-52). Primary radiotherapy is usually given in daily

fractions of 2 Gy up to a maximum of 66-70gy (i.e. 33-35 fractions), while postoperative doses are typically lower (1, 53-55). Lower doses may be used in early stage laryngeal cancer (e.g. 50-55gy in 16-20 fractions)(55, 56). Altered fractionation techniques may also be used. These can be either accelerated fractionation, where larger daily or weekly doses are used to reduce the overall treatment time, or hyperfractionation, where smaller doses are given more than once a day. Interestingly, there is evidence that altered fractionation techniques can improve survival in HNSCC (57). Transoral endoscopic approaches to the larynx and oropharynx, combined with laser or robotic resections, are an alternative treatment option in appropriately skilled hands (58-60).

1.1.5.2 Treatment of late stage disease

Locally advanced, resectable, stage III or IV tumours (i.e. T1-4a, N1-3) may be treated either with surgery and adjuvant radiotherapy (+/- platinum based chemotherapy), or alternatively with concurrent chemoradiotherapy (+/- neoadjuvant chemotherapy), whereas for unresectable disease, chemoradiotherapy is standard treatment (1, 61). Resectable oral cavity primaries are generally treated with primary surgery. This gives reasonable cosmetic and functional results, and can avoid chemoradiotherapy and their associated side effects. Advanced oropharyngeal, laryngeal and hypopharyngeal tumours are usually treated with primary chemoradiotherapy in an attempt to preserve organ function (62-64).

The addition of concurrent chemotherapy to primary radiotherapy regimens has been shown to improve survival in a number of phase III trials in all head and neck subsites, largely due to reduced loco-regional recurrence (61, 65-75). There is also evidence that the addition of chemotherapy to postoperative radiotherapy can improve outcomes, particularly in the presence of extra-capsular spread or involved resection margins (76-80).

Concurrent chemoradiotherapy became the standard of care for advanced stage oropharyngeal cancer following the publication of a French, multicentre, randomised controlled trial comparing radiotherapy with or without concurrent carboplatin and 5-flurouracil (5-FU), in 226 patients with stage III or IV OPSCC in 2004. Patients in the chemoradiotherapy group had improved 5-year overall and disease specific survival (OS 22% versus 16%, p=0.05; DSS 27% versus 15%, p=0.01) (68). The main side effects of adding chemotherapy were increased myelosuppression, mucositis related weight loss and feeding tube dependency. Interestingly, these increases did not reach statistical significance (68).

A meta-analysis of nearly 16500 cases of locally advanced HNSCC has shown that the addition of chemotherapy to their treatment improves 5-year survival by 4.5%. This survival benefit was only statistically significant for concurrent chemotherapy, with an absolute benefit of 6.5% at 5-years (81). Interestingly, the survival benefit associated with concurrent chemotherapy seems to depend on the subsite of the tumour, being 8.9%, 8.1%, 5.4% and 4% for oral cavity, oropharyngeal, laryngeal and hypopharyngeal tumours respectively (82). The benefits of neoadjuvant chemotherapy regimens combining 5-FU with cis- or carboplatin are less clear, and in the same meta-analysis, the absolute survival benefit for neoadjuvant chemotherapy was only 2.4% (81). There is now, however, mounting evidence that the addition of taxane based agents to neoadjuvant regimens can further improve outcomes, with reported reductions in the risk of death of up to 30% compared to cisplatin and 5-FU alone (83-85).

Another more recent treatment option is the epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab. EGFR is upregulated in approximately 90% of head and neck cancers and its expression is a negative prognostic marker (86-88). The addition of cetuximab to radiotherapy has been shown to improve locoregional control and overall survival in advanced HNSCC, compared to radiotherapy alone (47% vs. 34% and 55% vs. 45% at 3-years, p=0.005 and 0.03) (89). To date, no trial has compared radiotherapy plus cetuximab with radiotherapy plus chemotherapy, though there are retrospective reports suggesting that the combination of radiotherapy and chemotherapy is superior (90). The Radiation Therapy Oncology Group (RTOG) trial 1016 has recently finished recruiting and aims to compare chemotherapy and cetuximab (both in combination with radiotherapy) in patients with HPV-positive OPSCC (91). In the United Kingdom, the National Institute for Health and Clinical Excellence (NICE) guidelines recommend cetuximab for the treatment of locally advanced HNSCC only if all forms of platinum-based chemotherapy are considered inappropriate (92).

1.1.6 Survival outcomes from HNSCC

Survival outcome is generally measured in terms of those patients alive at 5-years. 5-year survival rates vary by tumour location, from nearly 95% for early testicular cancer to less than 5% for pancreatic cancer (93). In HNSCC, 5-year survival rates are typically in the region of 50-60%, depending on subsite (40). There are two main reasons for these poor survival rates: Firstly, the rate of tumour recurrence in patients with HNSCC is relatively high, with up to 30% of patients suffering from recurrent disease, even in the presence of clear surgical margins (94). Secondly, there is a reasonably high rate of second primary tumours in HNSCC patients, which can occur anywhere in the upper aerodigestive tract, thought to be around 2-5% per year (95, 96). One of the proposed

causes of this is the concept of field cancerisation, discussed in section 1.3.3. Over the past 30 years survival rates for HNSCC in general have remained largely unchanged, despite improvements in diagnosis and treatment. However, a number of studies have recently shown that survival from OPSCC is increasing.

In a cancer registry based study in the United States, Carvalho *et al* found that 5-year survival for laryngeal cancer was 65.2% in 1974-76 and 63.5% in 1992-97 ($p>0.05$). When considered as a group, oral and oropharyngeal cancers showed only a very small improvement in survival over the same time periods, from 54.3% in 1974-76 to 56.3% in 1992-97 ($p=0.049$). However, when this group was analysed in more detail, 5-year survival in oral cavity cancer showed only a non-significant trend towards increased survival (1974-76 53.2%, 1992-97 56.7%, $p=0.063$) whilst oropharyngeal cancer showed a large and significant increase from 36.3% to 49.1% ($p<0.001$) over the same time period (31). In addition, a population based study from Norway demonstrated that 3-year survival from OPSCC improved from 32.2% to 57.9% between the periods 1981-1995 and 1996-2007, whilst that of other HNSCC subsites remained relatively static (3-year survival 1981-1995 53.5% vs. 1996-2007 57.6%) (97).

In another recent cancer registry based study, overall 5-year survival from base of tongue tumours in the United States found to have increased from 24.7% in 1980-1982 to 50.5% in 2000-2002, whilst that of tonsillar tumours increased from 28.2% to 60% over the same time periods. Patients diagnosed in 2000-2002 had a 40% reduction in the risk of death from with tumour type when compared to those diagnosed in 1980-1982 (HR 0.6, $p<0.001$; adjusted for age, gender, race, tumour stage, tumour grade and treatment) (98).

This improved survival in oropharyngeal tumours again appears to be related to HPV, and a number of studies have shown significantly improved outcomes for HPV-positive tumours compared to those that are HPV-negative (see section 1.5.9) (99-101). The reasons behind this improved survival are unclear and may be multifactorial. In a randomised controlled trial comparing standard and accelerated-fractionation radiotherapy for stage III and IV disease, Ang *et al* demonstrated a 3-year survival of 82.4% in HPV-positive OPSCC, compared to 57.1% in HPV-negative patients ($p<0.001$) (99).

In an analysis of SEER (Surveillance Epidemiology and End Results, a branch of the National Cancer Institute in the USA) data regarding oropharyngeal cancers diagnosed between 1984 and 2004, HPV-positive tumours had a median survival of 131 months compared to 20 months in HPV-negative tumours, more than a six-fold difference. Furthermore, median survival in HPV-positive disease increased significantly over this time period (1984-1989, 43 months; 1995-1999, 142.2 months), whilst that of HPV-

negative disease remained static (1984-1989, 20.9 months; 1995-1999, 27.7 months) (20). Thus, it appears that the increased survival from OPSCC seen in recent years is the result of the survival advantage associated with an increasing proportion of HPV-positive tumours.

1.2 Pathogenesis of Head and Neck Cancer

A common feature of solid tumours is the activation of oncogenes and the inactivation of tumour suppressor genes, resulting in an expansion of a clonal population of cells, which possess a growth advantage (102). Inactivation of tumour suppressor genes often occurs through a process known as loss of heterozygosity (LOH). All genes occur as two separate alleles. If one allele becomes inactivated, and one remains normal, then the two alleles are described as heterozygous. In the case of tumour suppressor genes the normal allele typically remains dominant and promotes tumour suppression. However, if the normal allele also becomes mutated, i.e. loss of heterozygosity, then the tumour suppressor function is lost (103). Both p53 and p16 are the product of tumour suppressor genes. Oncogenes, on the other hand, are genes promoting mitosis and cell division, for example those encoding cyclin D1. A mutated allele in an oncogene will usually function in a dominant manner and drive cells to divide, promoting tumour development (104). The end result of these mutations is the acquisition of a malignant phenotype, which has a number of characteristics including: limitless replicative potential (i.e. cellular immortalisation), alterations in growth factor signalling, ability to evade apoptosis, invasion and metastasis, and angiogenesis (105). The commonly seen alterations relating to these phenotypic characteristics are described below.

The pathogenesis of both HPV-positive and HPV-negative tumours involves alterations in pathways responsible for regulation of the cell cycle. Progression of any individual cell through the cell cycle, and subsequent cell division, relies on a number of regulators and checkpoints. Two important factors in cell cycle control are p53 and the retinoblastoma protein (pRb). Both of these interact with a number of cyclins and cyclin dependent kinases, and are frequently inactivated in malignancy.

1.2.1 Retinoblastoma

The retinoblastoma protein acts at the G1-S checkpoint, and serves to prevent cells from entering the S-phase of the cell cycle (106). It does so along with two similar proteins known as RBL1 and RBL2. These proteins all bind to, and inactivate, the transcription factor E2F, the function of which is to promote expression of S-phase genes (106). When a cell receives a mitotic stimulus, cyclin D1 complexes with either CDK4 or CDK6, and promotes phosphorylation of Rb. This results in the release of E2F, and hence its activation (106). One of the functions of E2F is to induce cyclin E. This then complexes with CDK2 and causes further phosphorylation of pRb, initiating entry of the cell into the S-phase. This process is negatively regulated by the protein $p16^{INK4A}$, which is encoded on chromosome 9p21 at the CDNK2A gene. Expression of p16

inhibits the function of the cyclin D1 – CDK4/6 complexes and results in cellular senescence (107). The relationships between the various cyclins, CDKs and inhibitors determine whether or not a cell can pass through the G1-S checkpoint, and progression often requires stimulation by growth factors (106-108). This pathway is summarized in figure 1.3.

1.2.2 p53

Another important cell cycle regulator is p53 (109, 110). This protein is also involved in the response to DNA damage and replication errors. DNA-damage sensors, such as ataxia-telangiectasia (TLM), phosphorylate the checkpoint kinases CHK1 and CHK2 which in turn phosphorylate p53 and lead to its activation (111). Activation of p53 results in the formation of p53 tetramers, which can then act as a transcription factor (112). One of the functions of the p53 tetramer is to induce expression of the CDK inhibitor $p21^{CIP}$, which negatively regulates cyclin A/CDK1, cyclin B/CDK1 and cyclin E/CDK2 complexes, preventing cell cycle progression (figure 1.3) (113, 114). In addition to this, p53 also functions to drive DNA damaged cells towards apoptosis, thus further preventing cell proliferation (40).

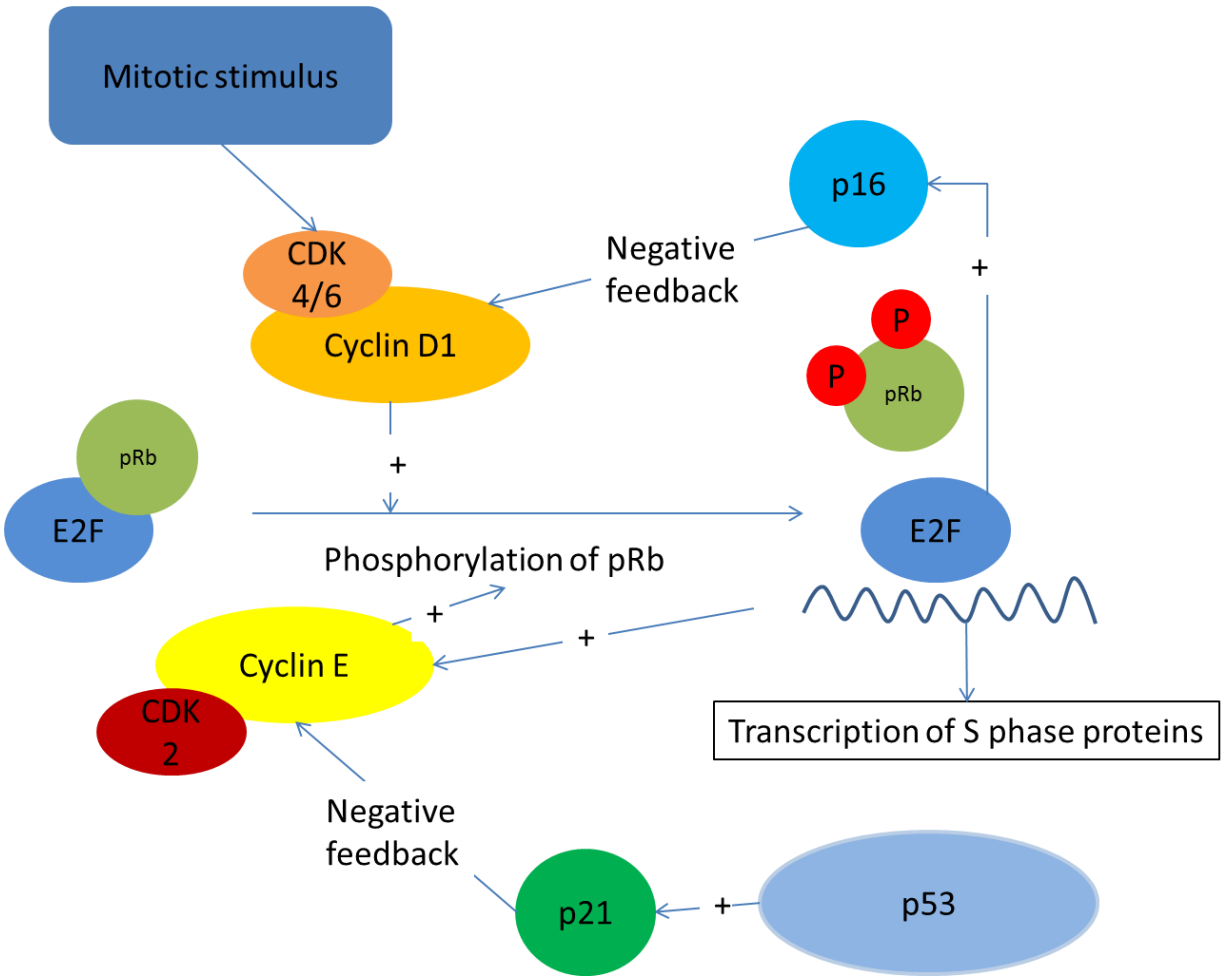


Figure 1. 3: Summary of the retinoblastoma and p53 pathways (adapted from Cancer Control 2004 H. Lee Moffitt Cancer Centre and Research Institute, accessed via www.medscape.com)

1.3 Pathogenesis of HPV-negative HNSCC

1.3.1 Common genetic features in HPV-negative HNSCC

1.3.1.1 Cellular Immortalisation

Two of the most common genetic abnormalities seen in HPV-negative tumours involve the pRb and p53 pathways (115, 116). In contrast, the oncogenic potential of high risk HPV types is related to the presence of two viral proteins, E6 and E7 which are able to interfere with these same key cellular processes (117, 118). In both cases, interference with the pRb and p53 pathways results in defects in apoptosis, abnormal DNA repair mechanisms and loss of normal cell cycle regulation. This leads to cellular immortalisation and eventually the development of a malignant phenotype within affected cells. The pathogenesis of HPV-related malignancy is dealt with in detail in section 1.4. This section will discuss the pathogenesis of non-HPV related tumours.

One of the most common characteristics of pathogenesis of any cancer type is the loss of regulation of the cell cycle (119). Two of the most critical regulators of the cell cycle are p53 and pRb pathways described earlier (section 1.2); these are both commonly affected in HNSCC (115, 116, 120). Point mutations in p53 and loss of heterozygosity of 17p13, where the TP53 gene is located are both common findings. These aberrations occur in over 50% of HNSCC cases and disruptive p53 mutations have been shown to be associated with reduced survival after surgical treatment of HNSCC (121, 122). Loss of heterozygosity of 9p21 is seen in 70-80% of HNSCC (123). The gene encoding p16, CDKN2A, is found at this location, and p16 inactivation, caused either by deletions, point mutations or promoter hypermethylation, is thought to be an early event in the development of HNSCC (124, 125). In addition, the overexpression of cyclin D1 (encoded by CCND1) due to amplification of chromosome 11q13, is found in over 80% of cases (126). There is evidence that overexpression of cyclin D1 is associated with a more aggressive tumour phenotype, including nodal metastasis, increased tumour recurrence and reduced overall survival (127, 128).

Interestingly, there is evidence that the levels of genetic alterations in HPV-negative HNSCC are variable and can have an effect on outcome. Smeets *et al* analysed 39 HPV-negative oral and oropharyngeal tumours for p53 mutations and used comparative genomic hybridisation (CGH) to classify tumours based on the number of chromosomal aberrations (129). CGH allows the visualisation of the presence or absence of chromosomes (or parts of chromosomes) in a tumour by using fluorescence microscopy (40). They found 3 groups of tumours based on the number of chromosomal aberrations; group 1 (n=8) had very few (mean 2.8 chromosomal

breakpoints); group 2a (n=26) had a moderately high number (mean 30 breakpoints) while group 2b (n=5) had a very high number (mean 52.8 breakpoints). Group 1 was characterised by lower levels of alcohol consumption, wild type p53 and a higher proportion of female patients. Indeed, all of the patients in group 1 had wild type p53 and 6 out of 8 had never consumed alcohol, suggesting a potential role for alcohol exposure in p53 mutations. They also found that the 3 groups stratified out in terms of survival, with those in group 1 having the best survival and those in group 2b the worst (p=0.004) (129). Mutations in p53 are thought to be an early event in the pathogenesis of HNSCC (see 1.3.2), however not all HPV-negative tumours have these mutations, as seen in Smeets' study. It is thought that these tumours either undergo p53 independent oncogenesis, or have mutations of other proteins in the p53 pathway (40).

The result of alterations in TP53, CDKN2A and CCND1, is the development of cellular immortalisation and unbounded cellular replication (130). Under normal circumstances, cell division results in shortening of telomeres, which is a form of cellular ageing. Functional telomeres are required for cells to undergo mitosis, and in time this shortening prevents further cell division. However, over 90% of tumours show reactivation of telomerase, an enzyme involved in maintenance of telomeres, leading to telomere immortalisation and subsequently the protection of these acquired genetic changes (131). It is considered likely that the overcoming of telomere shortening is an essential step for cellular immortalisation to become established (40).

1.3.1.2 Altered Growth Factor Signalling

Epidermal growth factor receptor (EGFR) is thought to play an important role in the development of HNSCC, and is overexpressed in up to 90% of tumours (87, 132). Binding of epidermal growth factor (and other ligands) to EGFR leads to activation of a number of signalling pathways, including those involved in the regulation of cell proliferation, apoptosis and angiogenesis (133). All of these can drive tumour cell survival, and tumour growth and metastasis (1, 40). One of the genes activated via EGFR is CCND1, which encodes cyclin D1, and is often amplified in HNSCC (126, 134). EGFR mutations, and amplification of chromosome 7p11.2 (where EGFR is encoded) have been reported in HNSCC, and ectopic expression of EGFR in keratinocytes can cause cellular proliferation (135-137). There is also evidence that EGFR expression, mutation and phosphorylation is associated with poor prognosis, and studies involving blockade of the EGFR pathway have shown benefit in HNSCC patients (see section 1.1.5) (86-89, 138).

Another important growth factor that has been implicated in the pathogenesis of HNSCC is transforming growth factor β (TGF- β) (139-141). TGF- β acts as an inhibitory growth factor in epithelial cells (142, 143). It signals via its receptor and downstream transcription factors such as SMAD2, 3 and 4 to activate target genes such as CDKN1A (encoding $p21^{CIP}$, a CDK inhibitor and part of the p53 pathway), resulting in reduced proliferation and increased apoptosis (40). TGF- β receptor expression is often found to be downregulated in HNSCC, possibly due to loss of chromosome 18q, where the genes for the TGF- β receptor, and the SMAD family are located (40, 139). Furthermore, it has recently been shown that SMAD4 knockout in the oral mucosa of mice can cause the development of spontaneous oral tumours, adding to the evidence suggesting a role for TGF- β in the development of HNSCC (144).

1.3.1.3 Evasion of Apoptosis

It is thought that phosphatidylinositol-3 Kinases (PI3Ks) play a role in the pathogenesis of many tumour types, particularly the class 1a PI3Ks (145). These are heterodimers, consisting of a catalytic and a regulatory subunit. They are often associated with receptor tyrosine kinases such as EGFR (146). On activation, the catalytic subunit converts phosphatidylinositol bisphosphate (PIP_2) to phosphatidylinositol triphosphate (PIP_3) (147). One of the downstream consequences of this is phosphorylation of AKT, which in turn phosphorylates multiple proteins, including apoptosis inhibitors such as BAD, resulting in cell survival and proliferation (145). One of the PI3K catalytic subunits is p110α, which is encoded by PIK3CA at 3q26 – a locus that is frequently amplified in HNSCC (148). Mutations of PIK3CA, which result in increased p110α activity, have been found in up to 20% of HNSCCs (149, 150). The conversion of PIP_3 to PIP_2 is mediated by the tumour suppressor PTEN, and PIP_2 counteracts the activation of AKT to stop the PI3K pathway. Loss of PTEN, through mutations or deletions, has been described in approximately 10% of HNSCCs (151). In these cases, activation of the PI3K pathway becomes irreversible.

1.3.1.4 Tumour Invasion and Metastasis

Head and neck tumours typically spread via the lymphatics to cervical lymph nodes. In order for tumour cells to reach lymphatic vessels, they must first degrade, and then invade through the extracellular matrix. Initially it was proposed that this might be due to overexpression of matrix metalloproteinases (MMPs) by these tumours (152). However, there is equivocal evidence for this, and anti MMP treatments have not proved useful in HNSCC (152). It has recently been shown that certain gene expression profiles are predictive of invasion and metastasis, and that these profiles commonly

contain genes associated with epithelial-to-mesenchymal transition (EMT) (153-155). This is a process whereby epithelial cells can undergo a change of phenotype to resemble mesenchymal cells. It is thought that epithelial cells lack the cellular plasticity necessary to metastasise, and that EMT may confer a more metastatic phenotype (40). Another important consideration is the role of the stromal cells: there is now evidence that a tumour stroma rich in myofibroblasts can promote tumour invasion, and is associated with poor outcomes (156).

1.3.1.5 Angiogenesis

The ingrowth of new vessels to support tumour bulk and metastasis is essential for any tumour to progress beyond a few millimetres in diameter (157). This is regulated by many angiogenic factors, but one of the most important is vascular endothelial growth factor (VEGF) and its associated receptors (158). VEGF may be upregulated in approximately 40% of head and neck tumours, and VEGF expression is associated with reduced overall and disease specific survival, and with increased tumour recurrence (159).

1.3.2 The progression model of HNSCC

In colorectal cancer, it has been well established that the development of a malignant phenotype requires the accumulation of a number of genetic imbalances over a period of time in a 'multistep process'. This same progression model has also been established in a number of other solid tumours, including brain and bladder carcinomas (102, 160-162). To this end, Califano *et al* aimed to determine whether a similar model was applicable to head and neck malignancy (163).

This group assessed a range of genetic abnormalities in 87 head and neck lesions, ranging from benign squamous hyperplasia, through to dysplasia, in-situ carcinoma (CIS) and finally invasive carcinoma. They investigated 10 chromosomal loci known to be frequently affected in invasive head and neck malignancy. These were: 9p21 (encoding p16), 11q13 (encoding cyclin D1), 17p13 (encoding p53), 3p, 13q21 (an area with frequent LOH near to the retinoblastoma locus), 4q26-28, 14q31-32, 8p, 8q and 6p. They reported that the mean number of events of LOH increased with increasing histological progression. Samples of benign squamous hyperplasia had a mean of 0.7 loss events, dysplastic lesions had 2.7, in-situ carcinomas 3.3 and invasive lesions 3.6. In benign lesions, the most common loss event was at 9p21 (20% of cases), followed by 3p21 (16%) and 17p13 (11%), suggesting that mutations of p16 and p53 are early events in the development of HNSCC. The progression from benign squamous hyperplasia to dysplasia was found to be associated with an increasing

incidence of LOH at 11q13 (cyclin D1; 29%), 13q21 (32%) and 14q31 (23%). There was also an increase in the frequency of LOH at 9p21 and 3p21 in progression from benign hyperplasia to dysplasia, and a further increase from dysplasia to in-situ carcinoma, which was at a similar level to invasive tumours. This was felt to be consistent with an early role for LOH at 9p21 and 3p21 in the development of malignancy. Later events in progression were characterised by increases in the frequency of LOH between in-situ carcinoma and invasive disease. Chromosomal loci found to be involved later in tumour progression included 6p (19% in CIS and 38% in invasive disease), 8p (21% and 40%), 8q (20% and 38%) and 4q26-28 (21% and 47%).

Several of the samples analysed in this study had areas within them of differing histological grades, i.e. dysplasia in one area and CIS in another. To attempt to further determine the order of progression of genetic events, the authors dissected these areas and compared patterns of genetic abnormality between them separately. They found that the more advanced area contained the same pattern of LOH as the less advanced area, but in the majority of cases also contained further loci that demonstrated LOH. This was felt to be consistent with the theory that histopathologically distinct areas within an abnormal area of mucosa all arise from clonal expansion of the same parent cells and is important in the concept of field cancerisation (see section 1.3.3).

Based on these findings, Califano *et al* proposed a progression model for HNSCC, a representation of which is shown in figure 1.4. An important factor to consider in this model is that, although there is evidence that certain genetic aberrations typically occur earlier than others (e.g. LOH at 9p21), it is the accumulation of a number of different mutations that ultimately results in malignancy, rather than the order in which they occur (163).

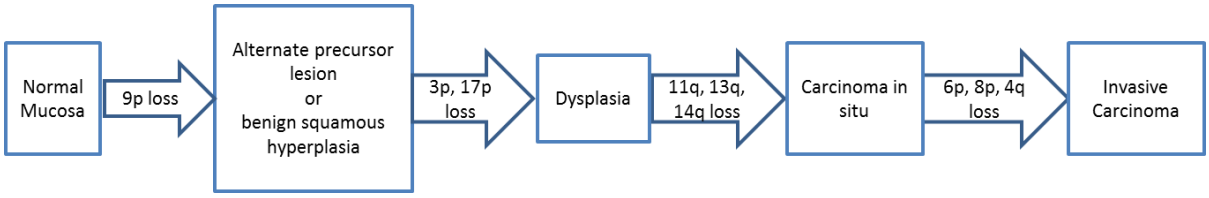


Figure 1. 4: A theoretical progression model of HNSCC as proposed by Califano et al (adapted from (163))

Further evidence of this progression model is added by the work of Tabor *et al* (164). They compared LOH in the primary tumour and adjacent macroscopically normal tissue in 10 patients with HNSCC. They found that in 6 of these patients, the primary tumour showed additional genetic alterations than those found in the "normal" tissue, and in 2

of them p53 mutations were present in the tumour but not in the surrounding tissue. These findings are consistent with Califano's descriptions of tumour progression. However, advances in the understanding of field cancerisation have led to a modification of this model and are discussed below.

1.3.3 Field Cancerisation

In 1953, Slaughter *et al* first introduced the concept of field cancerisation (165). They found that, in oral cancer resection specimens, there were often histological abnormalities in macroscopically normal tissue around an invasive tumour. These included areas of dysplasia, CIS or even additional areas of invasive disease. They suggested that, in response to a carcinogen such as tobacco smoke, an entire area (or field) of tissue could develop malignant or premalignant change, and that this field change could potentially explain the high rates of tumour recurrence and second primaries seen in head and neck cancer (163, 165-167).

Califano *et al* (discussed above) reported that histologically normal areas of mucosa had evidence of the early genetic changes seen in adjacent premalignant lesions. Furthermore, there was evidence that these cells were all derived from a common progenitor (163). This was the first genetic evidence for Slaughters field cancerisation hypothesis, and has been built on in recent years.

Tabor and colleagues examined genetic alterations in 28 patients who had undergone surgical resection of either oral or oropharyngeal tumours, and in whom the surgical margins were histologically clear of tumour (164). They examined for LOH at chromosomes 9p, 3p and 17p (based on the findings of Califano *et al,* these represented early genetic changes in premalignant lesions) in both the primary tumour and also in 5 biopsies of macroscopically normal mucosa taken from each resection specimen. Four of these were taken from within 5mm of the tumour, while one was taken from more than 5mm away. In patients in whom there was genetic evidence of field cancerisation (i.e. LOH in the macroscopically normal tissue), the surgical margins were also examined for genetic aberrations. In 10 out of the 28 patients (36%) there was LOH of at least one locus in at least one "normal" biopsy, i.e. there was evidence of field cancerisation. Furthermore, of the 10 patients with evidence of field change, LOH was found at the resection margin of 7, suggesting that the abnormal field had not been adequately excised. Comparisons of the genetic aberrations between the primary tumours and the "normal" biopsies demonstrated similar patterns of LOH, suggestive of a common clonal origin. Another study demonstrated the presence of cells that were clonally related to the primary tumour in the resection margin of over half of cases of HNSCC analysed for p53 mutations (168).

A follow up study by Tabor's group examined genetic alterations in 10 patients with HNSCC who developed second primaries, and compared p53 mutations and LOH profiles between each tumour pair (169). In 6 out of the 10 patients, the pattern of genetic alteration was in keeping with a common clonal origin of the two tumours, suggesting that at least a proportion of second primary tumours of the head and neck arise from the same area of field change as the original tumour. Interestingly, in all 10 patients, genetic aberrations were detected in the histologically normal resection margins of the primary tumour, emphasising the importance of field change at this location and suggesting that the presence of a genetically altered field at a resection margin is a risk factor for the development of a second primary tumour. Another important finding of this study related to the size of these altered fields. Of the patients in whom a common clonal origin was suspected, the two tumours were separated by a distance of up to 6cm. Once the tumour diameter was taken into consideration, this suggested that abnormal areas of field change could be in excess of 7cm in diameter.

These findings, amongst others, have led to a modification of Califano's HNSCC tumour progression model (170). In normal mucosa adjacent to tumours, small clusters of ~200 cells can be demonstrated which have mutated p53 (171, 172). These clusters are known as patches, and are thought to be a clonal expansion of a common progenitor cell, i.e. a stem cell (173). Braakhuis *et al* have proposed that if a stem cell acquires a mutation in p53 (or in the chromosome encoding it, namely 17p), then it can replicate and form a patch. They then hypothesise that the cells within these patches acquire further genetic anomalies, for example in 9p or 3p, and progress to form a field. This field has a growth advantage over the surrounding normal epithelium, and so expands and displaces it. As the field expands it may develop further mutations and form a number of subclones. There is evidence that these subclones exist, and that they share a common origin, though diverge genetically to have different mutations (164). Eventually one of these subclones develops enough mutations to form an invasive tumour. It seems that the progression to invasive disease requires amplification of 11q13, where cyclin D1 is encoded. This model builds on that of Califano *et al*, in that the development of malignancy requires the accumulation of a number of mutations. However, the identification of the presence of pre-neoplastic fields adds to Califano's model, by establishing a cause for second primary and recurrent tumours.

Thus, it can be seen that exposure to tobacco and alcohol results in alterations in a number of genes and their encoded proteins, and that these are commonly under-expressed, over-expressed or mutated in HNSCC. These aberrations are clearly related

to the development of characteristics required for malignancy and can exist in areas of seemingly normal mucosa over a large area of field change. Tumours can arise from anywhere within these fields. Some of these genetic aberrations are typically acquired earlier than others, and a “progression model” for HNSCC has been proposed in recent years. It must the noted that the important factor in the development of malignancy is not the order in which mutations are acquired, but the acquisition of multiple genetic defects. HPV related malignancies are characterised by a much lower rate of genetic alteration, and instead the development of a malignant phenotype, arises as a result of the expression of 2 viral oncoproteins, E6 and E7, which interact with the p53 and pRb pathways, and functionally alter them. This is discussed in more detail below.

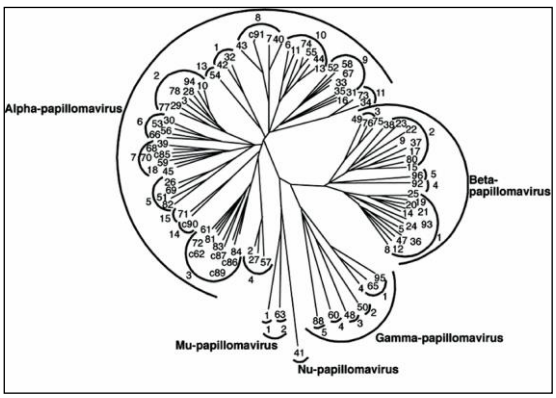
1.4 Human Papillomavirus – Infections and Oncogenesis

1.4.1 An Introduction to Human Papillomavirus

Human papillomaviruses are double stranded DNA viruses with an average size of approximately 8000 base pairs. More than 100 different types of HPV have been identified by DNA sequence analysis (174). Much of the literature regarding HPV lifecycle and oncogenesis comes from work in cervical carcinoma, where HPV is responsible for over 99% of cases. HPV is endemic in the population, and the lifetime risk of cervical HPV infection is in the region of 80% (175). Figures for levels of oral HPV infection are less clear, though a recent epidemiological study puts the prevalence of oral HPV infection in adult men at around 4% (176). HPV subtypes can generally be divided into low and high risk groups. Low risk groups, for example HPV-6 and HPV-11, are responsible for benign lesions such as cutaneous or genital warts and laryngeal papillomata. There are approximately 15 high risk types, for example HPV-16 and HPV-18, and it is these that are associated with malignancy (177, 178).

There are several evolutionary genera of HPV (figure 1.5). The main groups are the Alpha and Beta papillomaviruses. These account for approximately 90% of the known HPV subtypes. The Alpha papillomaviruses make up the largest group, and it is this group that contains the high risk mucosal subtypes responsible for both cervical carcinomas and HNSCC. HPV-16 is the most common high risk subtype in the general population, and is responsible for ~50% of cervical carcinomas and ~85% of HPV associated HNSCC (100, 177). Beta papillomaviruses are usually associated with benign cutaneous infections: However, in immunocompromised patients, these infections can be associated with the development of non-melanoma skin cancer. The remaining types of HPV belong to the Gamma, Mu and Nu genera. These cause cutaneous lesions and are not associated with malignancy.

Figure 1. 5: The evolutionary genera of HPV - from (177).



The structure of the Human Papillomavirus

Despite the large number of different types, all HPV share a similar viral structure. The virus consists of an icosahedral shaped protein capsid which contains a circular double-stranded DNA. The viral genome varies amongst types but typically contains somewhere in the region of 8000 base pairs. This genome encodes a number of different viral proteins, and typically has 8 or 9 open reading frames (ORFs). The proteins encoded are designated as “early” or “late” depending on where in the lifecycle they are expressed. The early proteins; E1, E2, E4, E5, E6 and E7 are involved in viral DNA replication and cell proliferation whilst the late phase proteins; L1 and L2, are involved in viral packaging and the formation of the capsid, with L1 playing the major role (177, 179, 180). Their roles are described in more detail below.

In addition to the ORFs coding for the various viral proteins, the HPV genome also includes a non-coding region known as the upstream regulatory region (URR) and two promoter regions (181). The HPV-16 genome is shown in figure 1.6.

1.4.2 Benign Human Papillomavirus Infection

The lifecycle of the papillomavirus takes place entirely within an epithelial layer, be it cutaneous or mucosal. With the exception of the E1/E2 complex (see 1.4.3 and 1.4.5), papillomaviruses do not contain the enzymes required for genome replication and thus rely on host cellular machinery. The majority of HPV infections are either asymptomatic or result in benign cutaneous or mucosal lesions, i.e. warts. In these infections, viral gene expression is highly regulated and viral proteins are produced at well-defined points in time, as the infected cell moves through the epithelial layer. The early stages of the lifecycle require epithelial cells that maintain the ability to divide, whilst the assembly and packaging of mature virions can only occur in terminally differentiated cells (177, 181, 182). The end result is production of further virions and thus maintenance of infection. Malignancy seems to be related to a loss of this strictly regulated gene expression and specifically, the over-expression of E6 and E7 proteins. There are a number of important steps in the viral lifecycle.

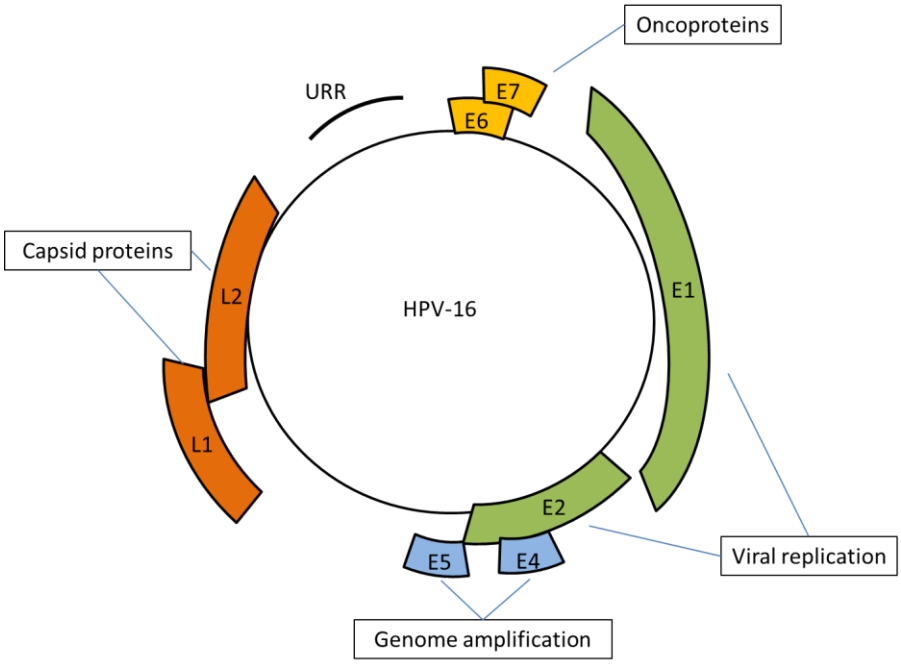


Figure 1. 6: Diagrammatic representation of the genome of HPV-16 (adapted from Malik H et al, Proteins and Peptides - Re-emergence in Prophylactics and Theraputics, http://esciencecentral.org/ebooks/advances-in-protein-chemistry/proteins-peptides.php)

1.4.3 Establishment of Infection

The first step in HPV infection is entry of the virion into an epithelial cell. Infection can only be established in the basal epithelial stem cells that maintain the ability to proliferate (183). These basal stem cells constantly divide to replace cells lost as a result of desquamation at the epithelial surface (184). In the cervix, infection occurs in the transformation zone, whilst in the oropharynx there is a strong predisposition for infection in the crypts of the palatine and lingual tonsils (185, 186). It is thought that micro-abrasions in the epithelial cell layer allow access of the virion to the basal layer (181). Entry of virions into the basal epithelial cells is an active process, though there is some debate as to the exact receptor(s) involved. It is thought that heparan sulphate proteoglycans, and in particular syndecan-1, play a role in initial binding and that a secondary receptor (possibly beta6 integrin) is then required for efficient infection (177, 187, 188). Following receptor binding, HPV particles enter the cell via endocytosis. They are then disassembled in lysosomes and the genome is transferred to the nucleus, a process that is facilitated by the L2 capsid protein (189). Once a cell is infected, the viral genome is established within the nucleus of the host cell as an episome, i.e. the viral genome is not integrated with that of the host cell. This process is thought to be reliant on the expression of the viral proteins E1 and E2 (177).

1.4.4 Initiation of Viral Gene Expression

The expression of the viral genes is initiated by two major promoter regions. The first, or early, promoter is found upstream of the E6 ORF, and is responsible for transcription and translation of the early viral proteins. This promoter can initiate transcription independently of the differentiation of the host cell. The second, late, promoter region is responsible for the transcription and translation of the L1 and L2 proteins; this activation is dependent on the differentiation of the host cell and this therefore restricts viral packaging to the upper layers of the epithelium (181).

1.4.5 Replication of the Viral Genome

Replication of the HPV genome depends on the reproductive cellular processes of the host and can only take place in dividing cells. Genome replication is mediated by the viral E1 and E2 proteins. E2 binds to the non-coding region of the viral genome and forms a complex with E1, thus bringing E1 into approximation with the origin of replication. E1 is a DNA helicase, responsible for unwinding the viral DNA double helix in the early stages of replication. It in turn binds to cellular proteins required for DNA replication including replication protein A and DNA polymerase α primase (190-192). The actions of these host cellular proteins, amongst others, result in replication of the

viral DNA. This occurs during the S-phase of the cell cycle with the replicated genome being partitioned equally during cell division. E2 plays a role in anchoring viral episomes to host mitotic chromosomes, a step that is vital for correct episomal segregation, ensuring that each daughter cell contains a set of viral genomes. These combined actions of E1 and E2 assist in the establishment of 20 to 100 copies of the viral episome within *each* basal cell (193).

1.4.6 Mediation of Cell Proliferation

As previously mentioned, HPV virions are entirely reliant on host cellular proteins for reproduction of their genome, which occurs during the S-phase of the cell cycle. In the basal epithelial layers, where cells are naturally dividing, this can occur freely. However, as cells ascend through the epithelial layer, they normally begin to differentiate into mature keratinocytes and exit the cell cycle (194). One of the key features of HPV infection is the increase in cellular proliferation in the basal and supra basal levels (177). The viral oncoproteins E6 and E7 are responsible for this increased proliferation, which results in an increase in the small number of cells initially infected. The actions of both E6 and E7 serve to push cells into S-phase. E6 interacts with the tumour suppressor gene p53, while E7 interacts with the oncogene pRb. These interactions are described in detail below, however both ultimately serve to produce more infected cells, which in turn go on to produce further virions.

E5 is also thought to play a role in stimulating cellular proliferation. It is a small trans-membrane protein usually found in association with the endoplasmic reticulum. It prevents acidification of early endosomes and thereby alters the recycling of EGFR on the cell surface. This leads to an increase in EGFR signalling and the maintenance of a replication supporting environment in cells of upper epithelial layers (195, 196). Interestingly, the E5 ORF is often lost as episomal DNA becomes integrated with that of the host (a step thought to be important in malignant transformation and discussed below) and therefore E5 is unlikely to be important in HPV associated malignancy (179).

Interestingly, the degree of cellular proliferation is dependent on the specific HPV lesion: For example, in benign cutaneous warts caused by the low risk HPV-1, proliferating cells are restricted to the basal epithelial layer. However in high risk infections such as HPV-16 this proliferating layer is several cells thick. Moreover, in malignant lesions, certainly in the cervix, the thickness of these cell layers increases with the grade of neoplasia. This is paralleled by a decrease in the extent of epithelial differentiation (197).

1.4.7 Late events in the Viral Lifecycle

As outlined above, the actions of E6 and E7 ensure that infected cells in the suprabasal layers remain mitotically active. Infected cells can therefore re-enter the S-phase of the cell cycle, resulting in on-going viral DNA replication and subsequent amplification of the viral genome. This further increases the number of potential virions. As infected cells ascend through the epithelium they begin to differentiate, though this process is delayed compared to uninfected cells. This differentiation of the host epithelial cell leads to activation of the late promoter region and subsequent translation of the L1 and L2 capsid proteins (177). Once translated, these proteins assemble into icosahedral capsids. It is not known whether the genome enters the capsid during or after its assembly, however encapsulation is assisted by L2 and possibly also by E2 (198). The encapsulated genome represents the mature HPV virion. At the most superficial layers of the epithelium, the plasma membrane of the epithelial cell is replaced by the so called *cornified envelope.* This consists of keratins enclosed in an insoluble protein mixture and surrounded by a lipid envelope (199). Within this cornified envelope, viral E1/E4 protein complexes are thought to interact with keratin networks and cause them to collapse. This then results in the release of mature virions and the propagation of infection.

1.4.8 The role of the E7 Oncoprotein

The potential for HPV genomes to cause cellular transformation was first recognised in the mid-1980s in experiments on rodent fibroblast lines and kidney cells (200). Further studies showed that expression of the HPV genome was capable of inducing cellular immortalisation and preventing differentiation in human epithelial cells, including those of the upper respiratory tract (201-203). Mutational analysis subsequently revealed that E7 was responsible for much of this potential and was dependent on the co-expression of E6 (204). Accordingly, E6 and E7 are the only two proteins consistently expressed in HPV associated cervical carcinomas (182). At present it is unclear whether this is the case for HNSCC. E7 plays a vital role in inducing on-going proliferation in epithelial cells that would normally undergo growth arrest. In benign infections, this allows for on-going viral replication, however when expression of E7 is dysregulated, it plays a central role in HPV induced oncogenesis (182). The ability of viral E7 to promote proliferation and impede differentiation varies between the HPV subtypes, and fits with the high/low risk classification. The E7 protein of HPV6, for example, has vastly reduced transformation potential compared to HPV16 and HPV18 (205).

The major role of E7 in stimulating cell proliferation involves its interaction with the pRb/E2F signalling pathway (see section 1.2.1). E7 proteins are all small polypeptides of approximately 100 amino acids. They share similarities with adenovirus (Ad) E1A and simian vacuolating virus 40 (SV40) large tumour antigen (T Ag) in that they all have a conserved region in their amino acid sequence that is capable of binding to pRb (206, 207). Ad E1A and SV40 T Ag are not associated with human tumours though may cause tumours in rats (207). After binding, E7 induces proteosomal degradation of pRb via an ubiquitin dependent pathway (208, 209). This replaces CDK dependent phosphorylation in freeing the transcription factor E2F. Dissociated E2F can then induce activation of the genes required for the entry into S-phase. Interestingly, E7 preferentially associates with E2F bound pRb rather than free pRb (210). Knockout studies have demonstrated that this ability for E7 to bind to pRb is vital in inducing cellular transformation (211). The association with, and degradation of, pRb and subsequent release of E2F allows for G1 exit and S-phase entry, resulting in the proliferation required for viral genome replication. The degradation of pRb by E7 also results in an upregulation of the CDK inhibitor $p16^{INK4A}$, and the expression of this protein can be used as a surrogate measure of E7 expression by tumour cells (40, 207).

As previously mentioned in section 1.2, CDKs play an important role in driving the cell cycle. CDKs are positively regulated by cyclins, and negatively regulated by CDK inhibitors (CDKIs). These processes are affected by E7. The expression of cyclins A and E, regulatory subunits of CDK2, is induced by E2F and thus is increased by E7 (212). Conversely, E7 has been shown to interact with and abrogate the CDKIs p21 and p27, both of which negatively regulate CDK2 (213, 214). This ability of E7 to increase cyclin A and E, and to reduce CDKI activity, ensures that epithelial cells remain in a state that enables DNA replication. In addition, E7 can directly interact with CDK2/Cyclin A and CDK2/Cyclin E complexes and increase CDK2 activity (215).

There are a number of additional mechanisms by which E7 is thought to promote cellular proliferation and transformation (216-219). These include:

- Interaction with class I histone deacetylases – these act as transcriptional repressors and their association with E7 results in increased levels of E2F dependent transcription.
- Interfering with p53 mediated cell cycle arrest in response to DNA damage: However, the main interaction between HPV and p53 is via the E6 oncoprotein).
- Inhibiting negative regulatory signals from circulating cytokines such as TGF-β

1.4.9 The role of the E6 Oncoprotein

E6 is another small polypeptide, approximately 160 amino acids in length (220). It too has the ability to transform human epithelial cells. The main functional role of E6 is via its ability to bind to, and induce degradation of, the tumour suppressor protein p53 (118, 221, 222). As discussed above, this protein acts at the G1/S checkpoint and targets cells for apoptosis in the event of DNA damage. Following binding, E6 and p53 form a trimer with E6-associated protein (E6AP), though there is evidence that E6 can degrade p53 even in the absence of E6AP. E6AP induces ubiquitination of p53 and thus targets it for proteosomal degradation (108, 223-225). E6 can also down-regulate signalling targets of p53, such as Notch1 (226). In the absence of functional p53, the integrity of replicated DNA is compromised and mutations can accumulate. The normal drive towards apoptosis in these cells is lost and this then results in uncontrolled proliferation or tumour development (118).

As with E7, the ability of E6 to bind to p53 varies between low and high risk HPV types (108). Although low risk HPV E6 can bind to p53, it does so with reduced affinity and is unable to bind to E6AP or induce p53 degradation (227, 228). Indeed cells infected with HPV-11 have been shown to *accumulate* p53 after DNA damage and undergo growth arrest in the G1 phase of the cell cycle (228).

In addition, several functions of high risk E6 enable inhibition of apoptosis in a p53 independent manner. E6 interacts with, and causes degradation of, the pro-apoptotic protein Bax (229). It can also bind to the TNF receptor-1 (TNFR1) and subsequently inhibit TNFR1 induced apoptosis (230). Furthermore, HPV-16 E6 has been shown to activate NF-κB, resulting in increased expression of IAP-2, an inhibitor of apoptosis of epithelial cells (231).

High risk E6 is capable not only of transforming host cells, but also inducing cellular immortality. In normal somatic cells, each cell division results in shortening of telomere length, a form of cellular ageing. E6 from high risk HPVs is capable of increasing expression of the catalytic subunit of human telomerase reverse transcriptase (hTERT). This increased hTERT expression prevents telomere shortening and immortalises the cell (118, 232, 233).

Another important function of E6 is its ability to interact with proteins containing a PDZ (PSD95/Dlg/ZO-1) domain. A number of these proteins are involved with cellular signalling, maintenance of cellular polarity and normal cell adhesion. E6 from high risk HPV types can bind these proteins and target them for degradation (118, 177). This results in cellular transformation due to loss of cell-cell contact and loss of polarity,

and may play a role in tumour metastasis by interrupting normal cell adhesion. Low risk HPV E6 does not contain a PDZ binding domain (118, 177, 234).

Thus E6 and E7 are capable of transforming epithelial cells in the basal and suprabasal layers and promoting differentiation. In benign infection this is tightly controlled at a transcriptional level: In malignancy, dysregulated expression plays a major role in uncontrolled cellular proliferation. The actions of E6 and E7 are summarised in figure 1.7.

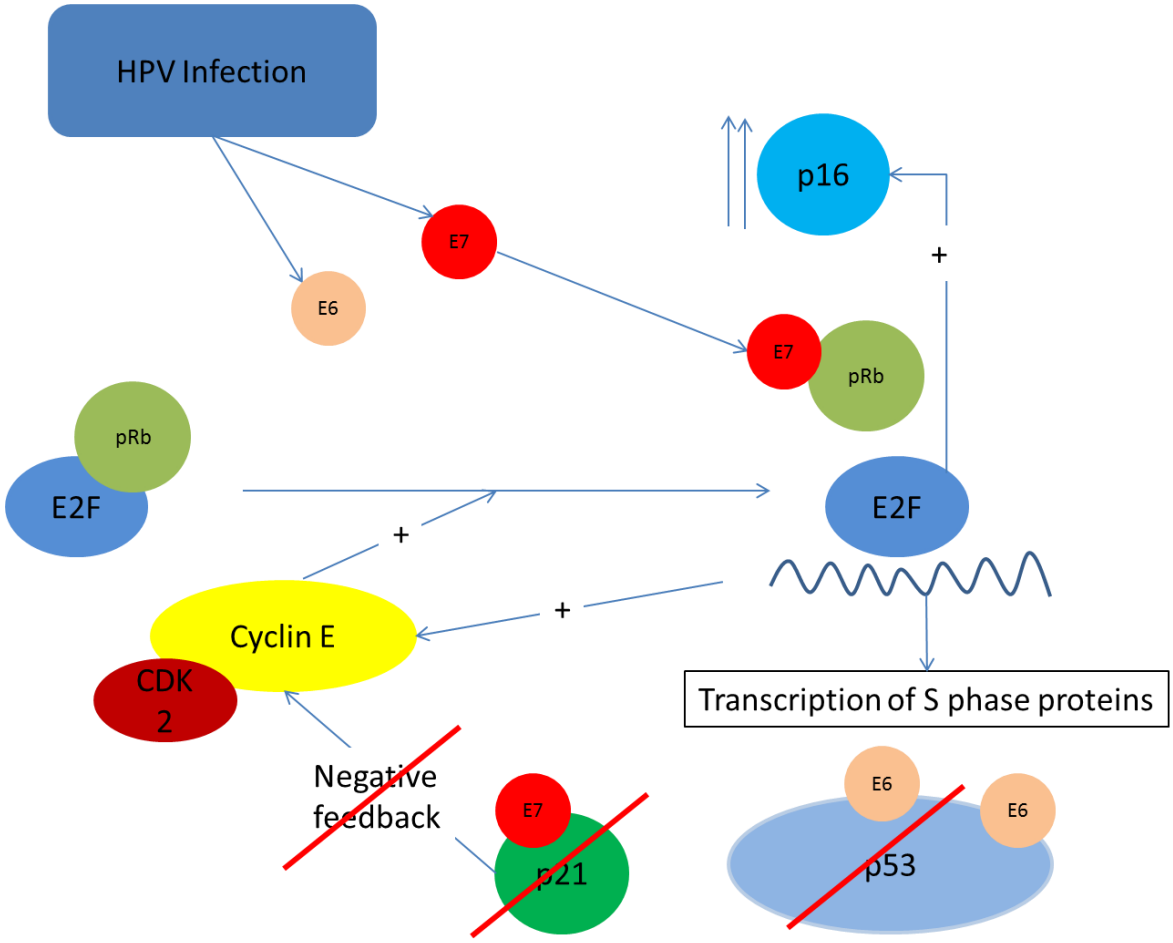


Figure 1. 7: Summary of the functions of the oncoproteins E6 and E7

1.4.10 Regulation of E6 and E7

In benign infections, the actions of E6 and E7 are tightly regulated. It is thought that E2 plays a key role in this regulation (235). At high levels, E2 binds to a site on the viral genome adjacent to the early promoter region. In doing so, it displaces the transcriptional activator SP1 and prevents E6/E7 expression (177, 192). One of the features of HPV associated malignancy is integration of the viral genome into the host,

during this process E2 is frequently lost. The subsequent deregulation of E6 and E7 is an important step in malignancy progression (236).

Therefore, the normal infective lifecycle of HPV is a highly regulated chain of events that results in the production of mature virions. When this sequence of protein expression is disrupted, or becomes dysregulated, the proliferative phase of the infection predominates, and can result in malignancy. This is described in more detail below.

1.4.11 Malignant progression

The actions of E6 and E7, abrogating p53 and pRb respectively, parallel a number of genetic aberrations seen in HPV-negative head and neck cancers. They have the same functional results, representing a parallel oncogenic pathway. However, only a small proportion of women who are infected with HPV go on to develop cervical cancer, and the same is likely to be true in head and neck malignancy, although the natural history in this patient group is less well established (179).

A pre-requisite of HPV-induced malignancy is a persistent infection with a high-risk HPV type. Therefore, viral persistence is arguably the most important factor in malignant progression (237). The majority of patients infected with HPV will clear the virus by immunological mechanisms. However, a minority do not, and may go on to develop malignancy. The importance of immune clearance of HPV is highlighted by the increased risk of HPV-associated malignancy in patients with chronic immunosuppression (238-240). In patients with normal immune responses it is unclear exactly why some progress to malignancy whilst others do not.

HPV can suppress the immune system in a number of ways. Keratinocytes infected with HPV have reduced expression of a numerous pro-inflammatory cytokines, including IL-1, IL-6, TNF-α and TGF-β, and also have increased expression of the anti-inflammatory cytokine IL-10 (117, 241-244). This altered cytokine profile reduces the ability of circulating immune cells to infiltrate the infected tissues. Furthermore, HPV can repress transcription of a number of genes involved in the interferon response pathway, a vital component of the innate immune system (245, 246).

It is now well established that clearance of HPV and HPV-related lesions is a specific immunological event requiring both a competent humoral and cell mediated immune system and characterised by an influx of $CD8^+$ and $CD4^+$ T-lymphocytes (247). This process requires antigen presentation by dendritic cells (DCs), and studies have shown that HPV is capable of altering DC function in a number of ways. Firstly the altered

cytokine expression discussed above inhibits the infiltration of infected tissues by DCs and the migration and maturation of DCs is reduced in the presence of HPV L2, as is cytokine production. Furthermore, E6 can reduce E-cadherin expression, and this is thought to interfere with interactions between DCs and keratinocytes (117, 248, 249). Recruitment of $CD8^+$ cytotoxic T-cells is, in part, mediated by the presentation of antigens on MHC-class I, which is expressed on all nucleated cells. There is evidence that HPV can down-regulate MHC class 1 expression by keratinocytes, probably due to the action of E5, and subsequently HPV-specific T-cell responses can be delayed in patients with HPV-infections (238, 250) . In addition to this, E7 is able to interfere with antigen processing, via the down-regulation of TAP, a transporter involved in the antigen processing pathway (117).

In primary human keratinocytes, expression of HPV oncoproteins results in cellular immortalisation and the cells exhibit many characteristics of premalignant lesions. However, they do not form tumours when injected into nude mice (241). This progression requires multiple passages in culture or the expression of additional oncoprotein (251-255). In addition, the development of cervical carcinomas in E6/E7 transgenic mice requires long term oestrogen exposure (256). Both of these are comparable to the long delay between exposure to HPV and development of cancer and suggest that, although the HPV oncoproteins are able to initiate carcinogenesis, further host mutations are required for the development of a malignant phenotype (182, 241).

There is evidence that hormones, for example oestrogen, may activate the HPV promoter and facilitate immortalisation of infected cells (179, 256-258). Whilst this may well be relevant to cervical cancer, it is unclear how this related to head and neck malignancy, especially given the male preponderance for HPV-positive HNSCC. Another possible confounding factor is the effect of other toxins, such as cigarette smoke (259). Mutagenic agents in these toxins may amplify persisting HPV DNA and facilitate malignant progression by altering cellular signalling cascades, and there is some evidence of differential patterns of genomic abnormality in HPV-positive smokers and non-smokers with HNSCC (179, 260). However, a significant proportion of patients with HPV-positive HNSCC have no history of tobacco exposure, so this does not explain malignant progression in these patients on its own.

It is possible that the further host mutations required for malignancy are in part caused by HPV. There is evidence that the expression of E6 and E7 can facilitate genomic instability, and a number of cytogenetic abnormalities have been detected in HPV immortalized keratinocytes (261-264). HPV infected cells are often aneuploid as a result of losses or gains of entire chromosomes during cell division (265). One of the

reasons for this is thought to be related to the induction of supernumerary centrosomes by HPV. In a normal cell division, cells contain two centrosomes that form the poles of the mitotic spindle and result in equal and symmetrical chromosome segregation between daughter cells (266). Both E6 and E7 are able to induce extra centrosomes, and this can subsequently result in abnormal division of chromosomes during mitosis (267, 268).

E7 is capable of inducing double strand DNA breaks, a factor that may well be important in viral DNA integration into the host genome (269). As previously mentioned, viral integration often results in the loss of E2 expression and subsequent overexpression of E6 and E7. In productive HPV infections, the expression of E6 and E7 is limited to the layers of the epithelium in which differentiation is occurring, and results in the production of mature virions. However in oncogenic infections, the loss of regulation by E2 results in E6 and E7 activity in the basal (i.e. stem cell) layer of the epithelium, resulting in abrogation of the usual cell cycle checkpoints and cellular immortalisation (108). The overexpression of E6 and E7 is vital in oncogenesis, and the inhibition of E6 in HPV-expressing cell lines results in apoptosis, as does the reintroduction of E2 (177))(270-274). The inactivation of p53 and pRb results in the loss of vital cell cycle checkpoints, predisposing cells to the development of further genetic aberrations and the progression to malignancy (177, 241).

It can be seen that HPV is well adapted to its primary aim of viral replication, and that each of the viral proteins plays a significant role. The virus is also well adapted to evading clearance by the host immune system, though is eventually cleared in the majority of cases (275). In the few cases where infection persists, and it is unclear what the reasons are behind this , the functions of the E6 and E7 oncoproteins encourage cellular proliferation and the accumulation of mutations, and eventually the development of malignancy.

1.5 HPV and Head and Neck Cancer

1.5.1 The epidemiology of oral HPV infection

A necessary precursor to HPV-induced malignancy is the presence of a persistent HPV infection with a high risk virus type (237). The epidemiology of cervical HPV infection is well established and, although vertical transmission via the birth canal has been reported, can essentially be considered a sexually transmitted disease (276). Cervical HPV is endemic. The lifetime risk of HPV exposure for sexually active women is in the region of 80%, and the prevalence of subclinical infection can be up to 40% of the female population (175, 277). The vast majority of these infections (>90%) are cleared by the immune system within 2 years of exposure (278). The remaining 10% of women become chronic carriers of HPV and are at significantly increased risk of progression to cervical pre-malignancy and malignancy (279).

The natural history of oral HPV infection is less well understood: There is increasing evidence that it too can largely be considered sexually transmitted. In children, oral HPV infection (i.e. the presence of HPV DNA in oral epithelial cells) is rare, with prevalence rates of less than 1% (280). Rates begin to increase around the teenage years, typically the age range for initial sexual contact (12-15 years 1.5%; 16-20 years 3.3%) and by adulthood are in the region of 4-7% (176, 280-283). Studies examining persistence of oral HPV are few in number, and have conflicting results. Some authors report similarities with cervical infections, with most being cleared within 24 months, whilst others reports low levels of viral clearance within this timeframe (25, 284, 285).

High risk sexual behaviours, and in particular increasing numbers of oral sex partners, have consistently been associated with HPV infections in cross sectional studies (25, 281, 286). Furthermore, partners of women with cervical cancer have a higher incidence of tonsillar cancer than the general population, again alluding to oro-genital contact as a mode of transmission (287). As previously discussed, oral HPV infection is rare in children. This is true of children born to mothers with or without a history of cervical cancer, arguing against vertical transmission of the disease. In addition, self-transmission from one site to another is uncommon (i.e. there is a poor correlation between cervical and oral HPV infections) (280, 288-290). Thus, there is now a reasonable body of evidence to suggest that oral HPV infection is sexually transmitted.

1.5.2 The prevalence of HPV-associated HNSCC

The prevalence of HPV-associated HNSCC is difficult to definitively establish, and rates of 0-100% have been reported in the literature (237). Several factors seem to be

important when considering the true prevalence of HPV-positive tumours. Firstly, tumour subsite and patient gender are important considerations, with the vast majority of HPV-positive HNSCCs occurring in the oropharynx of young men. HPV-positivity rates are increasing over time, probably due to changes in sexual behaviours, and vary by geographic location. Furthermore, the laboratory techniques used to define tumours as HPV-positive give varying results and so must be taken into consideration when trying to establish prevalence. The changes in HPV-positivity over time have already been discussed (1.1.3). Other factors affecting reported HPV-prevalence are discussed below.

1.5.2.1 The prevalence of HPV-positive tumours is highest in the oropharynx

Interpretation of the true prevalence of HPV-positive HNSCC is made difficult by inaccurate and inconsistent recording of tumour location between different studies. As described in section 1.1.1, the term HNSCC encompasses tumours affecting a large number of different sites within the head and neck region. In some studies, HPV prevalence is simply reported in a heterogeneous group of HNSCCs, whilst others divide tumour location up by subsite, e.g. larynx vs. oral cavity vs. oropharynx (237). Further confusion is added by categorising some "base of tongue" tumours as oral cavity, whereas they should be included with oropharyngeal tumours (101, 291). Several case series have been published which report significantly higher prevalence of HPV in the oropharynx compared to other sites (OPSCC 50-60% HPV-positive vs. 6-10% in non-oropharyngeal sites) (292, 293). A number of meta-analyses have also shown a higher prevalence of HPV in OPSCC as compared to generalised HNSCC. Taken in combination, these meta-analyses put the prevalence of HPV-positive HNSCC between 22 and 26% and that of OPSCC between 34 and 41% (100, 101, 294). In cases control series, the risk of a tumour being found to be HPV-positive is greatly increased by oropharyngeal location as opposed to other sites in the head and neck, with adjusted odds ratios of between 6 and 9 (23, 295).

Interestingly, there is evidence that HPV DNA in non-oropharyngeal locations is less likely to be biologically relevant. In one study, 71% of oropharyngeal tumours that were found to be HPV PCR-positive were also positive on more stringent testing with *in-situ* hybridisation, compared to only 8% of HPV PCR-positive tumours in the oral cavity, suggesting a bystander role for HPV in the rest of these oral cavity tumours (see section 1.5.5) (23).

Even within the oropharynx there are differing rates of HPV-prevalence, with the palatine and lingual tonsils containing the majority of HPV-positive tumours (5, 23). These subsites form part of the so-called Waldeyer's ring of lymphoid tissue. They

differ from other areas of the head and neck region in that their epithelium contains crypts in which the basal epithelial cells are readily exposed at the level of the squamo-columnar junction (237). This mirrors the "transformation zone" of the cervix, and indeed there is evidence that many HPV-positive tonsillar tumours begin with integration of the HPV-virus in these crypts (185, 186).

1.5.2.2 The prevalence of HPV-positive tumours depends on geographic location

The subsite location of head and neck SCCs varies worldwide, with higher rates of oropharyngeal tumours in developed countries, and higher rates of oral cavity cancers in the developing world, in part due to the high prevalence of tobacco chewing (296). The reported prevalence of HPV-positive OPSCC is also variable depending on the population studied, and the worldwide prevalence is estimated to be approximately 12% (296). A systematic review by Kreimer *et al* reported that the proportion of OPSCC attributable to HPV was 47% in North America, 46% in Asia, 36% in South/Central America, Australia and Africa, and 26% in Europe (101). A more recent systematic review puts North American and European prevalence rates at 59.9% and 39.7% respectively. For tumours diagnosed after 2005, the difference between North America and Europe is reversed, with prevalence rates of 69.7% and 73.1% respectively, suggesting that the incidence of HPV-positive OPSCC is increasing more rapidly in Europe than in North America (297). A recent study from China demonstrated a prevalence of just 16.7% in 66 oropharyngeal tumours, whilst another from Korea found that only 35% of 55 patients were HPV-positive, significantly lower than estimates from Europe (298, 299). Another study comparing HPV-DNA PCR in Hong Kong, Australia and the Jilin Province of China found positive rates of 29%, 46% and 0% respectively (300).

1.5.3 Gender difference in HPV-associated HNSCC

The male:female ratio of both HPV-positive and HPV-negative tumours is more than 2:1 (29). In HPV-negative disease, the reasons for this are thought to be higher rates of tobacco and alcohol consumption amongst men. However, in HPV-positive tumours the reasons are less clear (25). One theory is that although genital HPV infection is common amongst both sexes, HPV DNA copy number might be higher in the cervix and vagina, and thus males may be more likely to acquire oral HPV infection when performing oral sex (25). There is some support of this theory from one study that suggests that HPV transmission during vaginal intercourse is higher from the cervix to the penis than vice versa, though this study has small numbers (25 couples) and relatively short follow up (mean 7.5 months) (301).

1.5.4 Risk factors for HNSCC

A number of case control studies have demonstrated strong epidemiological evidence that HPV can cause head and neck cancer, and furthermore there are distinct differences in the risk factors associated with HPV-positive and HPV-negative tumours (291, 302, 303).

1.5.4.1 Sexual Behaviour

As discussed previously (section 1.5.1), there is significant evidence that both cervical and oral HPV infection are sexually transmitted diseases. It follows, therefore, that the greatest risk factor for exposure to HPV is an increasing number of sexual partners, and a number of case control studies have shown that the odds of developing oropharyngeal cancer are increased with an increasing number of sexual partners and younger age of first intercourse. Schwartz *et al* report a 3-fold increase in the risk of developing oral cancer (which in their study included oropharyngeal cancer) in males who first experience sexual contact under the age of 18, and a 2-fold increase in risk in males with more than 15 sexual partners (ORs 3.4 and 2.3 respectively, adjusted for age, smoking and alcohol exposure) (304). Interestingly odds ratios in women were not significantly raised according to these same boundaries. Similar results were found by D'Souza *et al* for oropharyngeal tumours alone (302).

A strong association has also been found between increased risk of oropharyngeal tumours and increasing numbers of oral sex partners: D'souza *et al* found a greater than 3-fold increased risk of OPSCC in patients with a history of more than 6 oral sex partners (302). This is in keeping with oral-genital contact being the main cause of oral HPV infection (25). The strength of these associations is increased when considering HPV-positive OPSCC. For example, the 3-fold increase in risk of OPSCC for patients with greater than 6 oral sex partners rises to a nearly 9-fold increase when considering HPV-positive OPSCC (ORs: all OPSCC 3.4, $p=0.002$; HPV-positive OPSCC 8.6, $p<0.001$) (302). Indeed, when stratified by HPV-status, these sexual practises were only found to increase the risk of HPV-positive OPSCC, and had no effect on HPV-negative disease (for example, ORs using descriptor of >11 sexual partners: HPV positive OPSCC 6.4, $p<0.001$; HPV-negative OPSCC 0.74, $p=0.58$) (303).

Certainly, there is evidence that sexual behaviours have changed over past decades, with a younger age of first intercourse, increasing number of sexual partners and increasing prevalence of oral sex (305, 306). Furthermore, the prevalence of genital herpes simplex viruses 1 and 2 and genital warts (known surrogate markers for oral sex, high risk sexual behaviour and HPV exposure) have increased in recent years (20, 307-309). However, whilst there is undoubtedly a strong association between sexual

behaviours and HPV-positive OPSCC, there is currently insufficient evidence of a definite causal relationship, and further studies are required.

1.5.4.2 Exposure to HPV

Exposure to HPV is a pre-requisite for HPV infection and tumour development. When the odds ratios for the sexual behaviours described above are adjusted for serological evidence of HPV exposure they lose significance, indicating that sexual practices are merely a surrogate measure of high risk HPV-exposure (302). Seropositivity to HPV-16 L1 is associated with a 4 to 30-fold increase in risk of development of OPSCC, and when HPV-16 E6 or E7 seropositivity is considered (indicating transcriptionally active HPV) this risk rises to between a 50 and 60-fold increase (and in some reports 180-fold!) (291, 302, 310). Again, when stratified by tumour HPV-status, these risk increases are only seen in HPV-positive disease (303). Mork *et al* recruited 900 000 patients to a nested case-control study across Scandinavia, and collected serum samples at recruitment. 292 patients subsequently developed HNSCC, of which 26 were in the oropharynx. Compared to control subjects (5 for each case subject), patients who were seropositive for HPV-16 L1 protein at study entry had a 14-fold increase in the risk of subsequently developing OPSCC (311). This is the only study that demonstrates that HPV-16 acquisition predates the development of OPSCC.

1.5.4.3 Oral HPV Infection

One limitation of HPV-serology is that it only demonstrates evidence of exposure to HPV, regardless of mucosal site. It does not specifically show that HPV infection is present in the oral cavity or oropharynx (312). It is generally considered that an association between oral HPV infection and malignancy is more robust evidence of a direct link between HPV and OPSCC. Certainly, patients with HPV-positive OPSCC are more likely to have evidence of oral HPV infection than matched controls (303, 304, 313). Furthermore, there is considerable evidence that patients with oral HPV infection are at increased risk of developing OPSCC (e.g. D'souza *et al*: adjusted OR for developing OPSCC if evidence of any oral HPV infection = 12.3). This risk is even greater when high risk HPV types, and specifically HPV-16 are detected (e.g. D'Souza *et al:* OR if evidence of HPV-16 oral infection = 14.6) (302, 310, 314). There is no evidence that oral HPV-infection increases the risk of HPV-negative OPSCC (303).

When considered together, there is significant evidence that sexually acquired oral HPV infection is a strong risk factor for oropharyngeal cancer, and that this risk is relevant to HPV-positive OPSCC only (312). There is a long delay in the progression from HPV infection to malignancy. Mork *et al* reported that 73% of patients who developed

HNSCC in their nested case-control series had been enrolled, and thus had evidence of positive HPV-16 serology, for at least 5-years (311). Thus a change in sexual behaviours since the 1960s could explain the current increasing incidence in HPV-related tumours (25).

1.5.4.4 Exposure to tobacco and alcohol

The interplay between HPV and traditional risk factors for HNSCC is a complicated one. In non-smokers, the majority of cases of malignancy are unsurprisingly now caused by HPV (5). However, both smokers and non-smokers (and similarly alcohol drinkers) are at equal risk of exposure to HPV, and thus these factors do not preclude the development of HPV-positive HNSCC. Whilst HPV-positive HNSCC has been associated with lower rates of smoking and drinking than HPV-negative disease in several case series, fewer than 20% of patients with HPV-positive tumours neither smoked nor consumed alcohol. Indeed, up to 30% of HPV-positive tumours were in patients with a history of heavy tobacco and alcohol consumption (295, 303).

There are mixed reports of the combined effects of tobacco, alcohol and HPV on the risk of developing malignancy. Smith *et al* found that heavy exposure to alcohol and tobacco in combination with evidence of oral HPV infection further increased the risk of developing HNSCC compared to control subjects (e.g. oral HPV infection/non-smoker/non-drinker OR 2.3 vs. oral HPV infection/>30 pack years/>21 drinks per week OR 26.2) (295). In contrast to this, D'Souza *et al* found no such evidence of a combined increased risk of OPSCC development (e.g. oral HPV-16 infection/<20 pack year/<15 drink year OR 16.0 vs. oral HPV-16 infection/>20 pack year/>15 drink year OR 11.0) (302). It is significant when comparing these studies, that Smith *et al* reported findings for all HNSCC whilst D'Souza *et al* only reported on oropharyngeal tumours. Furthermore the two studies compare different levels of alcohol and tobacco exposure, and use different qualifiers of oral HPV-infection (high risk HPV in Smith's study vs. HPV-16 in D'Souza's), and do not compare HPV-negative and HPV-positive tumours.

1.5.4.5 Different risk factors for HPV-positive and HPV-negative OPSCC

Gillison *et al* conducted a large scale case control study comparing the effects of a number of factors on the risk of developing HNSCC, and demonstrated markedly different risk factor profiles for HPV-positive and HPV-negative tumours (303). The findings that sexual behaviours and HPV (both seropositivity and evidence of oral

infection) increased the risk of HPV-positive, but not HPV-negative, tumours has already been discussed (1.5.4.1-1.5.4.3). In addition to this, it was also found that exposure to marijuana was also associated with an increased risk of developing HPV-positive, but not negative, tumours. The authors suggest that a possible mechanism for this risk is related to the immunomodulatory effects of marijuana.

In HPV-negative tumours, the risk of developing malignancy was increased by increasing measures of alcohol and tobacco exposure, as might be expected, and by poor oral hygiene (ORs: >50 drink years vs. non-drinker 2.8, p=0.03; >50 pack years vs. non-smoker 4.9, p<0.001; tooth loss all vs. none 2.8, p=0.001). None of these factors increased the risk of developing HPV-positive tumours.

1.5.5 Molecular evidence of a causal role of HPV in oropharyngeal cancer

The potential association between HPV and HNSCC was first suggested in a paper by Syrjanen *et al* in 1983 describing cytological abnormalities in a sinonasal papilloma that were consistent with HPV infection. The group were also able to demonstrate the presence of HPV antigens in the nuclei of a few cells using immunoperoxidase staining (315). In 1985, de Villiers *et al* used Southern Blot Hybridisation to demonstrate the presence of HPV-16 DNA in two tongue tumours (316). The last 25 years has seen an exponential growth in the literature surrounding this field, and there is now a strong and consistent molecular evidence base for a causal role of HPV in OPSCC. Indeed, in 2009 HPV-16 was recognised as a causal agent in the development of oropharyngeal cancer (24).

Numerous studies have demonstrated the presence of HPV DNA in OPSCC, using different techniques including PCR and *in-situ* hybridisation. With the use of *in-situ* hybridisation, HPV DNA is located within the nuclei of tumour cells, whilst being absent in surrounding normal tissue (23). Furthermore, there is evidence that HPV DNA is transcriptionally active, as demonstrated by elevated p16 expression in tumour cells. As previously discussed, approximately 45% of HNSCCs have mutations in the p53 gene, and expression of upstream regulators of pRb function (such as p16) is also commonly altered in HNSCC (120, 317, 318). Thus, the functional interference with these pathways by E6 and E7 represents an alternative molecular pathway with similar end results. Indeed, much of the molecular evidence for a causal role of HPV in OPSCC revolves around differential expression of both p53 and members of the retinoblastoma pathway between different groups of tumours.

One of the earliest studies exploring the link between these pathways and HPV was published in 1998 by Andl *et al.* They described a group of tumours (23/208) that

were preferentially localised to the tonsils and were characterised by reduced or absent expression of pRb. This same group of tumours also displayed reduced expression of cyclin D1, and overexpression of p16. The majority of these tumours had wild type p53 expression, and an absence of p53 gene mutations. They were able to demonstrate the presence of HPV DNA in 11 out of 12 these pRb-deficient tumours, but in none of 9 pRb-positive tonsillar tumours ($p<0.001$) (319). Furthermore, despite the fact that all of these HPV-positive tumours were poorly differentiated and with nodal metastases at the time of presentation, these patients had positive clinical outcomes, a fact that the authors attributed to increased radiosensitivity. Other authors have also demonstrated an association between HPV-positivity and reduced expression of pRb, cyclin D1 and p53, and overexpression of p16 (320, 321)

Gillison *et al*, in one of the most widely cited papers on the subject, also found a group of tumours that had wild-type p53, and were more likely to be associated with evidence of HPV DNA. Indeed, only 10% of oropharyngeal tumours with evidence of HPV DNA harboured p53 mutations, compared to 67% of those that were HPV-negative ($p=0.001$). In this paper, the authors examined tumour tissues from 253 patients for the presence of HPV DNA using three different techniques, namely PCR, southern blotting and *in-situ* hybridisation. They detected HPV DNA in 25% of cases and showed that it was specifically localised to the nuclei of affected tumour cells. Southern blotting patterns were consistent with viral integration, a step generally considered essential for HPV related oncogenesis (23). Of the tumours with evidence of HPV DNA, 90% were positive for the high risk HPV-16.

Weinberger and colleagues elegantly demonstrated the importance of biologically active HPV-infection in the pathogenesis and prognosis of OPSCC. They examined 79 oropharyngeal tumours with PCR and IHC (p16) for HPV DNA and functional E7 respectively. Tumours were then stratified into 3 groups based on their co-expression: Class I tumours were HPV-negative/p16-negative, class II HPV-positive/p16-negative and class III HPV-positive/p16-positive. They found that only tumours in class III had reduced expression of pRb and p53, and that only class III tumours were associated with improved survival (5-year survival: class III 79% vs. class I 20% and class II 18%). There was no difference in survival based on the presence of PCR-positive HPV DNA alone (322). Interestingly patients in class II had higher rates of tumour recurrence than those in class I, a finding that the authors attribute to a possible additive effect of HPV super-infection in tobacco induced malignancy. In a follow-up study by the same group, 77 oropharyngeal tumours were stratified according to the 3 class system and then analysed for a number of different tumour progression markers. They found significant differences in expression of EGFR, VEGF and β-catenin between class III

tumours and those belonging to classes I and II ($p=0.009$, $p=0.028$ and $p=0.009$) (323).

The findings of Weinberger *et al* suggest that evidence of HPV-infection alone is insufficient to label a tumour as HPV-driven. There must also be evidence of HPV activation for the molecular changes associated with HPV to be seen, as well as the survival benefits. Further strength is added to this argument by the findings of other authors. Dai *et al* examined the presence of p53 mutations in 35 HPV DNA-positive oral and oropharyngeal tumours, and compared them to 35 matched HPV-negative tumours. They also measured peripheral E6 antibody levels as a marker of E6 expression by the tumour in HPV-positive patients. Of the 35 HPV DNA-positive tumours, 14 also had E6 antibodies, and all of these patients had wild type p53. Patients with HPV DNA-positive tumours, but no antibodies against E6, had similar levels of p53 mutations to patients who were HPV DNA-negative. This suggests that the HPV in these E6 antibody negative patients was unlikely to be biologically active. Furthermore, patients who were HPV DNA-positive and had a history of smoking were more likely to harbour p53 mutations, though this was not statistically significant, again suggesting a possible additive effect of HPV and tobacco (324). In another study by Braakhuis *et al*, 16.7% of 143 oral and oropharyngeal SCCs were positive for HPV DNA, yet only half of these expressed E6 and/or E7 mRNA. There were no p53 mutations in 12 tumours expressing HPV DNA and E6/E7 mRNA, whilst 3 out of 8 tumours that were HPV DNA-positive yet E6/E7 mRNA negative had mutations in p53 (325). Westra *et al* also describe a lower rate of p53 mutations in HPV-positive tumours (25% vs. 52% in HPV-negative), whilst adding that disruptive mutations were only seen in HPV-negative tumours (326).

More recently, proteomic analysis has revealed differential protein expression between HPV-positive and HPV-negative tumours, albeit in a small number of cases (327). Slebos and colleagues performed proteomic analysis on 10 HPV-positive and 10 HPV-negative oropharyngeal tumours, and also 10 normal control samples. They found 31 differentially expressed proteins between the three groups. HPV-negative tumours tended to express proteins involved in epithelial cell development, keratinisation and extracellular matrix organisation, whilst HPV-positive tumours expressed proteins involved in DNA initiation and replication, and cell cycle control. HPV-positive tumours also expressed higher levels of the transcription factors E2F1 and E2F4, related to the effects of E7 of pRb (327).

1.5.6 Genetic Differences between HPV-positive and HPV-negative tumours

As discussed previously, the pathogenesis of HPV-negative HNSCC is characterised by the accumulation of a number of mutations in key cell cycle control genes, whilst HPV interferes with these same processes through the actions of the oncoproteins E6 and E7. There is considerable evidence that HPV-positive tumours have much lower rates of genetic aberrations than HPV-negative disease.

Braakhuis *et al* examined 143 consecutively treated HNSCCs for the presence of HPV DNA and E6/E7 mRNA by PCR, and then correlated HPV status with mutations in the gene encoding p53 (TP53) and loss of heterozygosity in 28 microsatellite markers in a number of chromosomes commonly found to be affected in HNSCC, including those in chromosomes 9p and 17p that harbour genes involved in the p53 and pRb pathways. They found that tumours that expressed both HPV DNA and E6/E7 mRNA had significantly lower levels of p53 mutations than tumours that were HPV DNA negative (0% vs. 75%, $p<0.001$) and that patients with HPV DNA-positive but E6/E7 mRNA negative tumours made up a third group in terms of p53 mutations (discussed above in section 1.5.5). They also found that HPV DNA-positive/mRNA-positive tumours had generally lower levels of LOH than HPV DNA-negative tumours. In the HPV DNA-negative tumours, most LOH was found on chromosomes 3p, 9p and 17p, and 81% of HPV DNA-negative tumours exhibited LOH at these locations, compared to only 14% of HPV-DNA/mRNA positive tumours ($p<0.05$). Interestingly, the HPV DNA-positive/mRNA-negative tumours had significantly higher levels of LOH than the HPV DNA-positive/mRNA-positive tumours (LOH in 3p, 9p and 17p 75%, $p<0.05$) which did not significantly differ from those tumours that were HPV DNA-negative (325). The lower levels of loss at chromosome 9p are significant in that CDKN2A, the gene encoding p16, is located here, and is commonly inactivated in tobacco related HNSCC (328). Furthermore, LOH at 3p, 9p and 17p is generally considered to be an early event in tobacco induced HNSCC carcinogenesis, and the absence of allelic loss here in HPV DNA-positive/mRNA positive tumours would suggest that HPV is involved early on in the pathogenesis of these group of tumours (325). This study again highlights differences between active and inactive HPV within tumours, as tumours with evidence of HPV DNA but without E6/E7 mRNA had similar levels of LOH to HPV DNA negative tumours. Again this raises the possibility that HPV is either a bystander in these tumours, or is a synergic carcinogen in combination with tobacco and alcohol. In this study there was no difference in tobacco exposure between the HPV DNA-negative and the HPV DNA-positive/mRNA-positive patients, unfortunately the authors do not comment as to whether this was also the case for HPV DNA-positive/mRNA-negative tumours.

As well as having lower levels of genetic aberrations, there is also evidence that HPV-positive tumours have a different distribution of DNA gains and losses than HPV-negative tumours. Klussmann *et al* assessed genetic changes in 60 oropharyngeal tumours, of which 28 were HPV-positive as determined by a combination of p16 IHC and HPV ISH. They confirmed the findings of Braakhuis *et al,* in that HPV-positive tumours generally had lower levels of chromosomal alterations (HPV-negative 12.1 vs. HPV-positive 8.9, p=0.03) and also lower levels of amplifications (presence of at least one amplification HPV-negative 38% vs. HPV-positive 14%, p=0.04). They also describe a different pattern of genetic imbalance for HPV-positive and HPV-negative tumours. HPV-negative tumours had more losses at 3p, 5q, 9p, 15q, and 18q, and more amplifications at 11q13 (p=0.002, 0.03, <0.001, 0.02, 0.004, and 0.001, respectively) while HPV-positive tumours had more losses at 16q and gains at Xp (p=0.002 and 0.003). Furthermore, chromosome amplification was a negative prognostic marker for both overall and disease free survival (amplification: yes vs. no; 5-year survival 40% vs. 64%, p=0.05; and 34% vs. 66%, p=0.01 respectively) while the 16q loss seen predominantly in HPV-positive tumours was an independent marker of positive outcome (16q loss yes vs. no: 5-year survival OS 100% vs. 49%, p=0.008; DSS 100% vs. 48%, p=0.01) (329). Jung *et al* also identified loss at 16q as being a common feature of HPV-positive tumours, and significantly found that one of the consequences of this was reduced expression of APP-BP1. APP-BP1 is responsible for inhibition of p53 transcriptional activity, and the authors speculate that its down-regulation in HPV-positive tumours might contribute to the restoration of p53 functions such as radiation induced apoptosis, thus contributing to the survival benefits seen in HPV-positive tumours (330).

Dahlgren *et al* analysed the pattern of DNA losses and gains in 25 tonsillar tumours, of which 60% were HPV-positive by PCR, and found that 73% of HPV-positive cases had gains at chromosome 3q, compared to 40% in HPV-negative tumours (p=0.049), and that 40% of HPV-negative tumours had gains at chromosome 7q compared to 0% in HPV-positive cases (p=0.017) (331). Interestingly the gain on chromosome 3q in HPV-positive tumours adds strength to the causal effect of HPV in these tumours, as gain at this chromosome is a frequent early event in cervical cancer (332). Furthermore, the human telomerase gene maps to chromosome 3q, and there is evidence that the HPV E6 protein is able to transcriptionally activate telomerase (233). Amplification of 11q13 (encoding cyclin D1 and commonly amplified in HPV-negative tumours) has also be shown to occur at significantly lower rates in HPV-positive tumours (333).

It can be clearly seen that HPV-positive and HPV-negative tumours have differing risk factors, molecular biology and genetic changes. All of which add together to demonstrate that HPV-positive OPSCC is a distinctly different disease process, a fact

that is reflected in the clinical presentation, pathological features and response to treatment of these tumours.

1.5.7 Clinicopathological differences between HPV-positive and HPV-negative HNSCC

Both clinical presentation and pathological features are dissimilar between HPV-positive and HPV-negative tumours. Patients with HPV-positive tumours are generally younger than those with HPV-negative tumours, and with distinctly different risk factor profile, as discussed previously. As mentioned above, HPV-positive tumours are most commonly found in the oropharynx, specifically the tonsils and base of tongue, whilst HPV-negative tumours can arise from any region of the upper aerodigestive tract. Whilst HPV-negative tumours present at a range of T- and N-stages, most HPV-positive tumours tend to be small primaries (commonly T1 or T2) but with advanced nodal stage (334). Metastatic lymph nodes in HPV-positive tumours are commonly cystic and often affect multiple lymph node levels (335). This subsequently means that HPV-positive tumours tend to have a higher overall AJCC stage at presentation, with most presenting at stage III or IV.

It is possible to detect evidence of HPV in metastatic nodes as well as in primary tumours. In a recent pathological study, metastatic lymph nodes from 20 patients with HPV-positive oropharyngeal tumours were examined for the presence of HPV DNA by PCR and p16 over-expression: they were found to be positive for both in all cases (336). Given the strong associations between HPV and oropharyngeal tumour location, it may be possible to use the HPV status of metastatic lymph nodes to target diagnostic efforts in patients presenting with cervical lymphadenopathy at an unknown primary tumour site.

Pathological features of HPV-positive and negative tumours can also markedly differ. HPV-negative tumours are usually classified as moderately differentiated, keratinising squamous cell carcinomas. They have polygonal shaped cells with abundant cytoplasm, distinct cells borders and intercellular bridges. They frequently have a prominent stromal reaction, with marked desmoplasia, and an infiltrative growth pattern consisting of angulated nests with tapered extensions (337, 338). They are frequently associated with dysplasia in surrounding tissues which can often involve large areas, a concept known as field change (40, 164). By contrast, most HPV-positive tumours are non-keratinising tumours that usually have basaloid morphology. They tend to lack the dysplasia and field change commonly seen in HPV-negative tumours, and often contain a prominent lymphocytic infiltrate. They also have differing growth patterns, with a lobular architecture and a ‘pushing border’ with little stromal response. The

cells are frequently ovoid or spindle shaped and have hyperchromatic nuclei, a lack of prominent nucleoli and indistinct cell boundaries (337-339).

The majority of non-keratinising oropharyngeal tumours are HPV-positive, with one recent study demonstrating that 69% of 42 non-keratinising OPSCCs were positive for HPV DNA by ISH, and 100% were positive for p16 expression, furthermore non-keratinising OPSCC had significantly better overall and disease specific survival that keratinising OPSCC, mimicking the survival benefits of HPV-positive tumours (338). HPV-positive tumours are frequently described as poorly differentiated (23), and although their microscopic appearance is markedly different from the normal squamous epithelium that lines the oral cavity and oropharynx (the standard by which differentiation is based), it is highly similar in appearance to the epithelium lining tonsillar crypts, from where most HPV-positive tumours are thought to arise (185, 186, 312). Therefore, it is very well differentiated, but not compared to oral mucosa. Another histological feature of HPV-positive tumours that has caused some contention is their description as basaloid, causing some confusion with the basaloid variant of squamous cell carcinoma, a subtype of HNSCC with classically aggressive behaviour. It has recently been shown that "basaloid" HNSCC consists of two groups, and HPV-positive group with excellent survival, and an HPV-negative group with a poor prognosis (339). These different pathological features have led to some authors calling for an update of tumour grading, arguing that the established system of well/moderately/poorly differentiated should be replaced with keratinising vs. non-keratinising, that the term basaloid should be avoided, and that all oropharyngeal tumours should be characterised for HPV (312). Indeed, a recent study of radiation oncologists in the United States found that only 40% routinely tested for HPV in patients with oropharyngeal carcinoma. Furthermore, nearly two-thirds of those who did not routinely test for HPV reported that they had no plans to do so (340).

Many of the features of HPV-positive tumours, especially the advanced stage and poor differentiation, would typically be expected to result in poor survival outcomes. Indeed advanced stage and poor tumour differentiation are well established negative prognostic indicators for HNSCC. However, the vast majority of published studies demonstrate a significant survival advantage of HPV-positive tumours compared to those that are HPV-negative. This is discussed in more detail in section 1.5.9.

Table 1.1: Differences between HPV-positive and HPV-negative HNSCC. Adapted from (5) and (40)

	HPV-positive tumours	HPV-negative tumours
Anatomical Site	Tonsil, Base of Tongue	All sites
Histology	Non-keratinised, poorly differentiated, basaloid	Keratinised, moderately differentiated, associated dysplasia
P53 mutations	Rare	Common
Field Cancerisation	Unknown	Common
Age	Younger mean age	Older mean age
Gender	3:1 male:female	3:1 male:female
Stage	III, IV Tx, T1-2	All stages
Risk factors	High risk sexual behaviour	Alcohol and tobacco
Incidence	Increasing	Decreasing
Survival	Improving	Unchanged

1.5.8 Diagnosis of HPV-positive HNSCC

Currently there is no accepted, standard protocol for the detection and classification of HPV-positive HNSCC (5). A number of different techniques have been used to classify tumours including: consensus and type specific PCR for viral early or late DNA or mRNA (E6/7 or L1); real time PCR to quantify viral load; demonstration of serum antibodies directed against viral antigens; *in-situ* hybridisation for HPV-16 specific or consensus high risk HPV DNA; and immunohistochemistry for p16 overexpression. PCR and *in-situ* hybridisation both allow demonstration of the presence of HPV DNA, whilst immunohistochemistry for p16 overexpression acts as a surrogate measure for the expression of E7 due to the interactions between E7 and the pRb system (discussed above). The demonstration of antibodies against HPV capsid antigens is not generally useful in diagnosis of HPV-driven malignancy, as these antibodies may be present in any patient who has been exposed to HPV in the past, regardless of the site of mucosal infection (312). Antibodies directed against viral oncoproteins (E6 and E7) are more

useful as they suggest transcriptionally active virus. They may potentially be useful as a prognostic marker, but this is yet to be firmly established (312).

All diagnostic techniques have their benefits and drawbacks. To call a tumour truly HPV-driven requires demonstration of the HPV viral DNA and also evidence that the virus is transcriptionally active, i.e. is expressing one, or both, of the viral oncoproteins E6 and E7. To this end, quantitative PCR for E6 and E7 mRNA is generally considered to be the gold standard in diagnosis, however this requires fresh tissue and, although an established technique in the research setting, is generally not useful in a diagnostic laboratory, where formalin fixed and paraffin embedded (FFPE) tissues are the norm (341, 342). Similarly PCR for viral DNA ideally requires fresh or frozen tissue samples and a high level of technical skill and time, and so is not useful in standard clinical, FFPE based practise. Furthermore, PCR for DNA is likely to overestimate the number of tumours that are HPV-driven due to detection of HPV DNA that is not transcriptionally active, i.e. tumours in which HPV is simply a bystander (5). For example, in a recent study comparing a number of different detection methods, 20% of tumours that were positive for HPV-16 E6 DNA qPCR were negative for E6 RNA qPCR(341).

In-situ hybridisation goes some way towards distinguishing between biologically relevant and irrelevant virus, and can be performed on paraffin embedded tissues. In truly HPV-driven tumours, HPV DNA can be seen in all tumour cells, indicating a clonal expansion. In tumours where HPV is simply a bystander, or perhaps there is contamination, then DNA is seen in low copy number and in only a small number of tumour cells. Furthermore, *in-situ* hybridisation allows distinction between integrated and episomal DNA due to differential staining patterns. Punctate nuclear staining demonstrates viral integration, a vital step in HPV-induced malignancy, whilst diffuse nuclear staining indicates episomal DNA that is unlikely to be oncogenic (343). *In-situ* hybridisation is also highly specific, and signal amplification steps allow the identification of single viral copies per cell (5, 343).

As discussed previously, the HPV E7 oncoprotein inactivates pRb. One of the consequences of this is upregulation of p16 expression. This can be demonstrated using standard immunohistochemical techniques (319). As a result of this, there have been suggestions in the literature that p16 immunohistochemistry should be a standard surrogate technique for the detection of HPV-positive tumours (185, 344, 345). Indeed, there is some evidence that p16 and HPV are independent prognostic factors in OPSCC (346). However, it is generally considered that simply demonstrating p16 overexpression is insufficient to truly call a tumour HPV-positive as it does not take into account overexpression of p16 for other reasons, for example mutational inactivation of the retinoblastoma protein. Interestingly, around 5-10% of p16 positive

tumours may be HPV-negative, depending on the HPV-detection assay used (5, 185). In some cases, this discordance is due to the use of an HPV-detection assay that is specific for HPV-16 (responsible for ~90% of HPV-positive HNSCC), and the possibility that the tumour is caused by another HPV type. However there remain cases where p16 is overexpressed and no evidence of HPV can be demonstrated, even with the use of consensus PCR or *in-situ* hybridisation. One study, using expression of E6 and E7 mRNA as a gold standard for HPV involvement, found that p16 expression was 100% sensitive but only 79% specific as a surrogate marker of HPV infection (347). Furthermore, there is evidence that p16 expression may be detected by immunohistochemistry in around a quarter of normal tonsils, particularly in crypt epithelium and lymphoid follicles, with no evidence of HPV infection (185, 348).

To overcome the relative drawbacks of individual HPV detection techniques, various combinations of diagnostic techniques have been suggested. Schache *et al* recently compared a number of different techniques (E6 DNA qPCR, p16 IHC, HPV ISH – alone and in combination) with the gold standard of E6 mRNA qPCR, both in terms of sensitivity and specificity, and also in terms of the effects of a positive test on survival outcome. They found that the combination of p16 IHC and E6 DNA qPCR had the best sensitivity and specificity compared to the gold standard (97% and 94% respectively) and that stratification using this combination gave the greatest prognostic significance in terms of both overall and disease specific survival ($p=0.002$ and $p=0.005$), outperforming E6 mRNA qPCR ($p=0.003$ and $p=0.005$) (341). They therefore recommend this combination as the test of choice in future clinical trials serving to stratify patients according to HPV-status. This reflects the approach of other authors, whereby specimens are tested for p16 overexpression by immunohistochemistry, and then those that are positive should be evaluated for the presence of HPV DNA by either PCR or *in-situ* hybridisation. This reduces the number of tumours falsely attributed to HPV based on p16 alone, and has a reported sensitivity and specificity approaching 100% (347, 349). In routine clinical practise, *in-situ* hybridisation probably represents the more practical of the two HPV-detection techniques, a fact eluded to by Schache *et al* (341). The combination of p16 immunohistochemistry and HPV consensus *in-situ* hybridisation was the technique we chose to use in this study to classify tumours as HPV-positive or HPV-negative.

1.5.9 HPV-positive HNSCC and survival

As discussed previously, survival from HNSCC in general is largely unchanged in recent years: However, that of OPSCC has consistently increased. There are several characteristics that have been identified as positive prognostic factors for OPSCC survival. These include: younger age at diagnosis, non-smokers, low exposure to

alcohol, good performance status and absence of significant comorbidity. All of these factors are the same as those associated with HPV-positive tumours (5).
Gillison *et al* were among the first authors to identify a positive survival benefit in patients with evidence of HPV in their tumours, reporting a 59% reduction in disease specific death compared to HPV-negative patients in a retrospective study (23). Several other retrospective studies had similar findings, and there is now significant evidence that HPV-positive tumours have greatly improved survival compared to those that are HPV-negative, with reductions in the risk of death of up to 80% (100, 350-352). This is despite the fact that many HPV-positive tumours are poorly differentiated (according to classical descriptors of differentiation) and often present at an advanced disease stage. Furthermore, the majority of published studies report lower rates of treatment failure and disease recurrence in HPV-positive tumours, possibly accounting for increased survival rates (237). This survival advantage is maintained seemingly regardless of treatment modality. Interestingly, there is some evidence that the survival benefit of HPV-positive tumours is limited to those located in the oropharynx (23).

There are few studies that directly compare HPV-stratified survival among different treatment types. However, one such study was published by Hong *et al* in 2010 (353). They reported a retrospective analysis of 195 oropharyngeal tumours and compared the effect of HPV-status on survival depending on treatment; either surgery and post-operative radiotherapy (n=110), radiotherapy alone (n=24) or concurrent chemoradiotherapy (n=47). Only 14 patients were treated by surgery alone and this group was deemed too small for multivariate analysis. When all patients were considered as a whole, HPV-positive tumours had improved locoregional control, event free survival and overall survival on multivariate analysis (HR relative to HPV-negative 0.27, 0.23, 0.29 respectively, $p<0.001$ for all). When analysed separately, these improved responses were maintained regardless of treatment. Interestingly, the effect of HPV on overall survival was found to be weakest in the group of patients treated with chemoradiotherapy (HR relative to HPV-negative: surgery + adjuvant radiotherapy 0.11, $p<0.001$; radiotherapy 0.11, $p=0.004$; chemoradiotherapy 0.37, $p=0.03$) (353).

Fischer and colleagues compared survival in patients treated with either primary surgery or primary radiotherapy. They found that 59% of 85 oropharyngeal tumours were HPV-positive, and that HPV-positive tumours had improved overall survival compared to those that were HPV-negative (5-year survival 57% vs. 27%, $p=0.007$). In multivariate analysis, including adjusting for HPV-status, there was no difference in survival between patients treated with surgery and radiotherapy (HR relative to surgery: 1.69, $p=0.15$) (354). It should be noted that the authors do not comment on the numbers of surgically treated patients who received post-operative adjuvant therapy.

A number of studies of individual treatment modalities have also reported a survival benefit with HPV-positive tumours, and these are discussed below.

1.5.9.1 Radiotherapy

The first reports of improved survival among HPV-positive tumours began to appear by the late 1990s. Lindel *et al* retrospectively analysed 99 oropharyngeal cancers treated with radiotherapy for the presence of HPV using PCR and found that those that were HPV-positive had improved locoregional control and overall survival compared to those that were HPV-negative (p=0.05 and 0.046 respectively) (351). O'Sullivan *et al* recently reported on a retrospective analysis of 207 patients with OPSCC and treated by radiotherapy alone between 2001 and 2008, of which 71% were HPV-positive. They found significant improvements in overall and disease free survival, as well as local and regional control in HPV-positive patients at 3 years (74% vs. 39%, $p < 0.001$, 83% vs. 56%, $p = 0.001$, 92% vs. 71%, $p < 0.001$ and 93% vs. 76%, $p < 0.001$ respectively). There was no difference in the rate of distant metastasis between HPV-positive and HPV-negative tumours (12% vs. 9%, p=0.29) (355).

Lassen *et al* prospectively classified 156 patients with oropharyngeal (stage I-IV) or supraglottic laryngeal (stage II-IV) cancer according to HPV-status in a subgroup of patients from the Danish Head and Neck Cancer Group (DAHANCA) 5 trial. These patients were in the control arm of the study and received conventionally fractionated primary radiotherapy (66-68gy in 33-34 fractions) as sole treatment of HNSCC. HPV-status was classified by p16 immunhistochemistry and reported to be positive in 22% of tumours. A subgroup of tumours (n=32) were also subjected to HPV *in-situ* hybridisation and the authors report good concordance between p16 and HPV ISH. Within the oropharynx, 34% of tumours were HPV (p16) positive. HPV-positive tumours had improved locoregional control, overall and disease specific survival compared to HPV-negative tumours (5-year rates: 58% vs. 28%, p=0.005; 62% vs. 26%, p=0.0003 and 72% vs. 34%, p=0.0006 respectively) and HPV-status was an independent predictor of both overall and disease specific survival on multivariate analysis (HR 0.44 and 0.36 respectively). Unfortunately, the authors did not analyse outcomes for oropharyngeal and laryngeal tumours separately (356).

Another prospective trial to show a survival benefit in HPV-positive patients treated with radiotherapy alone is RTOG 9003. In this trial, 1113 patients with HNSCC were randomised to 4 different radiotherapy regimens (standard fractionation, hyperfractionation and accelerated fractionation with either split or concomitant boost). Of these, 190 patients had oropharyngeal tumours, of which 39% were HPV-positive. The HPV-positive patients were evenly split between the 4 treatment groups.

HPV-positive patients had improved overall and progression free survival (5-year survival 49% vs. 19.6% and 43.6% vs. 19%, both p<0.001) and reduced rates of locoregional failure (28.9% vs. 54.9% at 5-years, p<0.001). Rates of distant metastasis and second primary tumours did not differ between HPV-positive and negative groups (357).

1.5.9.2 Chemoradiotherapy

One of the first prospective analyses to examine the effect of HPV on survival was by Fakhry *et al* in 2008 (358). They assessed the effects of HPV on response the therapy and survival in 96 stage III and IV laryngeal and oropharyngeal cancers in a phase II trial (ECOG 2399) of neoadjuvant chemotherapy with carboplatin and paclitaxel followed by concurrent standard fractionation radiotherapy and weekly paclitaxel for responders, and surgical resection for non-responders. HPV status was determined using a combination of PCR and *in-situ* hybridisation, and was positive in 40% of all tumours, and 63% of oropharyngeal tumours. When the group were considered as a whole, HPV-positive tumours had improved response to both neoadjuvant chemotherapy and concurrent chemoradiotherapy (82% vs. 55%, p=0.01 and 84% vs. 57%, p=0.007 respectively) and also improved overall survival (2-year survival 95% vs. 62%, p=0.005) and progression free survival (2-year PFS 86% vs. 53%, p=0.02). HPV-positive tumours had a 64% reduction in risk of all-cause mortality (HR 0.36, p=0.02) and 73% reduction in risk of disease progression (HR 0.27, p=0.01) adjusted for age, stage and performance status. When analysis was limited to only those tumours arising from the oropharynx, 2-year overall survival was 94% for those that were HPV-positive, compared to only 58% in HPV-negative tumours (p=0.004) and the risk of death was reduced by 61% (adjusted HR 0.39, p=0.06) whilst the risk of disease progression was reduced by 62% (adjusted HR 0.38, p=0.09).

One of the most widely cited examples of the improved survival in HPV-positive patients is that of Ang *et al*, reporting the phase III RTOG 0129 trial and already discussed briefly in section 1.1.6 (99). They retrospectively investigated the association between HPV-status and survival in 323 patients in a prospective trial comparing standard fractionation and accelerated fractionation radiotherapy, both in combination with cisplatin. All patients had stage III or IV oropharyngeal cancer, and 63% were HPV-positive, as determined by HPV *in-situ* hybridisation and p16 immunohistochemistry. Patients with HPV-positive tumours had better 3-year overall survival rates than HPV-negative (82% vs. 57%, p<0.001) and a 58% reduction in the risk of death (HR 0.42) after adjustment for a number of potential confounding factors, including age, race, TNM stage, smoking history and treatment group.

The phase II trial UMCC 9921 assessed response to neoadjuvant chemotherapy as a strategy for selecting definitive treatment in advanced stage oropharyngeal cancer. All patients received one cycle of neoadjuvant chemotherapy with either cisplatin or carboplatin with 5-flurouracil. Those that responded (defined as greater than 50% response of the primary tumour) received chemoradiotherapy (70gy in 35 fractions with concurrent cisplatin or carboplatin) whilst those with less than 50% response received surgical resection and postoperative radiotherapy. Patients with HPV-positive tumours (27/42, 64%) had an improved response to neoadjuvant chemotherapy and concurrent chemoradiotherapy ($p=0.003$, $p=0.005$) and improved overall and disease specific survival ($p=0.007$, $p=0.008$) compared to HPV-negative tumours (359).

A retrospective analysis of the phase III TAX-324 trial showed similar results. TAX-324 was designed to assess the addition of docetaxel to neoadjuvant chemotherapy with cisplatin and 5-FU, followed by concurrent chemoradiotherapy with carboplatin (83). Of the 264 patients with OPSCC in the trial, pathological material was available for HPV-assessment in 111 cases, of which 50% were HPV-positive by E6/E7 PCR. After a median follow-up of 83 months, overall survival and progression free survival were 79% and 73% for HPV-positive tumours, compared with 31% and 29% for HPV-negative tumours ($p<0.0001$). There was an 80% reduction in all-cause mortality for HPV-positive tumours (HR 0.2, $p<0.0001$) and a reduction in loco-regional failure rates (13% vs. 42%, $p=0.0002$) (360).

Another phase III trial to investigate the effects of HPV-status on outcome was TROG 02.02. Over 800 patients with stage III or IV HNSCC were randomised to receive concurrent cisplatin radiotherapy with or without a hypoxic cytotoxic, tirapazimine. Of these patients, pathological material was available for HPV assessment (by p16 IHC) for 185 patients with OPSCC. Of these, 57% were p16-positive and had improved overall and disease free survival rates (2-year survival: 91% vs. 74%, HR 0.36, $p=0.004$ and 87% vs. 72%, HR 0.39, $p=0.003$). Interestingly, although tirapazimine had no effect on survival in unstratified disease, there was evidence of improved overall survival and reduced recurrence rates in HPV-negative patients in the tirapazimine arm, though this was not statistically significant (361).

1.5.9.3 Surgery

As discussed previously, the standard of care for advanced stage oropharyngeal cancer is generally considered to be combinations of chemotherapy and radiotherapy. In addition to this, most HPV-positive tumours present at an advanced stage, usually due to nodal disease and so tend not to be surgically treated. As a result of these factors, there is less evidence of a role of HPV in altering survival after surgical treatment of

OPSCC than there is for chemoradiotherapy. However, small studies do exist, and again show a survival benefit with HPV-positive tumours.

Perhaps the most widely cited study of surgical treatment of OPSCC and classification by HPV-status is by Licitra *et al*. They retrospectively assessed the effect of HPV on outcome in 90 patients treated surgically for oropharyngeal cancer between 1990 and 1999, of which 64% received post-operative radiotherapy. Of the 90 patients, 17 were positive for HPV-16 by PCR (19%) and had improved overall survival compared to HPV-negative tumours (p=0.0018) and also had lower rates of tumours recurrence and second primary tumours (p=0.037 and p=0.015 respectively) (362).

In another retrospective analysis, Joo *et al* report on 55 surgically treated oropharyngeal tumours, all receiving post-operative radiotherapy. Tumours that were HPV-positive (35%) had improved 5-year disease specific survival compared to those that were HPV-negative (93% vs. 56%, p=0.03) (299).

Cohen *et al* recently reported on a retrospective analysis of 50 patients with OPSCC treated with transoral robotic resections (TORS). The majority of patients received adjuvant therapy with either radiotherapy (27%) or chemoradiotherapy (57%). Of the 50 patients, 37 (74%) were HPV-positive. Although no statistically significant effect on survival was seen in this cohort, perhaps due to small sample size, trends were seen towards improved overall and disease free survival in HPV-positive patients (60).

There are very few reported studies of HPV-status in triple modality therapy (i.e. combinations of chemotherapy, radiotherapy and surgery). One such study is a phase II trial by Jo *et al* in 2009, reporting on 31 patients with resectable stage III or IV HNSCC. All patients received 2 cycles of neoadjuvant chemotherapy with docetaxel, cisplatin and 5-FU. Responders then received a third cycle prior to surgical resection, while non-responders proceeded to surgery after cycle 2. All patients received post-operative radiotherapy. HPV-status was assessed in 24 patients, of whom 14 had oropharyngeal tumours. Of the whole cohort, 58% were HPV-positive (oropharyngeal tumours 93% HPV-positive), there was no relationship between response to neoadjuvant chemotherapy, and HPV-positive tumours had a trend towards improved overall and progression free survival compared to HPV-negative tumours (HRs 0.14 and 0.15, p=0.10 and 0.06 respectively) (363). The authors do not report separately on response rates for oropharyngeal tumours, and this study suffers from a lack of adequate power to show significant survival differences, however trends towards improved survival are again seen for HPV-positive tumours.

The survival benefits of HPV-positive tumours have been clearly demonstrated, certainly with regards patients treated with radiotherapy and/or chemotherapy. It is difficult to truly assess the benefits in surgically treated disease as the vast majority of patients in reported studies have also received adjuvant therapy. The advanced presentation of many HPV-positive tumours precludes their treatment by surgery alone, and to date no clinical trials exist of sole surgical treatment for HPV-positive tumours. The underlying reasons behind the prognostic advantage of HPV-positive tumours are unclear, though some theories are discussed below.

1.5.10 Why do patients with HPV-positive tumours have improved survival?

At present, the precise mechanisms underlying the survival advantage of HPV-positive tumours remain unclear. In HPV-positive tumours the p53 and pRb tumour suppressor pathways are functionally intact but dormant due to the expression of E6 and E7. There is evidence that functional p53 is involved in radiosensitivity and it is possible that the p53 mutations commonly seen in HPV-negative tumours may render them relatively radio-insensitive (109, 364). Although HPV-positive tumours have reduced p53 levels due to abrogation by E6, p53 mutations are rare in this group, possibly due to reduced exposure to tobacco, and it is possible that any remaining p53 is still functionally able to exert effects on abnormal cells (5, 326). Two prospective clinical trials have demonstrated that HPV-positive tumours respond well to chemotherapy (358, 365). Indeed, there is some evidence that the reduced p53 expression seen in HPV-positive tumours can be partially reversed by exposure to cisplatin, possibly due to a reduction in viral E6 expression (366, 367). However, responses of HPV-positive cell lines to chemotherapy and radiotherapy are variable. There is evidence that HPV-positive cell lines undergo apoptosis in response to platinum based chemotherapy, in both a p53 dependent and independent manner (366, 368). In contrast to this, some HPV-16 positive cell lines have been found to be highly resistant to both radiotherapy and cisplatin chemotherapy (369, 370). Studies of senescence in fibroblasts have demonstrated a role for p16 in maintaining growth arrest, and some authors have suggested that the overexpression of p16 by HPV-positive cancer cells may result in a senescent phonotype following treatment (322).

Another potential mechanism for increased radiosensitivity in HPV-positive tumours is related to the “suppressor with morphogenetic effect on genitalia”, or SMG-1. SMG-1 is a member of the PI3-kinase family and is thought to play an important role in regulating radiation induced DNA damage through regulation of the G1 cell cycle checkpoint (371). Gubanova and colleagues recently showed that SMG-1 is expressed at lower levels in HPV-positive than HPV-negative tumours (with 85% of 23 HPV-positive having weak or negative SMG-1 expression), and that SMG-1 expression

correlated significantly with both overall and disease specific survival. Cellular experiments demonstrated that SMG-1 expression is reduced by HPV E6 and E7 due to promoter hypermethylation, and that under expression of SMG-1 conferred an increased sensitivity to irradiation (372).

A common feature of HPV-negative tumours is the presence of genetic abnormalities in cells in the vicinity of the tumour, also known as field change. It has been suggested that field cancerisation may be absent in HPV-positive tumours, due to the clonal expansion of a number of HPV infected cells rather than the exposure of large areas of mucosa to the toxic effects of tobacco and alcohol (23, 40). Field cancerisation is thought to be associated with a high risk of tumour recurrence and second primary tumours and its absence in HPV-positive tumours is likely to be beneficial (40, 164). In keeping with this, a number of the studies discussed above have shown reduced rates of second primary tumours in HPV-positive OPSCC. Furthermore, HPV-positive tumours are inversely associated with some other markers of poor survival, such as EGFR (365, 373).

HPV-positive oropharyngeal tumours are commonly permeated by infiltrating lymphocytes (5, 339, 374). This observation is potentially associated with improved survival and makes up a significant part of this thesis. The relationships between tumour infiltrating lymphocytes (TILs) and cancer outcomes in discussed in more detail in section 1.6.

1.5.11 Factors influencing survival in HPV-positive tumours

1.5.11.1 Smoking

Although the majority of non-smokers with oropharyngeal cancer are HPV-positive, it is not true to say that the majority of patients with HPV-positive tumours are non-smokers, with up to 80% of patients in this group having some history of tobacco exposure (section 1.5.4) (295, 303). This has led to interest in the effects of smoking on survival in HPV-positive tumours, and although some studies report no effects, there is increasing evidence to suggest that HPV-positive smokers have poorer survival outcomes than HPV-positive non-smokers (99, 334, 357, 375).

Fakhry *et al* reported no difference in survival of HPV-positive smokers and non-smokers, compared to HPV-negative tumours (HR 0.36, 95% CI 0.14 to 0.87 and HR 0.36, 95% CI 0.02 to 5.9, respectively). However, they did not qualify smoking by amount (358). Ang *et* al, in reporting the RTOG 0129 trial, classified oropharyngeal tumours into three risk categories, based on a combination of HPV-status, smoking

and nodal stage. They found that HPV-positive patients with N2b or N3 disease and a greater than 10 pack year smoking history were at intermediate risk of disease specific death, with 3-year survival rates of 70.8% (95% CI 60.7-80.8). This compared with 3-year survival of 93% (95% CI 88.3-97.7) in the low risk group (HPV-positive, <10 pack year smoking history or HPV-positive, >10 pack year smoking history, N0-N2a) and 46.2% (95% CI 34.7-57.7) in the high risk group (HPV-negative, >10 pack years or HPV-negative, <10 pack years, T4 tumour). The intermediate risk group also contained HPV-negative tumours with <10 pack year smoking history and T2 or T3 tumours (99). In this same trial, when analysing survival in only those patients who had HPV-positive tumours, marked differences were seen dependent on smoking status. HPV-positive patients with >20 pack year smoking history had a 5-year survival of 80%, compared to 95% in HPV-positive patients with <20 pack years of smoking history (376). Based on the results of RTOG 0129, a number of other studies have also shown that the 10 pack year cut off can stratify survival in HPV-positive OPSCC (355, 377).

Gillison's group recently expanded on these findings in publishing further analysis from both RTOG 0129 and RTOG 9003 (a trial comparing several different radiotherapy regimens without chemotherapy) (357). In these two trials, and adjusting for HPV-status, patients with more than a 10 pack year smoking history had more than a twofold increase in the risk of death (HR 2.10, $p<0.001$) than those with less than 10 pack years, and there was more than a 30% difference in 5-year survival between the two groups. When amount smoked was considered as a continuous variable they found that the risk of disease progression and death increased by 1% per pack year smoked (HR for both 1.01, $p=0.002$) and 2% per year of smoking (HR for both 1.02, $p<0.001$) (357).

There is evidence that the survival differences in HPV-positive smokers and non-smokers are related to a higher rate of disease recurrence (i.e. locoregional failure, distant metastasis and second primary tumours), suggesting an additive pathogenic role of HPV and smoking. In both RTOG 0129 and RTOG 9003, rates of locoregional treatment failure and second primary tumours were higher in heavy smokers, again even when taking HPV-status into consideration (357). Maxwell *et al* examined disease recurrence rates in 124 patients with oropharyngeal cancer between 1999 and 2007. They found that HPV-positive patients with a history of smoking (either current or ex-smokers) had an increased risk of disease recurrence compared to HPV-positive never smokers (ever smoker 35% vs. never smoker 6%, HR 5.2, 95% CI 1.1-24.4, $p=0.04$) (375). Peck *et al* have recently reported that HPV-positive never smokers have a lower second primary tumour rate than HPV-positive ever smokers (HR compared to HPV-negative: HPV-positive smoker 1.05, 95% CI 0.43-2.58; HPV-positive never smoker 0.31, 95% CI 0.09-1.11; p 0.09) (378). It must be pointed out that these hazard ratios

do not reach statistical significance (though did so once modelling was used to account for missing data) and HPV-status was determined using serology, so may not be an accurate representation of true tumour HPV-status. However this does add to the evidence of a role for smoking in increasing tumour recurrence rates in HPV-positive patients.

1.5.11.2 EGFR and HPV

As discussed previously, EGFR expression is known to be a negative prognostic marker in HNSCC, and there has been much interest in the association between HPV and EGFR. A number of studies have demonstrated an inverse relationship between HPV-status and EGFR overexpression, with HPV-positive tumours having lower levels of EGFR than those that are HPV-negative. The effect of EGFR overexpression in HPV-positive tumours is less clear (186, 365, 373, 379).

Young *et al* recently reported on the relationships between p16 and EGFR in 240 head and neck cancers, of which 65% were oropharyngeal, from 3 successive clinical trials of varying combinations of chemoradiotherapy. In the oropharyngeal tumours they found a marked inverse correlation between EGFR gene copy number by fluorescence-ISH (positive FISH indicating either EGFR polysomy or gene amplification) and p16, with only 1.6% of tumours positive for both markers. Tumours that were p16-positive had a significant survival advantage over those that were p16-negative after adjustment for EGFR FISH status, whilst EGFR FISH did not predict for survival after adjustment for p16-status. However, it is difficult to assess fully whether EGFR FISH positivity stratifies HPV-positive disease, as the numbers in this group were very low (n=2) (379).

Reimers *et al* retrospectively analysed 96 oropharyngeal tumours for EGFR expression by immunohistochemistry and correlated expression with p16-status. They found that only 34.5% of p16-positive tumours expressed EGFR, compared to 54% of p16-negative tumours. They found that the strongest predictor of survival was a combination of EGFR and p16-status, with p16-positive/EGFR-negative tumours having the best disease specific survival (93% at 5-years vs. p16-negative/EGFR-positive 39%, p=0.003). Unfortunately the authors do not report survival rates for p16-positive/EGFR-positive and p16-negative/EGFR-negative tumours, however, on multivariate analysis (including adjustment for p16-status), they found no significant effect of EGFR status on either disease specific or overall survival (HRs relative to EGFR-negative 1.12 and 1.14 respectively, p=0.78 and p=0.69) (373).

Kumar *et al* also examined the relationships between HPV and EGFR expression by IHC. Similarly to Reimers *et al* they found an inverse relationship between HPV-positivity

and EGFR expression and that the combination of p16-positive staining and EGFR negative staining was predictive of both overall and disease free survival. However, in contrast to Reimers *et al*, they found that EGFR was predictive for both overall and disease free survival even after adjustment for HPV-status (p=0.03 and 0.04 respectively (365). Other authors have also found that EGFR negatively effects survival in HPV-positive HNSCC. Kong *et al* report a 5-year survival of only 30% in patients who were HPV-positive and with strong EGFR staining on IHC, compared to 83% in those with weak EGFR staining (p=0.05), though it must be noted that there was a strong inverse correlation between HPV and EGFR, with only 5 tumours positive for both (374). In contrast to these findings, Hong *et al* found that EGFR-positivity only conferred a negative effect on survival and tumour progression in HPV-negative patients (overall survival EGFR-positive vs. EGFR-negative: HPV-positive HR 0.58, 95% CI 0.09-3.76; HPV-negative 5.20, 95% CI 1.18-22.85). Interestingly, Hong *et al* do report hazard ratios for HPV-positive/EGFR-positive and HPV-negative/EGFR-negative patients and show no difference in survival for either group compared to HPV-positive/EGFR-negative patients, again suggesting that EGFR does not affect survival in HPV-positive patients (overall survival relative to HPV-positive/EGFR-negative: HPV-positive/EGFR-positive 1.42, 95% CI 0.39-5.19; HPV-negative/EGFR-negative 1.10, 95% CI 0.17-7.04; HPV-negative/EGFR-positive 4.49, 95% CI 1.34-15.03) (380).

1.5.11.3 Other prognostic factors in HPV-associated OPSCC

As discussed above, both cigarette smoking and EGFR-status have been suggested as potential factors that can affect the positive survival outcomes that are associated with HPV-positive OPSCC. There is also limited evidence to suggest other factors that also may influence outcome in this patient group.

One of the major pathogenic effects of HPV is via the interaction between the E7 oncoprotein and the retinoblastoma pathway, and specifically the abrogation of the pRb protein. Another factor involved in the retinoblastoma pathway is cyclin D1 (see section 1.2.1), which is overexpressed in up to 80% of HNSCCs and is associated with reduced survival in non-oropharyngeal head and neck tumours, especially those in the hypopharynx (381, 382). Hong *et al* retrospectively examined the interactions between HPV and cyclin D1 in 266 oropharyngeal cancers, of which 37% were HPV-positive. They found an inverse correlation between HPV-positivity and cyclin D1 expression, with only 27% of HPV-positive tumours also positive for cyclin D1, compared to 92% of HPV-negative tumours (p<0.0001). HPV-positive tumours had a 71% reduction in disease recurrence and a 67% reduction in disease specific death (adjusted HR 0.29 and 0.33, both p<0.0001). However, when HPV-positive tumours were analysed in conjunction with cyclin D1 status, it was found that expression of cyclin D1 markedly

increased the risks of both locoregional recurrence and disease specific death by up to 8 times (adjusted HR 8.10 and 5.67 respectively). Indeed outcomes for HPV-positive/cyclin D1 positive tumours were similar to those that were HPV-negative (383). However, the p-values for these HRs do not reach statistical significance (locoregional recurrence p=0.11, death p=0.18) and so must be treated with caution, in spite of their large size. The authors are unable to explain the overexpression of cyclin D1 in a minority of HPV-positive tumours. It is possible that these are patients with a significant tobacco exposure; however this data was not available in this study.

Another putative prognostic marker for HPV-positive OPSCC is the anti-apoptotic protein Bcl2, which has previously been identified as a negative prognostic factor in unstratified OPSCC treated with chemoradiotherapy (384). Nichols and colleagues examined 68 oropharyngeal, all treated with platinum based chemoradiotherapy, for the effects of Bcl-2 and HPV on survival. Of the 68 patients, 78% were HPV-positive and 35% expressed Bcl-2. There was no association between HPV and Bcl-2, and both HPV-positivity and low Bcl-2 expression were predictors of disease specific survival (p=0.005 and 0.039 respectively). When stratified by HPV-status, high Bcl-2 remained a strong predictor of poor disease specific survival (HR 7.6, p=0.004) and HPV-positive/Bcl-2 positive tumours had similar survival rates to HPV-negative/Bcl-2 negative tumours (385). Both the markers mentioned here are potential methods of stratifying HPV-positive tumours further, though both require validation in prospective trials. Bcl-2 status was not obviously affected by smoking, with similar rates of smoking in patients with Bcl-2 positive and negative tumours (385), whilst the relationship between cyclin D1 expression and smoking requires further investigation.

1.5.12 Should treatment be dependent on HPV status?

There is now significant evidence of both a role for HPV in the development of a proportion of oropharyngeal tumours, and also a significant survival advantage for those tumours that are HPV-positive. Furthermore, traditional treatments for HNSCC are associated with a number of severe toxic effects and poor quality of life. This has led to discussions regarding the future treatment for oropharyngeal cancer, and whether there should be different treatment regimens for HPV-positive and HPV-negative tumours. At present there is insufficient evidence to alter treatments in standard clinical practice. However, there are a number of trials of treatment “de-escalation” in patients who are HPV-positive, in an effort to reduce long term side effects of treatment whilst still preserving survival outcomes. Young patients are most likely to be affected by HPV-positive OPSCC and minimising treatment morbidity is vital. Possible “de-escalation” options that have been suggested include reducing

radiotherapy dosages, withholding chemotherapy or replacing chemotherapy with alternative therapies such as the EGFR monoclonal antibody cetuximab.

The ECOG 1308 trial started in March 2010, and is currently recruiting. It aims to assess the effects of reducing radiotherapy dose in HPV-positive patients with stage III and IV OPSCC. All patients receive 3 cycles of neoadjuvant chemotherapy with cisplatin, paclitaxel and cetuximab and are then assessed for response. Those who respond are then treated with a reduced dose of radiotherapy (54gy IMRT in 27 fractions), whilst those who do not respond receive standard dose radiotherapy (70gy IMRT in 33 fractions). Both cohorts are also treated with concurrent cetuximab. The primary outcome measure is 2-year progression free survival (386).

Another trial currently recruiting is RTOG 1016. This began in June 2011 and aims to assess the effects of replacing concurrent cisplatin chemotherapy with cetuximab. Patients with stage III or IV HPV-positive OPSCC are randomised to receive either cetuximab or cisplatin, both in conjunction with standard dose radiotherapy (70gy IMRT over 6 weeks). In this case, the primary outcome measure is 5-year survival (91). Interestingly, neither of these studies is stratifying patients by smoking status prior to randomisation, though RTOG 1016 lists evaluating the effects of smoking status among it's secondary outcome measures.

In the United Kingdom, the De-ESCALaTE HPV trial is now recruiting (387). This trial randomises HPV-positive, stage III or IV OPSCC to receive either cisplatin or cetuximab, both in combination with radiotherapy. The primary outcome measure is rates of long term (i.e. 2-years post treatment) toxic effects, whilst overall survival and disease recurrence are secondary outcome measures. Of note, this trial excludes patients who were in the intermediate risk group in RTOG 0129 (namely those HPV-positive patients who have a smoking history of >10 pack years and N2b, N2c or N3 nodal disease) (99).

An interesting point of note is that neither De-ESCALaTE nor RTOG 1016 aim to classify patients according to evidence of EGFR-status. There is a well-documented inverse association between HPV-positive tumours and EGFR-status, and some limited evidence that EGFR is only associated with poor outcome in HPV-negative patients (379, 380). On the other hand there is evidence from one phase II trial that HPV-positive patients treated with neoadjuvant cetuximab have improved survival compared to HPV-negative patients, regardless of EGFR-status (388). However, in this trial patients also received neoadjuvant paclitaxel and carboplatin, so it is difficult to draw definitive conclusions as to the effects of cetuximab in HPV-positive patients. It will be interesting to see the results of these new de-escalation trials.

At present there are no prospective trials comparing surgical outcomes between HPV-positive and HPV-negative tumours, and no prospective trials comparing surgery and chemoradiotherapy for HPV-associated OPSCC. Retrospective analysis suggests that the survival benefit seen in HPV-positive patients is applicable to surgically treated disease, however a significant proportion of patients included in these analyses underwent post-operative therapy with either radiotherapy or chemoradiotherapy, and it is difficult to establish the effects of surgery alone on survival (60, 362). Transoral resections (whether with laser or robotic) represent an attractive treatment option for resectable disease, with no requirement for mandibulotomy and an absence of chemoradiation induced side effects. However, the majority of HPV-positive patients present with advanced nodal disease, and it is likely that multiple involved nodes and extracapsular spread will remain an indication for post-operative treatment, even following a neck dissection. Whether it is possible to alter post-operative therapies (for example reduced radiotherapy dosage or replacing cytotoxic agents with cetuximab) is yet to be established, as are the relative survival rates of surgical treatment (and whichever adjuvant therapy is chosen) versus (chemo)radiotherapy. Certainly in unstratified, advanced stage head and neck cancer, evidence would suggest that post-operative chemoradiotherapy is associated with improved outcomes over post-operative radiotherapy alone (78, 79). It is currently unclear whether this same finding applies specifically to HPV-positive OPSCC. The PATHOS trial (Post-operative Adjuvant Treatment for HPV-positive Tumours), aiming to compare a number of adjuvant therapies (both RT and CRT) in resectable HPV-positive OPSCC will hopefully help to answer this question (389).

One important caveat in the design of de-escalation trials for HPV-positive tumours must be taken into consideration. Although the prognosis of these tumours is generally excellent compared to their HPV-negative counterparts, with 2-year survival rates of ~80%, there still remains a significant proportion of HPV-positive patients who do not share this outlook (312). Although there is growing evidence that heavy smoking negatively impacts on prognosis in those with HPV-positive tumours, it is unlikely to be the only factor determining poor outcome in this patient group. One consideration for future work in the field of HPV-associated OPSCC, and indeed of this thesis, is to attempt to find a way of phenotyping these patients. This is important not only to attempt to maximise survival rates in HPV-positive patients, but also to avoid recruiting those with poor prognosis into treatment de-escalation studies.

1.6 Cancer and the immune system

1.6.1 T-Lymphocytes

The adaptive immune system, including the various sub-populations of T-lymphocytes, represents the major component of the immune system involved in tumour regulation and cancer progression. Stem cells in the bone marrow develop into immature T-lymphocytes which then undergo maturation in the thymus before being trafficked via the blood and lymphatics to various secondary lymphoid organs such as the spleen and regional lymph nodes. Here, antigen presenting cells (APCs; such as dendritic cells) can present antigens to naïve T-lymphocytes and stimulate their activation into either effector or memory T-cells. APCs express major histocompatibility complex (MHC) on their cell surface. Antigens are processed and presented in combination with MHC to the T-cell receptors (TCRs). Stimulation of T-lymphocytes requires a combination of MHC-TCR interaction and various co-stimulatory molecules (390).

All T-cells express the cell surface molecule CD3 (390, 391). In addition, cytotoxic T-cells (CTLs) also express CD8. CTLs are capable of directly destroying tumour cells. In contrast, helper T-cells (Th) co-express CD4 and secrete cytokines. CD4-positive T-cells are subdivided into type-1 cells (Th_1; involved in the stimulation of CTLs) and type-2 cells (Th_2; involved in the stimulation of antibody production by B-cells) (392). Th_1 cells play a prominent role in tumour regulation, compared to Th_2 cells (393). A third important group of effector T-lymphocytes are CD4/CD25/Foxp3 expressing regulatory T-cells (T_{regs}). T_{regs} supress both CTLs and Th-cells, and are important in the development of tolerance to self-antigens and thus the prevention of autoimmunity (394). In cancer, cytokines produced by tumour cells often result in the preferential accumulation of T_{regs} and they are thought to aid tumour progression by repressing the anti-tumour actions of both CTLs and Th-cells (395).

1.6.2 Cancer immunoediting

The concept of cancer immunoediting describes three distinct phases of interaction between tumour cells and the immune system in the course of tumour progression. In the first phase, known as elimination, tumour cells are completely eradicated by T-lymphocytes. The second phase is characterised by the emergence of a population of tumour cells that become resistant or 'invisible' to the immune system. In conjunction with this, non-resistant tumour cells are continually destroyed by the immune system, leading to a state of equilibrium that can last for many years. Over a period of time

tumours increasingly develop ways to avoid detection and destruction by the immune system, known as immune escape. This can be due to a number of factors, including downregulation of MHC class-1, reduction in co-stimulatory signals in antigen presentation, loss of tumour antigens and secretion of anti-inflammatory cytokines (390, 396, 397).

1.6.3 Tumour Infiltrating Lymphocytes

1.6.3.1 Tumour Infiltrating Lymphocytes and Cancer Outcomes

Tumour infiltrating lymphocytes (TILs) are commonly seen in several different solid tumour types, and are thought to respond to specific tumour antigens (398). A number of studies have shown that high numbers of TILs are a positive prognostic indicator, for example in ovarian and colorectal cancer, suggesting that TILs are capable of overcoming immune escape mechanisms and delaying tumour progression (399-402). Cell ratios, for example CD8:Foxp3 and CD8:CD4, may be more predictive of survival than absolute numbers of TILs alone (391, 403). A recent meta-analysis of 52 studies demonstrated a consistent survival advantage in patients with TIL^{high} tumours. Patients whose tumours were infiltrated by $CD3^+$ cells had a 42% reduction in the risk of death compared to those who did not have a significant infiltrate (HR 0.58, 95% CI 0.43-0.78) while patients with a CTL infiltrate ($CD8^+$) in their tumour, also had a survival advantage (HR 0.71, 95% CI 0.62-0.82). The greatest survival benefit was seen in tumours with a high CD8:Foxp3 ratio, with these patients having a 52% reduction in the risk of death (HR 0.48, 95% CI 0.34-0.68, $p<0.001$). Interestingly, high levels of T_{regs} did not have an adverse effect on survival in this meta-analysis (HR 1.19, 95% CI 0.84-1.67) (391).

1.6.3.2 Tumour Infiltrating Lymphocytes and HNSCC

The positive survival benefits of high levels of TILs seen in other tumour types are less well established in head and neck malignancy, with conflicting reports from small data sets in the literature. Some authors have reported that a prominent lymphocytic infiltrate, based on semi-quantitative H&E grading, is associated with improved survival and low levels of $CD3^+$ cells have been shown to correlate with poor survival (156, 404, 405). Other reports describe $CD3^+$, $CD4^+$ or $CD8^+$ lymphocytes do not significantly affect outcome (406, 407). However, within the literature there are data that show a survival benefit with increased levels of $CD4^+$, but not $CD8^+$ cells, whilst one study found reduced prognosis in tumours with CD4:CD8 ratios of greater than 1, where the ratio was attributable to a reduction in $CD8^+$ cells, and another still found

that low $CD8^+$ infiltration was associated with poor survival (408-410). The reported effects of T_{regs} also vary. One study of 84 newly diagnosed head and neck tumours found that the presence of high numbers of activated T-helper cells (CD4+CD69+) was associated with improved overall survival while high levels of T_{regs} were associated with improved locoregional recurrence (411). Other authors have also found that the presence of high levels of T_{regs} is associated with improved overall and disease specific survival (412). In contrast to this, there are also reports that tumours containing high levels of T_{regs} have a poor prognosis (413). A criticism of many of these studies is that they were generally small, not stratified by HPV-status and contained tumours from a number of different subsites. Thus, it is difficult to draw any valid conclusions from them.

1.6.3.3 Tumour Infiltrating Lymphocytes and HPV-associated oropharyngeal cancer

There is evidence that HPV-positive oropharyngeal tumours tend to have a more prominent lymphocytic infiltrate than those that are HPV-negative (5, 374). Furthermore, it has been shown that the majority of HPV-positive oropharyngeal tumours have a gene expression signature indicating the presence of an adaptive immune response (414). Although HPV is known to repress the host immune system, for example by downregulating MHC-class 1 expression (section 1.4), it would seem intuitive that expression of the E6 and E7 oncoproteins by tumour cells would invoke an adaptive immune response. In addition, there is evidence from cervical cell lines that radiotherapy can increase expression of both E6 and E7, and also increase MHC class-1 expression (415). Moreover, experiments in mice have demonstrated that an intact immune system is required for both cisplatin and irradiation to clear HPV-driven oropharyngeal tumours (369, 416). Therefore, It is possible that the survival benefit seen in HPV-positive OPSCC is due to increased immune surveillance (417). In support of this, circulating HPV 16 E7-specific CTLs have been demonstrated in patients with HPV-positive oropharyngeal tumours, as have elevated anti-HPV 16 antibodies, both of which have been linked to improved outcomes (418-421). However, there is limited information in the literature regarding the relationships between HPV-positive tumour and TILs, and regarding the effects of TILs on prognosis and outcomes in HPV-associated tumours.

Heusinkveld *et al* isolated TILs from 20 unselected head and neck tumours. They detected HPV-16 E6 and E7 specific T-cells in 6 out of 8 HPV positive oropharyngeal tumours, and in none out of 12 that were HPV-negative. Further analysis revealed that these HPV specific T-cells were a mixed population containing CTLs, Th1 and Th2 CD4 cells and also Tregs, which suggests a specific anti-tumour immune response (422).

Kong *et al* analysed CD3-positive T-cell numbers in 92 HNSCC specimens, of which 49 were oropharyngeal tumours. They found a positive correlation between HPV-positive tumours (44% of the total cohort) and higher infiltration with T-cells, with strong staining for CD3 observed in 67% of HPV-positive tumours, compared to only 35% of those that were HPV-negative (p=0.03). Higher levels of $CD3^{+}$ cells were associated with improved survival in the cohort as a whole and for HPV-negative tumours (5-year survival $CD3^{strong}$ vs. $CD3^{weak}$: 70% vs. 34%, p=0.005; 60% vs. 22%, p=0.04). However, survival amongst HPV-positive tumours did not vary according to $CD3^{+}$ levels (5-year survival $CD3^{strong}$ vs. $CD3^{weak}$: 75% vs. 64%, p=0.97) (374).

Rajjoub *et al* examined 48 oropharyngeal tumours for the presence of $CD3^{+}$ T-cells and classified tumours as HPV-positive or negative on the basis of PCR. Of the 48 tumours, 68.8% were HPV-positive. HPV-positive tumours were more likely to be $CD3^{high}$ than those that were HPV-negative (51.5% vs. 33.3%), though the authors do not report whether this relationship was statistically significant. $CD3^{high}$ tumours showed a trend towards improved overall and disease free survival (5-year survival, 77% vs. 51% and 71% vs. 37%, p=0.15 and 0.09) though this did not reach statistical significance. The authors only reported on the effects of CD3 on survival in the cohort as a whole, and did not stratify by HPV-status. An interesting finding was the HPV-positive $CD3^{high}$ tumours had a significantly lower rate of nodal metastasis at presentation than those that were HPV-positive and $CD3^{low}$ (37.5% vs. 87.5%, p=0.009). This was not reflected in HPV-negative tumours (406).

Finally, Wansom *et al* measured levels of CD4, CD8 and Foxp3 positive T-cells in 46 patients with stage III or IV oropharyngeal cancer, of which 66% were HPV-positive (though only 38 of the 46 had sufficient material for HPV-testing). There was a trend towards increased CD4, CD8 and CD4 *plus* CD8 levels, and reduced Foxp3 levels, in HPV-positive tumours, though none of these were statistically significant. HPV-positive tumours had a trend towards a lower Foxp3:CD8 ratio that approached significance (HPV-positive 0.74:1 vs. HPV-negative 1.27:1, p=0.09) caused by a higher mean Foxp3 count in HPV-negative tumours. In terms of prognosis; mean CD8 count, mean Foxp3 count, CD4:CD8 ratio and CD4 *plus* CD8 count were all associated with improved overall survival, even after adjusting for HPV-status (p=0.01, p=0.03, p=0.03 and p=0.03, respectively). Only CD8 count and Foxp3 were associated with disease specific survival after adjusting for HPV-status (p=0.03 and p=0.004) (423). The authors report that the absence of a significant association between TIL levels and HPV-status is a major finding and suggests that other factors may be responsible for T-cell infiltration. However, this conclusion has been drawn from only 38 patients and so must be interpreted with some caution. This study does however add strength to a potential positive benefit of TILs in oropharyngeal cancer.

1.6.4 Summary of Current Knowledge

It is clear that HPV-positive OPSCC represents a distinct disease entity from HPV-negative disease, in terms of epidemiology, aetiology and pathology. Furthermore, significant evidence exists for a survival advantage in most patients with HPV-positive tumours and efforts are underway to maximise both prognosis and long term function. Despite this, there remain a number of unanswered questions regarding HPV-positive OPSCC. These include optimum treatment combinations and, perhaps more importantly, ways of identifying the small but significant number of HPV-positive patients who have poor long term survival prospects. Ongoing work, including that presented here, is aiming to answer some of these questions, and the results of a number of studies will add greatly to the literature regarding HPV-positive OPSCC.

1.7 Aims of this project

The aims of this thesis are:

- to establish rates of HPV-associated OPSCC in the patient population treated by two major centres in the South of England.
- to identify key differences between HPV-positive and HPV-negative disease in terms of presentation, disease characteristics, and prognosis.
- to identify biomarkers that predict for survival in HPV-positive oropharyngeal cancer, and identify HPV-positive patients with poor survival outcomes.

2 Materials and Methods

2.1 Database Construction

This thesis sought to examine various molecular, pathological and immunological features of archived oropharyngeal cancers in the context of patient outcomes following treatment. Unfortunately, there was no available database of locally treated head and neck cancers to enable these correlations to be made. Therefore, a major part of the work for this thesis was the development of a highly detailed tumour database, which then formed the backbone of all further work related to the project. The aim of the database was to include all oropharyngeal and oral cavity tumours treated in University Hospital Southampton NHS Foundation Trust (UHS) and Poole Hospital NHS Foundation Trust (PFT) between 1st January 2000 and 31st December 2010, with the main focus being on oropharyngeal tumours. The database was constructed using Microsoft Excel for Windows Version 14.0.6112.5000. Continuous numerical data was recorded to two decimal places, where appropriate, and categorical data was coded numerically to facilitate statistical analysis. All categorical codings are shown in Appendix 1.

2.1.1 The Human Tissues Act 2004

The human tissues act 2004, came into force on the 1st of September 2006 and, amongst other legislations, introduced a requirement for establishments storing tissue for research to be licensed. Furthermore, the act also introduced a necessity for patients to supply consent for the use of stored tissues for research, which had not previously been applicable.

> *"Consent is the cornerstone of the new legislation – the Human Tissue Act 2004 (HT Act). The HT Act requires that consent must be given for body parts, organs and tissue from the living or deceased to be removed, stored or used for certain specified purposes. The new legislation heralds a number of changes for both professionals and patients." (http://www.hta.gov.uk/media/mediareleases.cfm/ accessed 6th August 2012)*

The UHS consent form, signed prior to any surgical intervention, includes a statement permitting the use of excess tissues for research (see below). Archival tissues removed from patients after the 1st of September 2006 were thus able to be examined in this study.

> *"I agree that any tissue removed as part of the procedure or treatment, which is then surplus to my own care, may be used for audit, teaching, and/or research. Any sample used for such purposes would be done in an anonymous way so that my identity at the point of use would not be known. All research studies would be subject to Research Ethics Approval and would be subject to national standards of practice." (Southampton University Hospitals NHS Trust Consent Form 1)*

The PFT consent form contains no such statement, and thus we were unable to access tissues removed from patients after 1st September 2006. Prior to this date the HT Act did not apply. Therefore the PFT database and subsequent analysis was restricted to patients treated between the 1st January 2000 and the 31st August 2006.

2.1.2 Patient Identification

Patients from the two hospital sites were identified in different ways. In Southampton, two databases containing limited patient information were already in existence. These were held by Mr Andrew Webb, Consultant Oral and Maxillofacial Surgeon; and Mrs Caroline Hampton, MacMillan Head and Neck Cancer Nurse Specialist. The names, dates of birth and hospital numbers of patients who had been treated for Oropharyngeal and Oral Cavity Cancer between 2000 and 2010 were extracted from these databases to enable the retrieval of their hospital notes. In Poole, no such databases existed. However, since 2003, the names of all patients treated for Head and Neck Cancer had been recorded and entered onto the national Data for Head and Neck Oncology (DAHNO) register. Therefore, the local DAHNO co-ordinator, Mrs Jill Horton, was contacted and asked to supply names and hospital numbers for patients with oropharyngeal cancer treated between 2003 and 2006. To identify patients treated between 2000 and 2003, the Cancer Intelligence Service at the South West Public Health Observatory (SWPHO) were contacted and asked to provide the appropriate details.

2.1.3 Data Sources

The paper notes of all patients identified were retrieved from the hospital archives at UHS and PFT and retrospectively analysed to acquire the data required for the tumour database (see below). Clinic letters were checked in the first instance and then handwritten notes. In all cases, electronic hospital records (UHS: Electronic Documents System, eDOCS; PFT: Electronic Patient Records, EPR) were also checked to account for any missing information in the paper notes. In a small number of cases paper notes were not available, in these circumstances only the electronic records were used for data collection. Two further electronic resources were utilised in UHS to acquire data

relating to pathology and imaging reports. These were the eQUEST and PACS systems (eQUEST: Electronic Request System, PACS: Picture Archiving and Communication System). In PFT, this information was available from EPR. Approximately 30% of patients had been followed up in sites away from UHS and PFT, for example in Salisbury, Winchester, Dorchester, the Isle of Wight and the Channel Islands. For these cases, the trusts were contacted to request clinic letters from follow-up visits in an attempt to maximise long term outcome data.

2.1.4 Ethical Approval, Patient Anonymity and data protection

Patient anonymity is an essential consideration in medical research. However to enable correlation between tumour characteristics and patient outcomes, it was important to maintain a link between pathological tissues and the information in the tumour database.

The tumour database was established in a "linked anonymised" manner. The only identifiable data in the database was each patient's hospital number, date of birth and the unique pathology number of each tumour specimen. Patient names were not recorded. All patients were allocated an arbitrary study number, starting at 1, according to the order in which they were entered into the database. The study number and pathology number were the only identifiers subsequently used throughout the study. All pathological analyses were performed on "sub-databases" extracted from the main tumour database, and containing only the study number and pathology number. Once this analysis had been performed, the data was copied back into the main database to allow correlation with demographic, clinical and follow-up data. All statistical analysis was performed in an anonymous fashion. All patient information was stored in password protected files on a University of Southampton, Dell Latitude laptop fitted with Bitlocker Drive Encryption. Passwords were only known by myself, Dr Emma King and Professor Gareth Thomas. A backup copy of all data was stored on the University of Southampton secure internal network. Ethical approval had previously been granted for the study (UKCRN 8130; ISRCTN 71276356; REC references 09/H0501/90 and 07/Q0405/1).

2.1.5 Demographics, referrals and diagnosis

Demographic details were recorded for all patients. These included: treating hospital (UHS vs. PFT), hospital number, gender and date of birth. The date of referral (taken from the referral letter) was recorded where available, as was the date of first contact with any member of the Head and Neck Multidisciplinary Team (MDT), typically the first clinic appointment, and the date that treatment first started. The date of diagnosis was

taken as the date of authorisation of the pathology report for the diagnostic biopsy. Where original pathology reports were unavailable, the date of diagnosis was taken as the date of diagnostic biopsy. If this was unavailable then the date of diagnosis was taken from the first MDT after the diagnosis of malignancy had been confirmed. Patients' age at diagnosis was calculated as the difference between their date of birth and date of diagnosis, and was rounded to the nearest whole year. The time from referral to first contact, diagnosis and start of treatment was also calculated and recorded to the nearest whole day.

2.1.6 Risk Factors

Risk factors related to the development of HNSCC were recorded for each patient, namely smoking and alcohol history. Patients were coded according to their smoking and drinking status at the time of cancer diagnosis. Unfortunately, documentation of these risk factors was inconsistent within the patient notes. Therefore, a variety of data codings were used.

Where available, alcohol history was coded as: non-drinker; current drinker - less than 10 units per week, 10 to 20 units per week or greater than 20 units per week; or ex-drinker. In some cases alcohol history had been recorded as light, moderate or heavy, and these were coded in the less than 10, 10 to 20 and greater than 20 units per week groups, respectively. Alcohol history was generally poorly recorded, and thus for the majority of analysis, alcohol history was summarised as: non/ex drinker vs. current drinker.

Similar problems were encountered with smoking history. Where available, patients were classified according to the number of pack years smoked (where one pack year represents 20 cigarettes per day for one year), as less than 10, 10 to 20 and greater than 20 pack years. When only the number of cigarettes smoked per day had been documented, patients were grouped as less than 10 per day, 10 to 20 per day or greater than 20 per day. Patients recorded as light, moderate or heavy smokers were coded along with these "per day" groupings (i.e. light with <10, moderate with 10-20 and heavy with >20). Patients who had never smoked or were ex-smokers were recorded as such. In cases where patients smoked loose tobacco, the number of cigarettes per day was calculated according to the formula of ½ an ounce of tobacco being equivalent to 20 cigarettes (424). Recent analysis of the effect of smoking on survival in both HPV-negative and HPV-positive oropharyngeal cancers has shown that patients with more than a 10 pack year smoking history have more than a twofold increase in the risk of death (HR 2.10, $p<0.001$) than those with less than 10 pack years (357). Therefore, for all survival analysis, smoking was recoded to take this cut-

off point into account, with the data available. The "low smoking" group contained those that were coded as: non-smokers, ex-smokers, or current smokers who had less than 10 pack years, smoked less than 10 per day or were documented as being light smokers; while the "heavy smoking" group contained all those who were current smokers with a greater than 10 pack year history, who smoked greater than 10 per day or where documented as moderate or heavy smokers.

2.1.7 Tumour characteristics – location and staging

Tumour subsite was recorded as accurately as possible from clinic letters, handwritten notes and CT/MRI reports. Initially tumour location was coded as: tonsil, base of tongue, lateral pharyngeal wall, posterior pharyngeal wall, soft palate, tonsillolingual sulcus or oropharynx (if the exact subsite location was unclear). In subsequent analyses, tumour subsite was summarised as tonsil, base of tongue or "other oropharynx". Tumour side was coded as left, right or bilateral/midline as appropriate.

After analysing the first few sets of patient notes, it became clear that there were some inaccuracies in the documented tumour staging. Therefore, all tumours were re-staged according to AJCC criteria with the aid of the "Pocket Guide To TNM Staging of Head and Neck Cancer and Neck Dissection Classification" (41). TNM stage was recorded according to clinical, radiological and, where appropriate, pathological parameters. A "final TNM" stage was then calculated according to which of these was available. We considered pathological staging to be most accurate, followed by radiological and clinical staging. Therefore, if patients had undergone resection of their primary tumour and underwent a neck dissection, then the pathological TNM stage was used, if not, then the radiological TNM stage was used. An exception to this was in the cases where patients had received pre-operative chemotherapy, when the radiological staging was considered more accurate. Only in cases where imaging reports were unavailable and patients had not been surgically treated was the clinical TNM stage taken as the final stage. The final TNM stage was converted to an overall disease stage (I-IVc) according to the AJCC criteria.

TNM staging was first recorded in as much detail as possible (i.e. T1-T4, N0-N3, Stage I-IV) and then summarised in a number of analyses, due to small numbers in some subgroups. Overall disease stage and T-stage were classified as early (i.e. I/II or T1/T2) vs. late (i.e. III/IV or T3/T4). Nodal metastases were first classified as either present or absent, and then summarised according to the "high risk" nodal staging suggested by Ang *et al* for HPV-positive tumours (i.e. N0-N2a vs. N2b-N3) (99).

The maximum tumour diameter (in millimetres) was recorded, again according to clinical, radiological and pathological reports. If patients had undergone diagnostic cervical ultrasound scanning and fine needle aspiration cytology, then the results were recorded in the database. The presence and location of synchronous primary tumours and distant metastases were noted.

2.1.8 Pathological Details and TIL grading

The pathology reports for diagnostic biopsies and surgical resections were examined and standard pathological data were recorded. These included: depth of tumour invasion (in mm); grade of differentiation (initially recorded as: well, moderate, poor, undifferentiated; and then summarised for all analyses as well/moderate vs. poor/undifferentiated); cohesive nature of the invasive margin (cohesive, discohesive); and the presence or absence of perineural, intravascular and perilymphatic spread. In patients who had undergone surgical resection, the nature of the resection margin was classified as clear, close (tumour within 5mm) or involved. The presence or absence of dysplasia at the resection margin, a known negative prognostic marker related to field change, was also recorded. When patients had undergone a neck dissection, the total number of nodes, the number of involved nodes, the size of the largest involved node (maximum diameter in millimetres), the presence of extracapsular spread and the lymph node levels of the involved nodes were recorded.

Where available, the H&E stained slides for all biopsies and resections were retrieved from the histology archives at UHS and PFT. These were then reviewed by Professor Gareth Thomas, a Consultant Pathologist, and any data missing from pathology reports was recorded. Tumour grade was widely available from pathology reports. However; tumour cohesion, perineural, intravascular and perilymphatic spread was poorly reported in routine pathology reports, despite being part of the minimum dataset for reporting of head and neck malignancy, and in the majority of cases came from Professor Thomas' review. Tumour resection margins were not re-reported, so this data only came from pathology reports.

In addition to standard pathological markers, the presence and extent of tumour infiltrating lymphocytes (i.e. TILs) was graded as: low (infiltrate in less than 20% of the tumour), moderate (20-80% of tumour) or high (greater than 80%). This grading took into account infiltrate in both tumour islands and stromal tissues and formed a major component of subsequent survival analysis. This grading system was previously described by Marsh et al (156) and has been assessed by multiple histopathologists. Concordance between pathologists is >95%.

2.1.9 Assessment of HPV-status

For a large number of analyses in this thesis, comparisons are made between HPV-negative and HPV-positive tumours. HPV-status was determined using a combination of p16 immunohistochemistry, and HPV *in situ* hybridisation. Only tumours that were positive for both were considered to be HPV-positive. These techniques are described in detail in sections 2.3 to 2.5.

2.1.10 Treatment

The treatment modality of the primary tumour was recorded for each patient, i.e. surgery, radiotherapy or chemoradiotherapy. Neck dissections for cervical metastasis were also recorded. Patients who had undergone a diagnostic tonsillectomy followed by irradiation were coded as having been treated by radiotherapy. Similarly, patients who underwent a neck dissection prior to definitive radiotherapy or chemoradiotherapy were coded as having received (chemo)radiotherapy. In patients undergoing surgical resection, the method of resection (i.e. open surgery or trans-oral resection) was recorded, as was the use of post-operative radiotherapy. The side, dose and number of fractions were recorded for all patients receiving radiotherapy. For patients undergoing chemotherapy, the timing (neoadjuvant, concurrent or both), number of cycles, and chemotherapeutic agents received was recorded. Patients who had received no treatment, or only palliative chemotherapy, were coded as such.

Treatments were initially recorded in as much detail as possible, and later summarised for the majority of analyses. A treatment summary is included in Table 5.4 and a detailed breakdown is shown in appendix 2.

Interestingly, the centres involved in this study differed in their approach to treatment of OPSCC, with more patients being surgically treated at UHS. This allowed a direct comparison of survival according to treatment modality. Patients undergoing chemoradiotherapy received 2-6 doses of platinum based chemotherapy (either cisplatin or carboplatin) combined with 64-66Gy of radiotherapy given in 32-33 fractions. No patients received altered fractionation or intensity modulated radiotherapy. Neoadjuvant chemotherapy was 1-2 doses of either cisplatin or carboplatin, in conjunction with 5-flurouracil.

2.1.11 Treatment failure, recurrence and death

The following patient outcomes were recorded: treatment failure, tumour recurrence, second primary tumours (SPTs), distant metastases and death. Treatment was considered to have failed in any patient who still had evidence of tumour at the first post treatment follow up visit, and which did not spontaneously resolve. Tumour recurrence was deemed to have occurred in any patient who had had a documented tumour free interval following treatment but subsequently developed evidence of further tumour. The site of treatment failure or tumour recurrence was coded as local, regional (i.e. nodal) or locoregional. The time to recurrence was calculated as the difference, in months, between the end of treatment and the first documented evidence of tumour recurrence. The presence and site of second primary tumours and distant metastases was recorded, and the time to event calculated from the end of treatment. A summary variable was created to establish patients who had suffered from any form of recurrent disease, be it treatment failure, late recurrence or distant metastases. If patients did not receive treatment, or did not complete treatment, then treatment failure/disease recurrence/distant metastases were considered "not applicable". In patients in whom treatment failed and no further treatment was given, then tumour recurrence, distant metastases and second primaries were deemed "not applicable".

The date of death was obtained from the patient notes. If it was unavailable in the notes then the General Practitioner was contacted. Cause of death was typically established from the patients' notes. Patients were coded as either "died from disease" if there was evidence from the notes that the cause of death was related to the tumour, or "still alive/other cause of death" if the patient had not died from their tumour (e.g. had suffered an unrelated myocardial infarction). When the cause of death was equivocal, a consensus opinion was reached between myself and Dr King as to the most appropriate coding. In cases where the cause of death was unavailable, the SWPHO were again contacted and asked to supply this information. The survival outcomes used throughout the analysis were "Overall Survival" and "Disease Specific Survival". Overall survival was calculated as the time between the date of diagnosis and the date of death from any cause. Disease specific survival was the time between the date of diagnosis and the date of death from oropharyngeal cancer. Two variables were created to establish the number of patients who had died of their disease at 3- and 5-years. In these variables, patients who were still alive, but with follow-up of less than 3- or 5-years, were deemed to be missing. The 3- and 5-year survival rates were then taken from those patients where the status was known.

2.1.12 Quality of Life Outcomes

An important consideration in the treatment of head and neck malignancy is long term functional outcome and quality of life (QOL). As this was a retrospective study, it was impossible to obtain detailed QOL information. Therefore, as a surrogate measure of function and QOL, the use of assisted feeding (nasogastric tube or percutaneous gastrostomy) after treatment was recorded. In patients' who required a feeding tube after treatment, it was also recorded whether or not the tube was removed, and after how long. The requirement for tracheostomy after treatment was also noted.

2.1.13 Patient Numbers

A total of 418 patients with oropharyngeal cancer were identified and entered into the database from UHS (n=316) and PFT (n=102). However, when attempting to retrieve pathology specimens for these patients, it became clear that a number of pathology samples were unavailable. In the majority of cases, patients had undergone diagnostic biopsy in another centre; either Salisbury, the Isle of Wight, Jersey, Guernsey or Dorchester; and had then had chemo(radiotherapy) at either UHS or PFT. As our ethical approval did not cover these peripheral sites, we were unable to obtain archival material. In 11 cases at UHS and 12 cases at PFT there was either insufficient tissue remaining in the paraffin block for TMA construction, or the original slides and/or block were missing from the archives.

Of the 418 patients, pathological material was available for 267 tumours. To increase numbers, the archival material for 24 oropharyngeal tumours treated at Bart's and the London NHS Trust (BLT) was also included in the study. This material had been collected by Professor Thomas for a previous study, and was linked to a limited clinical database containing demographic, staging and survival information. All of these patients had been surgically treated, with or without postoperative radiotherapy. Of these additional 24 tumours, 1 had insufficient material remaining for any further analysis.

Thus, follow up data were available for 442 patients treated across three separate sites (i.e. UHS, PFT and BLT). Archival tissue was available for 290 tumours, which were used to create tissue microarrays for further analysis.

2.2 Tissue Microarray Construction

A tissue microarray (TMA) is a single paraffin block containing samples from a number of different specimens, in this case oropharyngeal tumours. These TMAs can then be sectioned and stained for a number of targets, allowing for rapid identification of potential biomarkers in a large number of tumour specimens. There are a number of steps involved in the construction of a TMA, including sample identification and retrieval, slide mapping, recipient block production, array design, array construction, and array tempering and sectioning. Specimen identification and retrieval, all array design and construction were performed by myself, while slide mapping and array sectioning were performed by others (acknowledged below).

2.2.1 Sample Identification

In this study, TMAs were linked to outcome data. Therefore, it was essential to accurately identify archival samples included in the arrays. The histology report for each patient contains a unique pathology number which identifies the tissue blocks and any slides produced for each individual tumour. Individual pathology numbers were recorded in the tumour database and used to retrieve the original diagnostic H&E slides from the archives at UHS and PFT. These slides were then used to identify the region of interest of the tissue block in each case (see below) and the appropriate block was subsequently also retrieved from the archives. Where patients had undergone surgical treatment, the resection specimen was preferentially used as it typically contained more tissue than the initial biopsy specimen. In patients who had undergone radiotherapy or chemoradiotherapy, only the biopsy specimen was available for TMA production.

2.2.2 Slide Mapping

The ultimate aim of TMA construction is to have a single paraffin block containing representative tissue from a number of different tumours (425). Any individual tissue block contains areas of tumour and often also areas of normal tissue. To ensure that only tumour tissue was sampled in the construction of the TMA, original diagnostic H&E slides were reviewed by Professor Thomas, and the areas of interest from within the tumour were marked using permanent ink. One of the criticisms often levelled at TMAs is that the tissue cores may not truly represent the status of the tissue as a whole in terms of protein or DNA expression, due to in-tumour heterogeneity (425). Furthermore, when a TMA is sectioned, tissue cores may be lost. A way of improving the accuracy of representation within the TMA is to take multiple cores from each donor block, and most studies reported have used between two and five tissue cores

per tumour (426, 427). We aimed to take a minimum of three cores from each tumour, and each H&E slide was marked at least in triplicate.

2.2.3 Recipient Block Production

The general principle of TMA production is to transfer cores of tissue from "donor" to "recipient" paraffin blocks. These are typically produced from low melting point paraffin (52-56°C) and care must be taken to avoid bubbles or cracks within the recipient block, as these can affect the integrity of the final array (425). Recipient blocks used in this study were 35mm in length, 25mm in width and 5mm in depth. A 3mm border around the periphery of each recipient block was demarcated and cores were only placed inside this border. Therefore the actual area available for utilisation in each recipient block was 32x22mm. The plastic cassette holding each recipient block was etched with the name of the TMA contained within it (see below), to enable ease of identification of each array.

2.2.4 Tissue Array Design

The design of each tissue array is perhaps the most important factor in TMA construction, and there are several factors that must be considered (425, 428). The size of the tumour core used depends on the quality and quantity of donor tissue available, and is ultimately a balance between the amount of tissue in each core and the total number of cores that can be contained in one array (i.e. the larger the core size, the fewer number of cores that will fit in each array). The generally accepted rule is that the larger the cohort, the smaller the core size needs to be, and the majority of published studies have used either 0.6mm or 1mm cores (425, 428). Another consideration regarding core size is that larger cores require greater spacing between each core. If there is not enough wax between each core, then cores have an increased tendency to be lost on sectioning. Spacing is taken as the distance between the centres of each adjacent core. In this study, a 1mm core diameter was used, with a distance between each core of 1.6mm. As previously mentioned, each tumour was replicated at least in triplicate. It becomes harder to ensure accuracy of scoring TMAs with larger numbers of cores; therefore we aimed to have a maximum of 50 tumours (and thus 150 cores) on each array.

The TMAs used in this study were prognostic ones, linked to a clinical tumour database. It was therefore vital that the cores in each TMA were ordered in such a way that it would be easy to identify which cores originated from which tumour. The TMAs were designed using Alphelys TMADesigner® 2 Version 1.0.0.11 (Mitogen, UK). The first step in the design process was the creation of an excel spreadsheet containing the

pathology number and study number of each tumour. This was then imported into the design software and converted to an alphanumerical grid. The rows in the grid were alphabetically labelled, and the columns numerically, such that the core in the top left hand corner was labelled A1, the next in the row A2 and so on. The recipient block details were entered into the software, which then allocated specific coordinates (i.e. X and Y, in micrometres, where X relates to the length along the block, and Y the width) in relation to the top left hand corner of the recipient block. On completion of the TMA construction process, the software created an excel spreadsheet that contained the alphanumeric location of each tissue core according to pathology and study number, enabling easy linkage to the tumour database.

When the sectioned TMA is stained and analysed, it is vital that the slide can be correctly orientated such that the A1 core is in the top left hand corner. The design software allows the allocation of specific spot locations as orientation spots. To this end, the four spots in the top left hand corner (i.e. A1, A2, B1 and B2) of each array were allocated as orientation cores. Instead of having tumour tissue in these locations, an alternative orientation tissue was used instead, either kidney or lung. This enabled easy orientation of the TMA slides once sectioned and stained.

2.2.5 Tissue Array Construction

There are a number of different arraying machines commercially available. These range from manual to semi- and fully-automated systems. All of these have a number of components in common. The donor and recipient blocks are placed on a platform that secures them in place. Tissue cores are taken using a hollow punch containing a metal stylet. When a core is taken, the punch is driven into the tissue block with downward pressure, and with the stylet in an elevated position to allow the core of wax to enter the punch. The punch is removed from the tissue block and the stylet is then advanced into to the punch and pushes the core out. All arrayers also have a detailed X-Y precision guide, typically linked to a digital micrometer, enabling precise placement of cores in the recipient block according to a predefined grid pattern.

The general process of constructing the TMA is a cyclical one. First, a core of tissue is removed from the recipient block, with the X-Y guide in the zero position, and discarded. Then a core of tissue is removed from the donor block and inserted into the recipient hole. This process is repeated until the array has been filled. Each time the process is repeated, the X-axis micrometer is moved a pre-defined distance to ensure accurate spacing of the next core. When the first row is complete, the X-axis is reset to zero and the Y-axis is advanced to create a new row.

For this study, the Alphelys MiniCore® 3 tissue arrayer and MiniCore® Control Station controller software (Mitogen, UK, figure 2.1) was used. This is a semi-automated system in which the taking of and transfer of cores is performed manually using a control handle, and the X-Y position of the cores is determined automatically according to the predefined coordinates designated in the array design process. The Minicore® 3 has a rotating circular carousel with eight block positions. The punch is secured in a spring loaded mount which is positioned over block position 1. When cores are taken, the mount is pushed downwards into the paraffin block, and when pressure is released the spring raises the punch back to its starting position. In the Minicore® 3 it is the mount that moves in the X-Y plane, rather than the block platform. The mount also contains a camera that shows the block in position 1 on a laptop connected to the Minicore® 3. The camera also shows, via a mirror, the pathology number written on the cassette of the donor block, to ensure that the correct block is being used. On the side of the punch mount is a control handle that has three settings: (i) recipient – in this position the stylet is held out of the punch to allow a core of tissue to be removed from the recipient block; (ii) donor – again the stylet is held out of the punch to enable coring; (iii) transfer – when the control handle is rotated into this position, the stylet descends within the punch and pushes the tissue core out of the needle. The control handle also contains a "coring" button. When this is depressed, the punch rotates within the paraffin block and facilities the removal of a tissue core. This button is pressed every time a core is taken. The final components of the punch mount are the depth stop adjusters. These are two small magnets, one mounted adjacent to the punch, and the other mounted behind the control handle. These ensure a consistent depth of coring in both the donor and recipient blocks, and also help prevent damage to the punch from excessive downwards pressure.

The sequence for creating a TMA using this equipment is as follows (figures 2.2 and 2.3). Firstly the pre-designed tissue array is imported from the design software into the array construction software. This array design contains the details of all the blocks to be sampled, the number of cores from each block, and the designated position in the recipient block of each core. Next the camera and X-Y positioning are calibrated using a calibration block. Once the array design has been loaded and the camera is calibrated, the recipient block is loaded into position 1 on the carousel. The first seven donor blocks are then loaded into the remaining positions on the carousel. The camera takes a photo of the recipient block and the edges are then marked on the array software using crosshairs. This defines the zero X-Y position for the first core, and the limits of the TMA construction area.

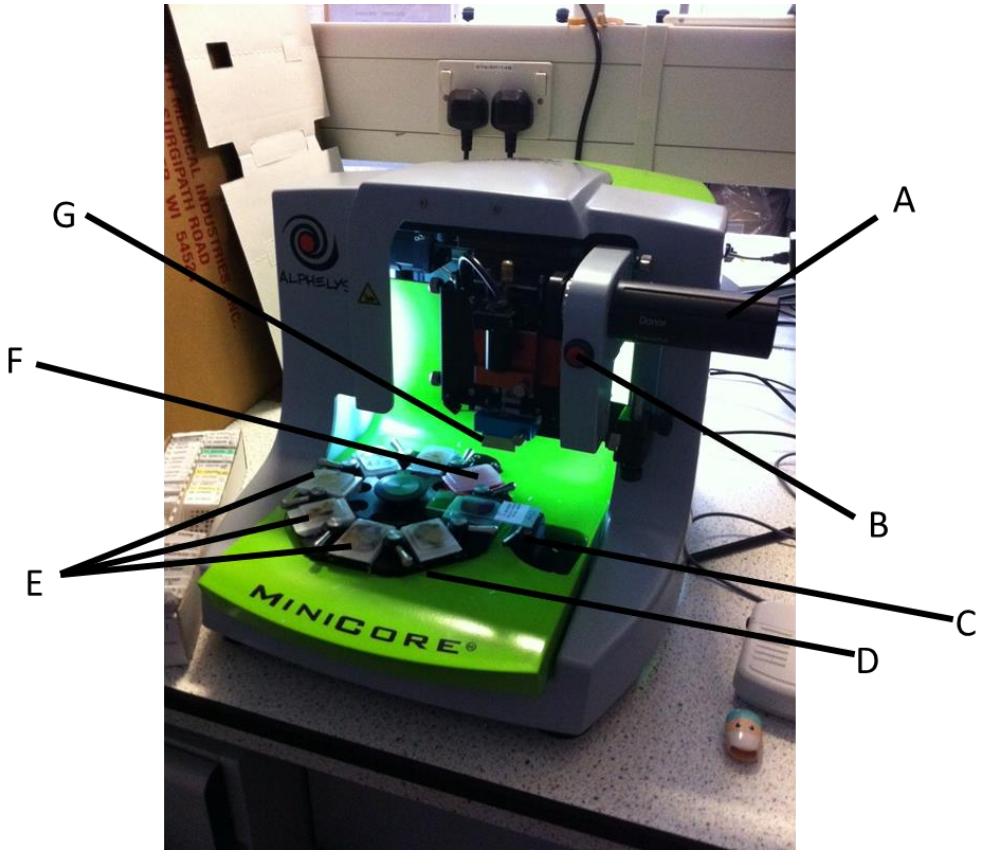


Figure 2. 1: The Alphelys Minicore 3 Tissue Arrayer. A: Control Handle B: Core Control Button C: Mirror D: Rotating Carousel E: Donor Blocks F: Recipient Block G: Coring Punch and Spacer

Next the carousel rotates so that the donor block in position 2 is under the camera. The H&E mapping slide with the core positions marked on it is placed on top of the block, and a photo taken. The core positions on the H&E slide are then marked with crosshairs and this defines where on the block the cores will be taken. This process is repeated until all 7 donor blocks have been marked on the array construction software.

When all donor blocks have been marked, the carousel rotates so that the recipient block is under the punch mount. With the control handle in the "recipient" position, the punch is driven into the recipient block and the "core" button depressed to take the first core, which is discarded. The carousel again rotates and the mount moves such that the first donor core location is under the punch. The same coring process is repeated, with the control handle in the "donor" position, and then the carousel rotates back to the recipient block. The core is transferred into the recipient block by depressing the mount and turning the handle to the "transfer" position. The camera then takes a photo of the recipient block to confirm that the core has been transferred successfully.

The mount then moves along the X-axis of the recipient block to the next defined core position, and a recipient core is again removed and discarded, before the second donor core is taken and transferred. This process is repeated until all donor cores have been taken and transferred to the recipient block, with the mount moving along the X and Y axes as appropriate to create the predefined array grid. The software then creates a final Excel spreadsheet that contains the alphanumeric location of each core, with the study number and pathology number of each location: This is used when scoring the TMAs (see section 2.5).

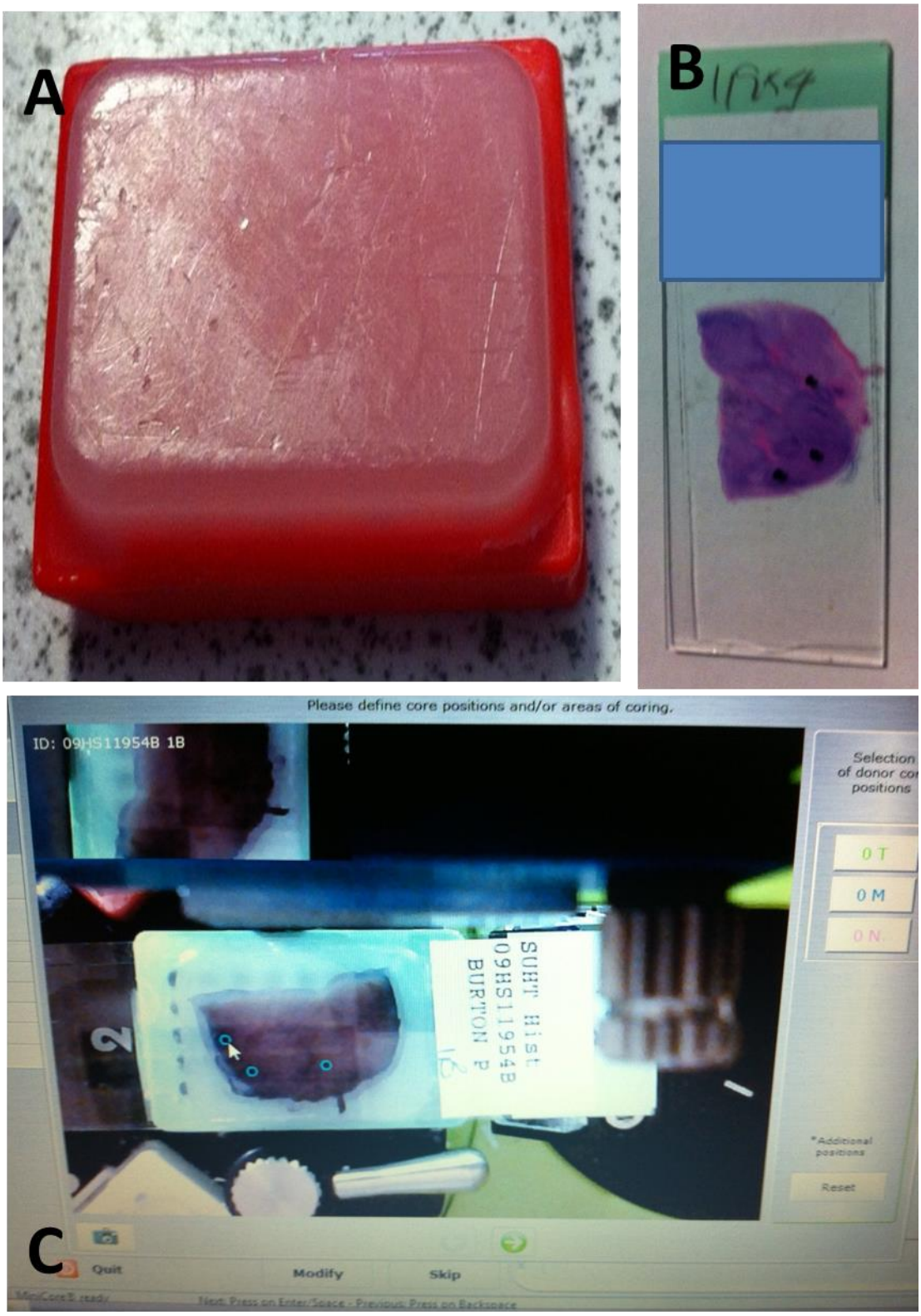


Figure 2. 2: Initial steps in TMA construction. A: An empty recipient block. B: A donor slide with the core locations marked (black dots). C: The donor slide is overlayed onto the donor block and the core positions marked on the TMA construction software

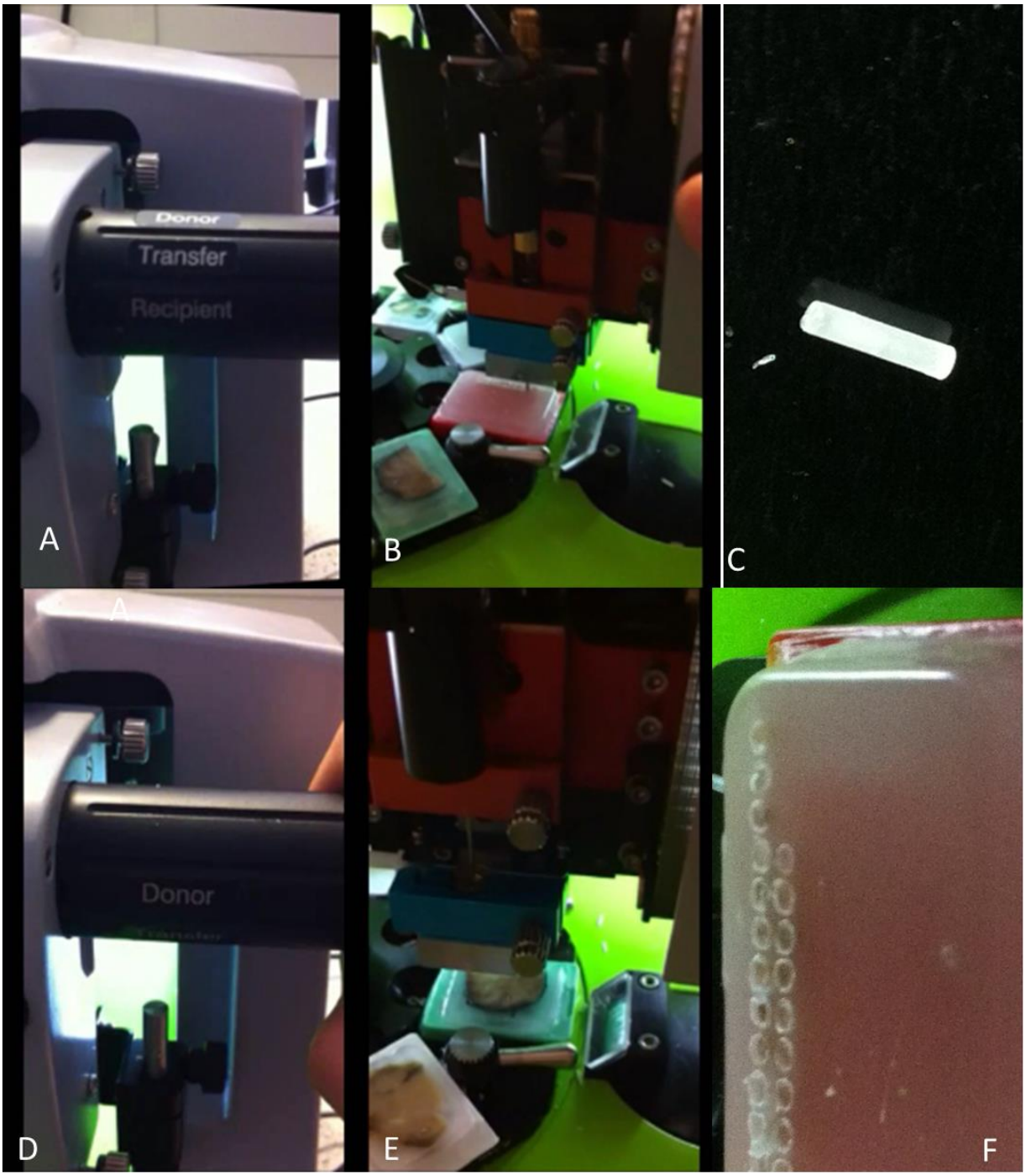


Figure 2. 3: TMA Construction. A: The control handle in "Recipient" position. B: Taking the recipient core. C: The core of wax taken from the recipient block (to be discarded). D: The control handle in “Donor” position. E: Taking the core from the donor block. F: A number of donor cores have been inserted into the recipient block

For the purposes of this study, 8 separate TMAs were created. The characteristics of these are shown in table 2.1. A small number of tumours (n=9) were inadvertently included in more than 1 array due to having multiple biopsies and thus a total of 290 tumours were arrayed in a total of 907 cores.

Array Name	Tumour Type	Number of Tumours	Total Number of Cores (not including orientation cores)
Tonsil 1	Tonsil	53	159
Tonsil 2	Tonsil	48	145
Oropharynx 1	Tongue base Other oropharynx	30	91
Oropharynx 2	Tongue base Other oropharynx	29	89
Oropharynx 3	Tongue base Other oropharynx	33	99
Poole Oropharynx 1	Tonsil Tongue base Other oropharynx	39	122
Poole Oropharynx 2	Tonsil Tongue base Other oropharynx	44	133
Bart's Oropharynx	Tonsil Tongue base Other oropharynx	23	69

Table 2. 1: The TMAs created during the course of this study

2.2.6 Array Tempering and Sectioning

After all donor cores have been transferred to the recipient block, the TMA needs to be heated gently and then cooled. This process, known as tempering, allows the wax to melt slightly and then harden again, and ensures that the tissue cores are securely held in place within the block. All TMAs were tempered at 37-40°C for 12 hours.

After tempering, the TMAs were cut into 4µm sections on a microtome by Toby Mellows, a member of the UHS histology department. A section of each array was stained with H&E to check for adequate tumour representation (figure 2.4), and subsequent sections were used for both IHC and ISH.

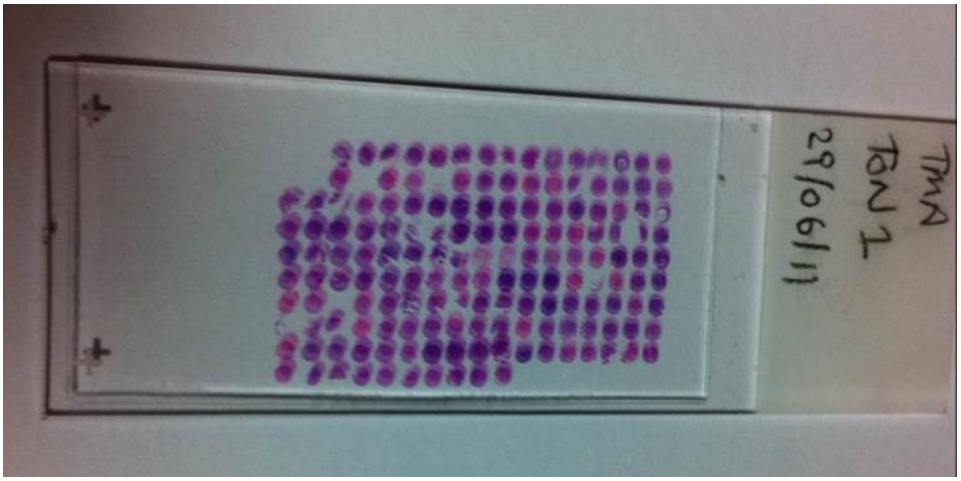


Figure 2. 4: An H&E strained section of TMA “Tonsil 1”. The orientation marking is visible in the upper left corner of the section.

2.3 Immunohistochemistry

The immunohistochemistry for this study was performed by Mr Toby Mellows, biomedical scientist in the cellular pathology department at UHS, a CPA-accredited clinical cellular pathology department using antibodies optimized to national diagnostic standards (NEQAS). A number of 4µm sections were cut from each TMA and mounted on Superfrost® Plus slides (Fisher Scientific, UK) then dried at 60°C for 20 minutes. All subsequent steps were performed on the Leica BOND-MAX™ automated stainer (Leica Microsystems, UK). This fully automated stainer is routinely used in diagnostic IHC in UHS, and was used to ensure consistency in staining results. All reagents come pre-mixed and were supplied by Leica (Leica Microsystems, UK).

Slides were placed in racks, covered with a cover tile and then loaded into the machine. The cover tile assists in the spreading of reagents over the surface of each slide, and allows smaller volumes of each reagent to be used. Sections were dewaxed using Bond™ Dewax Solution (Novocastra Reagents, Leica Microsystems, UK) and endogenous peroxidase activity blocked with 3% hydrogen peroxide for 10 minutes. Antigen retrieval was performed using either heat- or protein-induced epitope retrieval (HIER, PIER). HIER was performed using Bond™ Epitope Retrieval Solution 1 or 2 for 20 minutes at 98°C (Novocastra Reagents, Leica Microsystems, UK). Bond™ epitope retrieval solution 1 is a citrate based buffer and surfactant at pH 5.9-6.1, while Bond epitope retrieval solution 2 is an EDTA based buffer and surfactant at pH 8.9-9.1. PIER was performed using Bond™ Enzyme Pretreatment Kit (Novocastra Reagents, Leica Microsystems, UK) which contains a proteolytic enzyme concentrate and a Tris-buffered saline based diluent. One drop of concentrate was diluted in 7ml of diluent and slides were incubated at 37°C for 10 minutes.

Sections were incubated with primary antibody at 37°C for 20 minutes. All primary antibodies were diluted in Bond™ Primary Antibody Diluent (Novocastra Reagents, Leica Microsystems, UK). Optimal antibody concentrations and retrieval methods had been previously determined for antibodies that were in routine clinical practice (CD3, CD4, CD8, p16, α-SMA and CD1a). The remaining primary antibodies (Foxp3, MHC-1, EGFR and p53) were newly acquired for this study and were tested at varying concentrations and using different antigen retrieval techniques, which were then analysed and approved by Professor Thomas. The antibodies used, and their concentrations and antigen retrieval techniques are shown in table 2.2.

The detection and visualization of the primary antibodies was performed using the Bond™ Polymer Refine Detection kit (Novocastra Reagents, Leica Microsystems, UK). This kit consists of a post-primary “linker” rabbit anti mouse IgG antibody which is

used after the primary antibody. Sections were incubated with the post-primary antibody for 20 minutes at room temperature. Following this an anti-rabbit/polymer/HRP complex was added for 20 minutes at room temperature, which complexes with the linker antibody. The antibody detection was visualized with DAB (10 minutes at room temperature), which reacts with the HRP in the polymer to give a brown colour. Finally, the sections were counterstained with haematoxylin for 5 minutes and then dehydrated through graded alcohol and xylene before being coverslipped.

The antigenic targets chosen for IHC were as follows: p16 – a surrogate marker of HPV E7 expression; CD3 – pan T-Cell marker; CD4 – helper T-Cells; CD8 – cytotoxic T-Cells; Foxp3 – regulatory T-Cells; α-SMA – a myofibroblast marker; p53 – a cell cycle regulator; EGFR – a growth factor receptor; CD1a – a dendritic cell marker; MHC class 1 – a cell surface receptor involved in antigen presentation.

In some cases, tumour cores were missing from the sectioned slides, or there was insufficient tumour tissue remaining in the archival block for analysis. The total number of cases successfully stained for each antibody was as follows: p16 287 (99.0%), p53 269 (92.8%), EGFR 271 (93.4%), MHC-1 269 (92.8%), CD1a 274 (94.5%), SMA 276 (95.2%), CD3 279 (96.2%), CD4 272 (93.8%), CD8 279 (96.2%), and Foxp3 276 (95.2%). The results for each individual marker are discussed in the appropriate section throughout this thesis.

Antibody	Clone	Manufacturer	Concentration	Antigen Retrieval Technique
CD3	LN10	Novocastra	1:200	Bond Epitope Retrieval Solution 2
CD4	1F6	Novocastra	1:50	Bond Epitope Retrieval Solution 2
CD8	1A5	Novocastra	1:50	Bond Epitope Retrieval Solution 2
Foxp3	236A/E7	Ebioscience	1:20	Bond Epitope Retrieval Solution 2
α-SMA	αsm-1	Novocastra	1:50	Bond Epitope Retrieval Solution 1
P53	DO-7	DAKO Cytomation	1:50	Bond Epitope Retrieval Solution 1
EGFR	E30	DAKO Cytomation	1:50	Bond Enzyme Pre-treatment
CD1a	MTB1	Novocastra	1:60	Bond Epitope Retrieval Solution 2
MHC-1	EP1395Y	Abcam	1:500	Bond Epitope Retrieval Solution 2
P16	ink4a	CINtec	1:3	Bond Epitope Retrieval Solution 2

Table 2. 2: The antibodies used for immunohistochemistry in this study

2.4 In-Situ Hybridisation

All in situ hybridization in this study was performed manually by myself. As with IHC, 4µm sections of each FFPE TMA were mounted on Superfrost® Plus slides (Fisher Scientific, UK) and dried in a 60°C oven for 20 minutes. Initially samples were dewaxed in 2 washes of xylene for 3 minutes each. Following this, sections were hydrated through graded alcohol (100% x2, 70% x1) and distilled water for 2 minutes each. Pretreatment was performed with Target Retrieval Solution (Dako, UK), a citrate based solution at pH 6.0, in a water bath set at 95°C for 40 minutes, and then with Proteinase K (1:5000, Dako, UK) for 1 minute. Following pretreatment, sections were dehydrated through graded alcohol and left to air dry prior to adding the ISH probe. This was to prevent water from diluting the probe, and from interfering with probe-DNA interactions. Once dry, sections were incubated with Genpoint™ HPV DNA Probe Cocktail (Dako, UK). The Genpoint™ HPV DNA Biotinylated Probe Cocktail contains genomic clones of 13 high risk HPV types (including HPV-16 and 18) in the form of 500 base pair double stranded fragments. These fragments are biotinylated to allow detection by the Genpoint™ Tyramide Signal Amplification system (Dako, UK). Firstly, a denaturation step was performed at 92°C for 5 minutes, and this was followed by hybridization at 37°C for 20 hours. These steps were performed on a dedicated hybridizer, which consists of a highly accurate automated hotplate that is maintained in a humid environment (Dako, UK).

Following hybridisation, unbound probe was removed with Stringent Wash Solution (Dako, UK) at 55°C for 20 minutes then endogenous peroxidase activity blocked with 0.3% hydrogen peroxide for 10 minutes at room temperature. All subsequent detection steps were performed using the Genpoint™ Tyramide Signal Amplification System for Biotinylated Probes (Dako, UK). Firstly, slides were incubated with Primary Streptavidin-HRP for 30 minutes. The streptavidin binds with high affinity to the biotin that is conjugated to the HPV probe. Biotinyl tyramide is then added for 15 minutes as an amplification step. The HRP from the previous step catalyzes the oxidation of the biotinyl tyramide and results in the deposition of large quantities of biotin at the site of hybridization. A secondary streptavidin-HRP conjugate was then added for 15 minutes, and again the streptavidin binds to the biotin. Following this, staining was developed with DAB for 5 minutes and sections were counterstained with Haematoxylin. Slides were then dehydrated through graded alcohol (70% x1, 100% x2) and xylene for 2 minutes each before coverslipping. HPV *in-situ* hybridization was successfully performed in 287 cases (99.0%).

2.5 TMA Scoring

After all TMAs had been sectioned and stained, each antibody and probe was individually scored and the results recorded on the Excel Spreadsheet produced by the TMADesigner® 2 software. Initially, an H&E stained section of each TMA was examined by Professor Thomas and the presence or absence of tumour in each core was noted. For the various T-cell markers, a mean count per tumour was recorded (described below) while the remaining antibodies, and the HPV ISH, were scored by Professor Thomas on either a binary (i.e. positive vs. negative) or semi-quantitative basis. All scoring was performed on a Zeiss AxioCam MRc5 microscope. The scoring systems for each stain are described below.

HPV in situ hybridisation was recorded as positive or negative based on the presence of nuclear staining in tumour cells. The Genpoint HPV probe identifies the 13 most prevalent high risk HPV genotypes, including HPV 16 and 18 and allows for identification of 1-2 copies of viral DNA per cell nucleus. Integrated DNA is visualised as punctate nuclear staining, whilst episomal DNA is seen as diffuse nuclear staining. Therefore the nature of staining in positive tumours was also noted.

Staining for p16 was scored as negative or positive (strong staining in >70% of the tumour cells; (429)). Only patients in whom tumours were positive for both HPV ISH and p16 IHC were deemed to have HPV driven tumours, in keeping with recently proposed protocols (341, 347, 349).

Staining of EGFR, MHC class-1, SMA and CD1a was scored using a semi-quantitative scale described previously (156), as negative/low, moderate or high. EGFR staining was subsequently summarised as either negative/low or positive (moderate/high). p53 staining was scored as positive or negative, where negative tumours were those with fewer than 10% of tumour cells staining positive. Once all staining results had been recorded against the appropriate tumour in the TMA spreadsheet, they were transferred into the tumour database.

For the four T-cell markers an average count per high power field (x400) was established. The counts were performed using the Zeiss AxioCam MRc5 microscope and Zeiss Axiovision software (version 4·8·1·0). Groups of tissue cores belonging to each tumour were initially examined at x100 magnification and 3 representative areas of staining were chosen. These areas were then photographed at x400 (x200 magnification on the microscope and x2 on the camera) using the Axiovision software. The number of positive cells in each of these images was then counted using the "count events" function in the Axiovision software (nested under

measurement→interactive measurement→events) and recorded in the TMA spreadsheet. Only staining within tumour islands was counted, and staining in stromal tissues was ignored. A mean of the three counts was then calculated and recorded in the tumour database. The first TMA was scored in conjunction with Professor Thomas to ensure that only tumour islands were included. For subsequent TMAs scoring was performed solely by myself.

2.6 Statistical Analysis

Statistical analysis was performed with the advice of Dr Scott Harris, medical statistician at UHS. Relationships between categorical variables were assessed by cross tabulation and the use of either Fischer's exact test (for 2x2 tables) or Chi-squared test (for all other tables). Continuous variables were compared using the independent T-test for normally distributed data (e.g. age at diagnosis), and the Mann-Whitney U test for non-normally distributed data (e.g. follow up time).

Survival outcomes between variables were compared using Kaplan Meier analysis with log rank tests, and both Univariate and Multivariate Cox Proportional Hazards regression. Patients who did not receive any treatment, who underwent treatment with palliative intent (n=26), or who had follow up of less than 6 months were excluded from all survival and outcome analysis. The adjustments made for multivariate models are described in the individual results chapters. Overall survival was measured as the time between diagnosis of malignancy and death from any cause. Patients who were alive at the time of last follow up were censored at this time-point. Disease specific survival was measured as the time between diagnosis of malignancy and death from oropharyngeal cancer. Patients who were still alive at the time of last follow up, or who died for another cause, were censored at this time-point.

To evaluate the prognostic performance of relevant factors (i.e. the 3-year OPSCC death rate), the detection rate (DR; also known as sensitivity) and false-positive rate (FPR; also known as 1 minus specificity) were calculated. DR is the proportion of patients who died from OPSCC with marker-positive results; FPR is the proportion of patients who did not die from OPSCC with marker-positive results. Likelihood ratios (DR/FPR) were obtained, which indicate the strength of a marker (i.e. maximizing the DR whilst minimizing the FPR) (430).

A prognostic model for 3-year mortality in HPV-positive OPSCC was developed using binary logistic regression, with a backwards selection procedure (431). The initial variables entered into the model were: age, T-stage, N-stage, grade, HPV-status, TIL-level, alcohol history, p53, EGFR and smoking history. Variables were retained in the model if they were significant at the 5% level. The UHS cohort was used as the 'training' set to develop the model, with the PFT/BLT cohort acting as the 'validation' set. A ROC curve of the linear predictor was used to identify the optimum cut-point (balancing both sensitivity and specificity) (431). The prognostic ability of the final model was then assessed through the calculation of DR and FPR values in the 'validation' set (432). This model is presented in chapter 4.

All statistical tests were two-sided and a p-value of 0.05 taken as significant. All statistical analysis was performed by myself using SPSS for Windows version 19.0 (IBM, Portsmouth, UK).

3 HPV and Oropharyngeal Cancer

3.1 Introduction

HPV-positive oropharyngeal cancer is a rapidly increasing health problem across the western world and to a lesser extent, the developing world. There is now compelling evidence in the literature to show that HPV-positive tumours represent a separate disease entity with different epidemiological, clinical and biological features compared to HPV-negative OPSCC (5). While both HPV-negative and HPV-positive tumours predominantly affect men, HPV-positive tumours are typically diagnosed at a younger age than HPV-negative tumours (312). There are different risk factors for HPV-positive and HPV-negative OPSCC. HPV-negative OPSCC is strongly associated with heavy smoking and high levels of alcohol consumption, while HPV-positive tumours are more likely to occur in patients without a significant history of smoking or alcohol. Furthermore, HPV-positive OPSCC correlates significantly with high risk sexual practices, including multiple sexual partners and specifically, increasing numbers of oral sexual partners (303). HPV-positive tumours tend to present at an advanced disease stage, often due to the presence of bulky nodal disease, and are typically poorly differentiated (374). In addition, HPV-positive OPSCC is associated with a lower expression of p53 by tumour cells due to the actions of the viral oncoprotein E6 (221, 293).

Histological features can predict poor survival in HNSCC: These include poor differentiation, discohesive invasive margin, the presence of perineural and intravascular tumour spread, and extracapsular spread from tumour involved lymph nodes (433). It is well established that HPV-positive OPSCC is typically poorly differentiated (434). Despite this poor differentiation and also often presenting at an advanced disease stage, HPV-positive tumours consistently have improved survival outcomes, with a reduction in the risk of death of up to 60% compared to HPV-negative disease (100). It is possible that other histological features of HPV-positive tumour may confer a prognostic benefit: However, the relationship between HPV-status and these other histological markers is not well described.

3.2 Aims

The primary aim of this chapter is to compare the clinical and histological features of HPV-positive and HPV-negative tumours, and to assess the effect of these features on survival.

3.3 Objectives

The objectives of this chapter are:

- To describe the characteristics of the dataset.
- To create tissue microarrays of archival material.
- To establish rates of HPV-positive tumours in the cohort.
- To establish associations between HPV-status and clinicopathological features.
- To establish the effect of HPV on survival.
- To establish the effects of traditional histological features on survival.

3.4 Results

3.4.1 Characteristics of the Dataset

A total of 442 oropharyngeal cancers were included in the dataset. The demographics of these tumours are shown in tables 3.1 – 3.3. For the majority of analyses in this thesis, the entire dataset is treated as one cohort, however at times the UHS cohort and the PFT/BLT cohorts are compared, and thus the characteristics of these cohorts are separated and shown in appendix 3. Pathology samples for TMA creation and full histological assessment were available for 290 patients (UHS n=187, PFT/BLT n=103). The demographics of those with and without archival material available were broadly similar, and are shown in the appendix 3.

3.4.2 Patient Demographics and Risk Factors

Patient details are shown in table 3.1. In keeping with the published literature, males outnumbered females by a ratio of approximately 3:1, with a mean age of diagnosis of 59.2 years (SD 11.3). The majority of patients had some history of tobacco exposure, with approximately 50% of patients being current smokers at the time of diagnosis, while a further 20% were ex-smokers. For the smoking summary variable used in the majority of analyses, approximately half of the patients fell into the "less than 10 pack years" group and half into the "greater than 10 pack years". The vast majority of patients had some history of alcohol exposure, though unfortunately the level of consumption was generally poorly recorded.

		Entire Cohort N=442
		Number (%)
Hospital	*UHS*	316 (71.5)
	PFT	102 (23.1)
	BLT	24 (5.4)
Age	*<50*	86 (19.5)
	50-69	268 (60.6)
	>70	88 (19.9)
	Mean (SD)	59.2 (11.3)
Gender	*Male*	325 (73.5)
	Female	117 (26.5)
Smoking	*Never Smoked*	81 (19.3)
	Current Light	23 (5.2)
	Current Heavy	184 (41.6)
	Ex-smoker	95 (21.5)
	Not Known	59 (13.3)
Smoking (according to 10 pack year cut off)	*<10 pack years*	199 (45.0)
	>10 pack years	184 (41.6)
	Not Known	59 (13.3)
Alcohol	*Non Drinker*	35 (7.9)
	<10u/week	103 (23.3)
	10-20u/week	50 (11.3)
	>20u/week	118 (26.7)
	Ex-drinker	26 (5.9)
	Not Known	110 (24.9)

Table 3. 1: Patient Characteristics of the entire cohort

3.4.3 Tumour Characteristics

The tumour characteristics are shown in table 3.2, and summarised below.

3.4.3.1 Tumour Site

The majority of tumours were located in the palatine tonsils or the tongue base, with only 20% of tumours being found outside of these sites. The details of these "other oropharyngeal" tumours are shown in appendix 3.

3.4.3.2 Synchronous Primaries and Distant Metastatic Disease

Synchronous primary tumours, i.e. diagnosed at the same time as the primary, were identified in just 3.4% of cases (n=15), while distant metastatic disease was found in less than 2% of cases (n=7). There was no difference in either the rate of synchronous primaries, or the rate of distant metastases, according to primary site. Synchronous primary tumours were found in 4.1% of tonsillar tumours, 3.5% of base of tongue tumours, and 2.4% of "other oropharyngeal tumours" (p=0.76). The corresponding rates of distant metastasis were 1.4%, 2.7% and 2.4% (p=0.83).

3.4.3.3 TNM Staging

There was a reasonably even spread of T-stage across the cohort. In contrast, the majority of patients presented with nodal metastases, with less than a quarter being node negative at the time of diagnosis. Correspondingly, most patients in the study had advanced stage disease (i.e. AJCC stage III or IV) at the time of diagnosis, with only 16% having early stage tumours.

3.4.3.4 Histological Features

Full histological assessment was possible in the 290 tumours for which archival material was available. The histological features of tumours without available tissues were obtained from pathology reports. Thus, there is relatively wide variation in the number of tumours for which information was available for different histological features, depending on the information on the pathology reports and tissue availability. These features are shown in table 3.3.

Interestingly, more than half of all tumours were either poorly or undifferentiated, while only a third showed moderate differentiation. For those tumours with data available; the majority had a cohesive invasive margin, while perineural and intravascular spread were rare.

In the majority of cases, the resection margin was clear (58.0% of surgically treated patients), while close and involved margins were found in 18% and 15% of cases, respectively. Interestingly, the status of the resection margin was not reported in 13 (8.7%) surgically treated tumours. Dysplasia was found at the resection margin of 15 tumours (10% of those treated surgically). In over half of the surgically treated tumours, the presence or absence of dysplasia at the resection margin was not reported.

3.4.3.5 Patient follow up and outcomes

Patient outcomes are summarised in table 3.4. Treatment failure (i.e. evidence of residual disease at the end of treatment) was seen in less than 10% of patients, while late recurrence was seen in approximately 15% of cases. Distant metastatic disease and second primary tumours were both seen in approximately 8% of patients. In total, 125 patients (28.2% of total cohort) had residual, recurrent, or metastatic disease over the course of the study.

There were a total of 196 deaths over the time period encompassed by the study, of which 140 were due to OPSCC. In 6 cases (1.4%) it was not possible to ascertain whether the patient was still alive or not, whilst in 11 cases the exact cause of death was unknown, despite contacting the GP and the Cancer Registry. The 3-year survival rate was 63%, while the 5-year survival rate was 45.5%. The median follow up time was 49 months, with a minimum of 2 months and a maximum of 137.

		Entire Cohort N=442
		Number (%)
Tumour Site	*Tonsil*	236 (53.4)
	Base of Tongue	118 (26.7)
	Other Oropharynx	88 (19.9)
Tumour Side	*Left*	179 (40.5)
	Right	209 (47.3)
	Midline/Bilateral	29 (6.6)
	Not Documented	25 (5.6)
Synchronous Primary Tumour	*Yes*	15 (3.4)
	No	403 (91.2)
	Not Known	24 (5.4)
Disease Stage	*I*	31 (7.0)
	II	41 (9.3)
	III	61 (13.8)
	IV	305 (69.0)
	Not Known	4 (0.9)
T-Stage	*T1*	109 (24.7)
	T2	148 (33.5)
	T3	56 (12.7)
	T4	120 (27.1)
	Not Known	9 (2.0)
Nodal Metastases	*No*	104 (23.5)
	Yes	334 (75.6)
	Not Known	4 (0.9)
N-Stage	*N0*	104 (23.5)
	N1	61 (13.8)
	N2a	52 (11.8)
	N2b	127 (28.7)
	N2c	72 (16.3)
	N3	22 (5.0)
	Not Known	4 (0.9)
Distant Metastases at Presentation	*Yes*	7 (1.6)
	No	409 (92.5)
	Not Known	26 (5.9)

Table 3. 2: Tumour Characteristics of the entire cohort

	Entire Cohort N=442 Number (%)	
Tumour Grade	*Well differentiated*	13 (2.9)
	Moderately differentiated	155 (35.1)
	Poorly Differentiated	247 (55.9)
	Undifferentiated	3 (0.7)
	Not Known	24 (5.4)
Cohesion	*Cohesive*	176 (39.8)
	Discohesive	124 (28.1)
	Not Known	142 (32.1)
Perineural Spread	*No*	274 (62.0)
	Yes	14 (3.2)
	Not Known	154 (34.8)
Intravascular Spread	*No*	286 (64.7)
	Yes	23 (5.2)
	Not Known	133 (30.1)
	Patients Treated Surgically N=150 Number (%)	
Resection Margin	*Clear*	87 (58.0)
	Close (<5mm)	27 (18.0)
	Involved	23 (15.3)
	Not Known	13 (8.7)
Dysplasia at Resection Margin	*No*	66 (44.0)
	Yes	15 (10.0)
	Not Known	69 (46.0)
	Patients Undergoing Primary Neck Dissection N=100 Number (%)	
Extracapsular Spread	*No*	36 (36.0)
	Yes	54 (54.0)
	Not Known	10 (10.0)

Table 3. 3: Histological Features of the entire cohort

		Entire Cohort N=442
		Number (%)
Treatment Failure	*No*	323 (73.1)
	Yes	43 (9.7)
	Not Applicable	28 (6.3)
	Not Known	48 (10.9)
Late Recurrence	*No*	278 (62.9)
	Yes	68 (15.4)
	Not Applicable	44 (10.0)
	Not Known	52 (11.8)
Second Primary	*No*	340 (76.9)
	Yes	36 (8.1)
	Not Applicable	13 (2.9)
	Not Known	53 (12.0)
Distant Metastases	*No*	341 (77.1)
	Yes	33 (7.5)
	Not Applicable	17 (3.8)
	Not Known	51 (11.5)
Failure, Recurrence or Distant Metastases	*No*	257 (58.1)
	Yes	125 (28.3)
	Not Known	60 (13.6)
Life Status (at time of last follow up)	*Dead*	196 (44.3)
	Alive	240 (54.3)
	Not Known	6 (1.4)
Cause of Death	*Died from OPSCC*	140 (31.7)
	Still alive/Other cause of death	291 (65.8)
	Not Known	11 (2.5)
3-year Survival (known for 316 patients)	*Dead*	117 (37.0)
	Alive	199 (63.0)
5-year Survival (known for 246 patients)	*Dead*	134 (54.4)
	Alive	112 (45.5)

Table 3. 4: Outcomes of the entire cohort

3.4.4 Diagnosis of HPV-positive tumours

As discussed previously, tumours were only considered HPV-driven if they were positive for both p16 IHC and HPV ISH. A total of 153 tumours were positive for both p16 and HPV (table 3.5), and were deemed to be truly HPV-positive (52.8% of those with tissues available). Of those tumours that were p16 positive, 93.9% were HPV ISH positive, while 6.1% were HPV ISH negative. Of the tumours that were HPV ISH positive, 89% were p16 positive, and 11% p16 negative.

		Tumours with available tissue N=290 Number (%)
P16 IHC	*Negative*	124 (42.8)
	Positive	163 (56.2)
	Not Known	3 (1.0)
HPV ISH	*Negative*	114 (39.3)
	Positive	173 (59.7)
	Not Known	3 (1.0)
Final HPV Status	*Negative*	133 (45.9)
	Positive	153 (52.8)
	Not Known	4 (1.4)

Table 3. 5: Results of staining for HPV markers

3.4.5 Associations between HPV-status and demographic details

The associations between HPV-status and important clinicopathological variables are shown in table 3.6. There was no difference in the gender distribution of HPV-negative and HPV-positive tumours (p=0.35). HPV-positive tumours were more likely to be diagnosed in younger patients, with 26.1% of patients under the age of 50 at diagnosis compared to only 13.5% of those with HPV-negative tumours (p=0.005). The mean age of diagnosis for HPV-positive tumours was 56.9 years, compared to 61.1 years for HPV-negative tumours (p=0.002).

3.4.6 Risk factors for HPV-positive and HPV-negative tumours

Patients with HPV-negative tumours were more likely to have significant tobacco exposure than those with HPV-positive tumours. Only 7.5% of those with HPV-negative tumours were “never smokers”, compared to nearly 30% of those with HPV-positive

tumours (p<0.001). When classified according to the 10 pack year cut off, only 30.6% of patients with HPV-positive tumours were in the heavy smoking group (current smoker >10 pack years), compared to 67% of those with HPV-negative tumours (p<0.001). There was no difference in the number of patients who were current drinkers at the time of diagnosis (HPV-positive 82.8%, HPV-negative 82.3%, p=1.00), however patients with HPV-negative tumours were more likely to be heavy consumers of alcohol, with 56.3% of patients consuming greater than 20 units per week compared to just 19.8% of HPV-positive patients (p<0.001).

3.4.7 Tumour Characteristics – the effect of HPV-status

HPV-positive tumours were more likely to occur in the tonsil or base of tongue, with only 7.2% of tumours occurring outside of these sites, compared to 29.3% of HPV-negative tumours (p<0.001). Rates of synchronous primary tumours and distant metastases did not differ according to HPV-status (p=0.19 and p=0.32). HPV-positive tumours were more likely to present at an advanced overall TNM stage (stage III/IV: HPV-positive 92.8%, HPV-negative 67.7%, p<0.001) and to have nodal metastases at presentation (HPV-positive 89.5%, HPV-negative 59.4%, p<0.001). However, there was no difference in T-stage at presentation according to HPV-status (p=0.40).

HPV-positive tumours were more likely to be poorly differentiated (76.5%) than those that were HPV-negative (43.6%, p<0.001), and to have a cohesive invasive margin (HPV-positive 69.9%, HPV-negative 46.6%, p<0.001). There was no difference in the rates of perineural or perivascular spread according to HPV-status (p=0.30 and p=1.00, respectively), and no difference in the rates of involved resection margins, dysplasia at the resection margin, or extra-capsular spread (p=0.63, p=0.30, and p=0.13). HPV-positive tumours were less likely to express p53 than those that were HPV-negative and also had lower expression of EGFR (p=0.003 and p<0.001).

		HPV-positive N=153 Number (%)	HPV-negative N=133 Number (%)	P-value
Gender	*Male*	115 (75.2)	93 (69.9)	0.35
	Female	38 (24.8)	40 (30.1)	
Age at diagnosis	*<50*	40 (26.1)	18 (13.5)	0.005
	50-69	92 (60.1)	81 (60.9)	
	>70	21 (13.7)	34 (25.6)	
	Mean (SD)	56.9 (11.2)	61.1 (11.9)	0.002
Smoking	*Never Smoked*	44 (28.8)	10 (7.5)	<0.001
	Current Light	10 (6.5)	8 (6.0)	
	Current Heavy	41 (26.8)	77 (57.9)	
	Ex-Smoker	39 (25.5)	20 (15.0)	
Smoking according to 10 pack year cut off	*<10 pack years*	93 (60.8)	38 (28.6)	<0.001
	>10 pack years	41 (26.8)	77 (57.9)	
	Not Known	19 (12.4)	18 (13.5)	
Alcohol	*Never*	17 (11.1)	9 (6.8)	<0.001
	<10u/week	53 (34.6)	14 (10.5)	
	10-20u/week	20 (13.1)	11 (8.3)	
	>20u/week	23 (15.0)	54 (40.6)	
	Ex-drinker	3 (2.0)	8 (6.0)	
	Not Known	37 (24.2)	37 (27.8)	
Tumour Site	*Tonsil*	101 (66.0)	59 (44.4)	<0.001
	Base of Tongue	41 (26.8)	35 (26.3)	
	Other Oropharynx	11 (7.2)	39 (29.3)	
Synchronous Primary	*No*	145 (94.8)	111 (83.5)	0.19
	Yes	3 (2.0)	6 (4.5)	
	Not Known	5 (3.3)	16 (12.0)	
Distant Metastases (at presentation)	*No*	146 (95.4)	113 (85.0)	0.32
	Yes	1 (0.7)	3 (2.3)	
	Not Known	6 (3.9)	17 (12.8)	
T-Stage	*T1*	43 (28.2)	35 (26.4)	0.40
	T2	66 (43.1)	45 (33.8)	
	T3	14 (9.2)	15 (11.3)	
	T4	30 (19.6)	37 (27.8)	
	Not Known	0 (0.0)	1 (0.8)	
Nodal Metastases	*No*	15 (9.8)	53 (39.8)	<0.001
	Yes	137 (89.5)	79 (59.4)	
	Not Known	1 (0.7)	1 (0.8)	
N-Stage	*N0*	15 (9.8)	53 (39.8)	<0.001
	N1	23 (15.0)	16 (12.0)	
	N2a	31 (20.3)	8 (6.0)	
	N2b	55 (35.9)	31 (23.3)	
	N2c	20 (13.1)	18 (13.5)	
	N3	8 (5.2)	6 (4.5)	
	Not Known	1 (0.7)	1 (0.8)	

Table 3. 6: Clinicopathological features of the cohort according to HPV-status

		HPV-positive N=153 Number (%)	HPV-negative N=133 Number (%)	P-value
Disease Stage	*I*	2 (1.3)	20 (15.0)	<0.001
	II	8 (5.2)	22 (16.5)	
	III	21 (13.7)	17 (12.8)	
	IV	121 (79.1)	73 (54.9)	
	Not Known	1 (0.7)	1 (0.8)	
Tumour Grade	*Well Differentiated*	2 (1.4)	1 (0.8)	<0.001
	Moderately Differentiated	32 (20.9)	74 (55.6)	
	Poorly Differentiated	117 (76.5)	58 (43.6)	
	Undifferentiated	2 (1.3)	0 (0.0)	
Invasive Margin	*Cohesive*	107 (69.9)	61 (45.9)	<0.001
	Discohesive	46 (30.1)	70 (52.6)	
	Not Known	2 (1.5)	0 (0.0)	
Perineural Spread	*No*	139 (90.8)	115 (86.5)	0.30
	Yes	6 (3.9)	2 (1.5)	
	Not Known	8 (5.2)	16 (12.0)	
Intravascular Spread	*No*	137 (89.5)	110 (82.7)	1.00
	Yes	9 (5.9)	7 (5.3)	
	Not Known	7 (4.6)	16 (12.0)	
P53 status	*Negative*	76 (49.7)	43 (32.3)	0.003
	Positive	68 (44.4)	82 (61.7)	
	Not Known	9 (5.9)	8 (6.0)	
EGFR Status	*Negative*	79 (51.6)	34 (25.6)	<0.001
	Positive	64 (41.8)	91 (68.4)	
	Not Known	10 (6.5)	8 (6.0)	
Patients undergoing surgical treatment		**N=60**	**N=57**	
Margin Status	*Clear*	31 (51.7)	35 (61.4)	0.63
	Close	14 (23.3)	10 (17.5)	
	Involved	11 (18.3)	9 (15.8)	
	Not Known	4 (6.7)	3 (5.3)	
Dysplasia at Margin	*No*	33 (55.0)	19 (33.3)	0.30
	Yes	4 (6.7)	6 (10.5)	
	Not Known	23 (38.3)	32 (56.1)	
Patients undergoing neck dissection		**N=54**	**N=25**	
Extracapsular Spread	*No*	24 (44.4)	7 (28.0)	0.13
	Yes	25 (46.3)	18 (72.0)	
	Not Known	5 (9.3)	0 (0.0)	

Table 3.6 continued

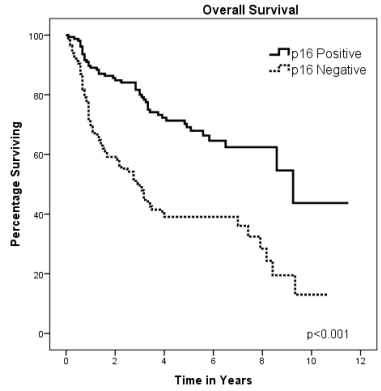
3.4.8 The effect of HPV-status on treatment outcomes and survival

On Kaplan Meier analysis, tumours that were positive for either p16, HPV ISH or both had improved overall and disease specific survival compared to those that were negative (all $p<0.001$, figure 3.1). This was reflected on cox regression analysis, shown in table 3.7. Although both p16 and HPV ISH were predictive of survival in isolation, the combination of the two had the greatest prognostic ability. When used in combination, tumours that were positive for p16 IHC and HPV ISH, i.e. those tumours that were deemed to be truly HPV-driven, had a 63% reduction in the risk of all-cause death, and a 67% reduction in the risk of death from oropharyngeal cancer (HRs: OS 0.37, $p<0.001$; DSS 0.33, $p<0.001$).

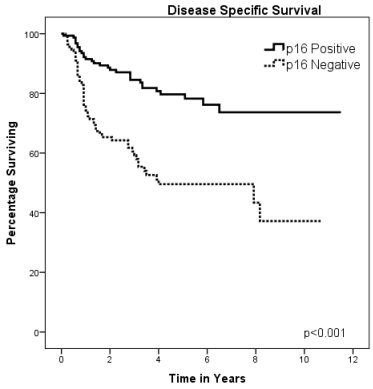
Interestingly, p16 status stratified both overall and disease specific survival in tumours that were HPV ISH positive. HPV ISH positive tumours that were also p16 positive had a 62% reduction in the risk of all-cause death, and a 63% reduction in the risk of tumour specific death compared to those that were HPV ISH positive but p16 negative (HRs: OS 0.38, $p=0.009$; DSS 0.37, $p=0.02$).

		Overall Survival		Disease Specific Survival	
		HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
p16 status	*Negative*	1		1	
	Positive	0.38 (0.26-0.54)	<0.001	0.34 (0.21-0.53)	<0.001
HPV ISH status	*Negative*	1		1	
	Positive	0.42 (0.29-0.61)	<0.001	0.38 (0.24-0.59)	<0.001
Final HPV status	*Negative*	1		1	
	Positive	0.37 (0.26-0.54)	<0.001	0.33 (0.21-0.53)	<0.001

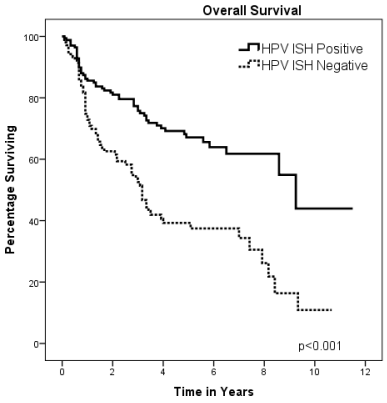
Table 3. 7: Hazard ratios for Overall and Disease Specific Survival from OPSCC according to p16-status, HPV ISH-status and Final HPV status



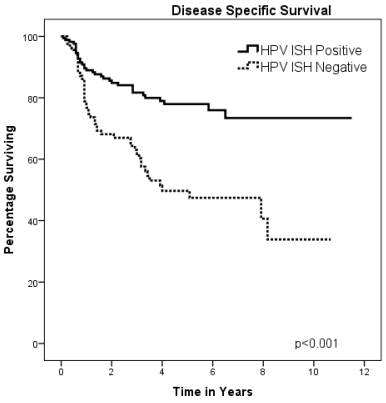
Time	0	2	4	6	8	10
p16 positive	156	116	76	33	12	2
p16 negative	115	61	33	18	7	2



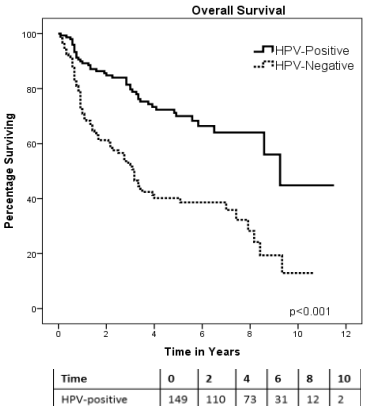
Time	0	2	4	6	8	10
p16 positive	156	116	76	33	12	2
p16 negative	115	61	33	18	7	2



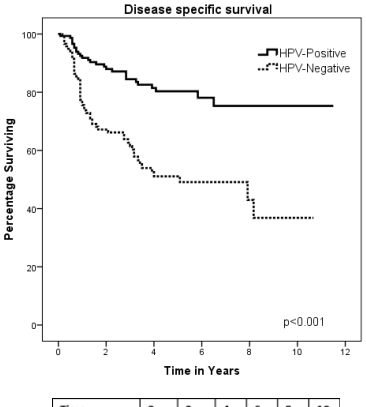
Time	0	2	4	6	8	10
HPV ISH positive	167	119	79	34	13	2
HPV ISH negative	104	58	30	17	6	2



Time	0	2	4	6	8	10
HPV ISH positive	167	119	79	34	13	2
HPV ISH negative	104	58	30	17	6	2



Time	0	2	4	6	8	10
HPV-positive	149	110	73	31	12	2
HPV-negative	121	67	36	20	7	2



Time	0	2	4	6	8	10
HPV-positive	149	110	73	31	12	2
HPV-negative	121	67	36	20	7	2

Figure 3. 1: Kaplan Meier survival curves for Overall and Disease Specific Survival from OPSCC according to p16-status, HPV ISH-status and Final HPV Status

A number of other treatment outcomes are shown in table 3.8. Patients with HPV-driven tumours had a significant reduction in the rates of residual disease following treatment (HPV-positive 6.7%, HPV-negative 12.3%, $p=0.05$), significantly lower levels of tumour recurrence (HPV-positive 8.7%, HPV-negative 27.0%, $p<0.001$), and a significant reduction in distant metastasis (HPV-positive 4.0%, HPV-negative 9.8%, $p=0.02$). When considered together, residual, recurrent, or metastatic tumour occurred in just 18.1% of HPV-positive patients, compared to 41.0% of those who were HPV-negative ($p<0.001$). Both the 3- and 5-year survival rates were significantly higher in HPV-positive than in HPV-negative tumours (3-year survival: HPV-negative 56.4%, HPV-positive 81.9%, $p<0.001$. 5-year survival: HPV-negative 34.2%, HPV-positive 69.1%, $p<0.001$).

Patients receiving no treatment excluded		HPV-Positive N=149 Number (%)	HPV-Negative N=122 Number (%)	p-value
Treatment Failure	*No*	130 (87.2)	81 (66.4)	
	Yes	11 (6.7)	15 (12.3)	0.05
	Not Applicable	1 (0.7)	4 (3.3)	
	Not Known	8 (5.4)	22 (18.0)	
Late Recurrence	*No*	120 (80.5)	56 (45.9)	
	Yes	13 (8.7)	33 (27.0)	<0.001
	Not Applicable	7 (4.7)	10 (8.2)	
	Not Known	9 (6.0)	23 (18.9)	
Second Primary	*No*	128 (85.9)	85 (69.7)	
	Yes	12 (8.1)	11 (9.0)	0.51
	Not Applicable	1 (0.7)	2 (1.6)	
	Not Known	8 (5.4)	24 (19.7)	
Distant Metastases	*No*	134 (89.9)	83 (68.0)	
	Yes	6 (4.0)	12 (9.8)	
	Not Applicable	1 (0.7)	3 (2.5)	0.02
	Not Known	8 (5.4)	24 (19.7)	
Failure, Recurrence or Distant Metastases	*No*	113 (75.8)	49 (40.2)	
	Yes	27 (18.1)	50 (41.0)	<0.001
	Not Known	9 (6.0)	23 (18.9)	
Life Status (at time of last follow up)	*Dead*	43 (28.9)	74 (60.7)	
	Alive	106 (71.1)	47 (38.5)	<0.001
	Not Known	0 (0.0)	1 (0.8)	
Cause of Death	*Died from OPSCC*	27 (18.1)	53 (43.4)	
	Still alive/Other cause	122 (81.9)	68 (55.7)	<0.001
	Not Known	0 (0.0)	1 (0.8)	
		N=116	**N=94**	
3-year Survival	*Dead*	21 (18.1)	41 (43.6)	
	Alive	95 (81.9)	53 (56.4)	<0.001
		N=81	**N=76**	
5-year Survival	*Dead*	25 (30.9)	50 (65.8)	
	Alive	56 (69.1)	26 (34.2)	<0.001

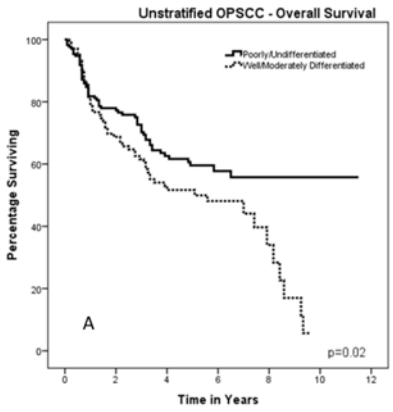
Table 3. 8 : Treatment outcomes according to HPV status

3.4.9 The effect of histological features on patient outcomes

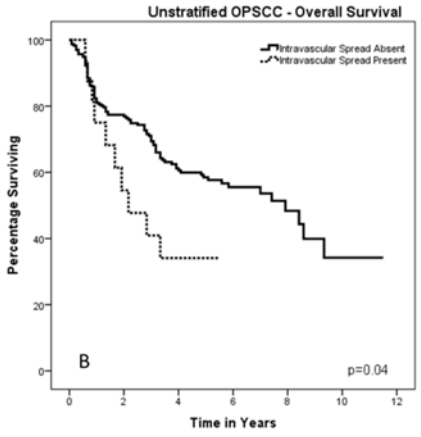
The impact of traditional histological features on survival was assessed in unstratified OPSCC, and then stratified according to HPV-status. Only patients with tissues available were included, to allow for stratification by HPV-status. On Kaplan Meier analysis of unstratified OPSCC, significantly reduced overall survival was associated with: well/moderately differentiated tumours ($p=0.02$), the presence of intravascular spread ($p=0.04$), and a discohesive invasive front ($p<0.001$, figure 3.2 A-C); while reduced disease specific survival was only significantly associated with tumours with a discohesive invasive front ($p<0.001$, figure 3.2 D). There was a trend towards reduced overall and disease specific survival in the presence of extracapsular spread without reaching statistical significance (OS $p=0.062$, DSS $p=0.075$, figure 3.2 E&F). In HPV-negative tumours, none of these histological features were significantly associated with either overall or disease specific survival. However, there was a trend towards reduced disease specific survival in poorly differentiated tumours ($p=0.09$). In HPV-positive tumours (figure 3.3), a discohesive invasive margin was associated with reduced overall and disease specific survival (OS $p=0.009$, DSS $p=0.005$), as was the presence of intravascular spread (OS $p=0.02$, DSS $p=0.01$) and involved resection margins (OS $p=0.03$, DSS $p=0.04$).

The hazard ratios for each of these histological features for unstratified OPSCC are shown in table 3.9. After adjustment for age, T-stage, N-stage and smoking, only the cohesive nature of the invasive margin remained a significant predictor of poor outcome, and patients with a discohesive tumour had a 2-fold increase in the risk of death, and a 2.3-fold increase in the risk of tumour specific death (HRs: OS 1.99, $p=0.001$; DSS 2.33, $p=0.001$). The hazard ratios stratified by HPV-status are shown in tables 3.10 and 3.11. In HPV-negative tumours, none of the histological features examined had a statistically significant independent effect on the risk of either all-cause or disease specific death. The marker that came closest to significantly predicting death was cohesion, with hazard ratios for all-cause and disease specific death of 1.49 and 1.74 respectively ($p=0.14$ and $p=0.08$).

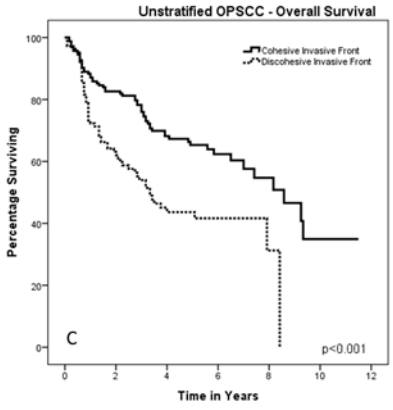
In HPV-positive tumours, discohesive tumours were at an increased risk of both all cause and disease specific death (HRs: OS 2.55, $p=0.007$; DSS 2.90, $p=0.01$), whilst the presence of intravascular spread increased the risk of all-cause, but not tumour specific, death (HRs: OS 3.34, $p=0.03$; DSS 3.15, $p=0.08$). No other histological features had a statistically significant effect on survival.



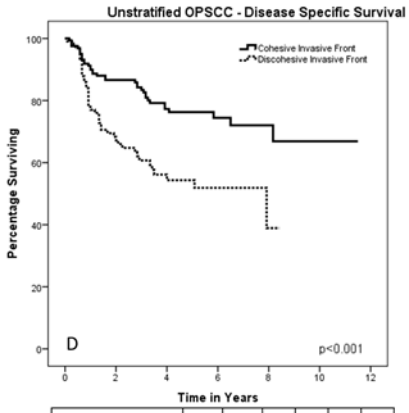
Time	0	2	4	6	8	10
Poorly/Undifferentiated	171	111	66	31	13	4
Well/Moderately Differentiated	103	68	44	21	6	-



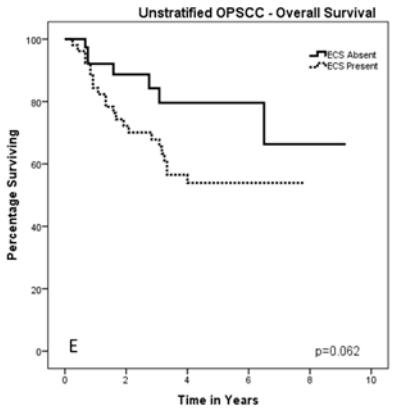
Time	0	2	4	6	8	10
Intravascular Spread Absent	233	156	97	45	16	4
Intravascular Spread Present	16	8	3	-	-	-



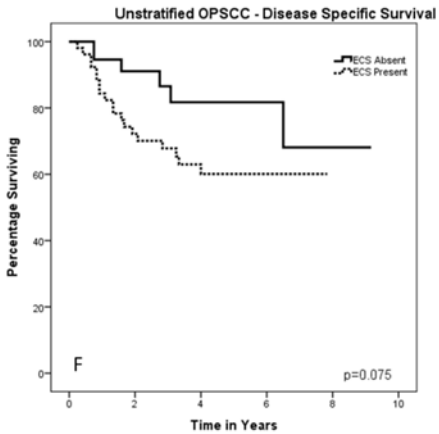
Time	0	2	4	6	8	10
Cohesive Invasion	163	120	79	36	16	4
Discohesive Invasion	109	59	31	16	3	-



Time	0	2	4	6	8	10
Cohesive Invasion	163	120	79	36	16	4
Discohesive Invasion	109	59	31	16	3	-

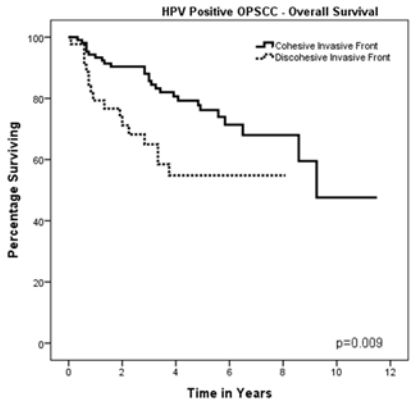


Time	0	2	4	6	8	10
ECS Absent	38	24	12	6	3	-
ECS Present	52	34	22	8	-	-

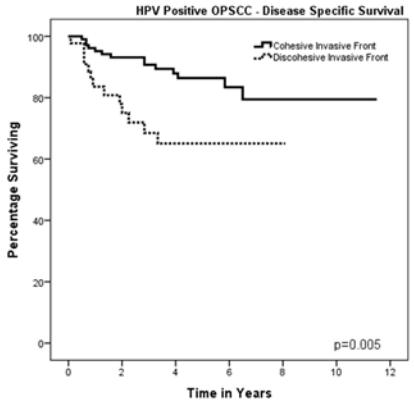


Time	0	2	4	6	8	10
ECS Absent	38	24	12	6	3	-
ECS Present	52	34	22	8	-	-

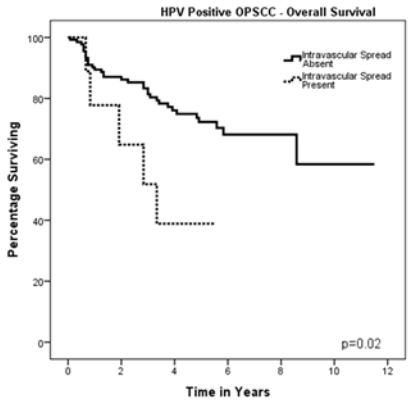
Figure 3. 2: Kaplan Meier Survival Curves for "Traditional" Pathological Factors in Unstratified OPSCC



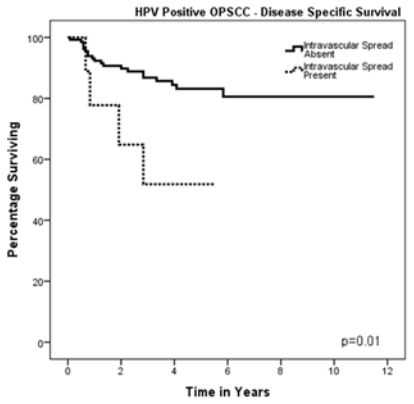
Time	0	2	4	6	8	10
Cohesive Invasion	105	83	59	24	11	2
Discohesive Invasion	44	27	14	7	1	-



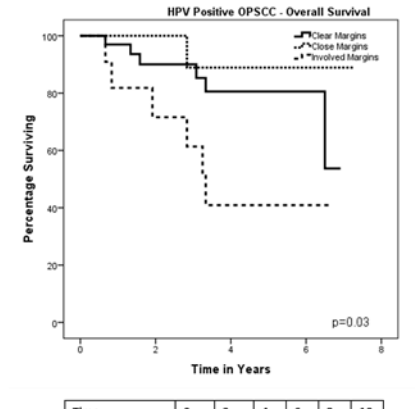
Time	0	2	4	6	8	10
Cohesive Invasion	105	83	59	24	11	2
Discohesive Invasion	44	27	14	7	1	-



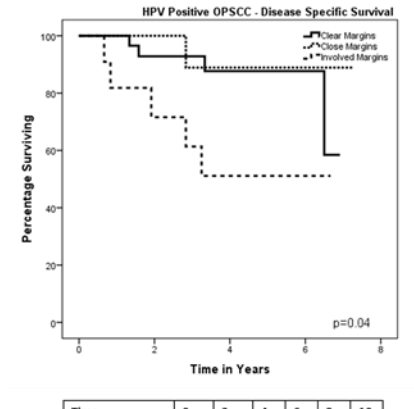
Time	0	2	4	6	8	10
Intravascular Spread Absent	133	99	67	27	11	2
Intravascular Spread Present	9	5	2	1	-	-



Time	0	2	4	6	8	10
Intravascular Spread Absent	133	99	67	27	11	2
Intravascular Spread Present	9	5	2	1	-	-



Time	0	2	4	6	8	10
Clear Margins	33	24	13	3	-	-
Close Margins	14	10	8	2	-	-
Involved Margins	11	7	2	1	-	-



Time	0	2	4	6	8	10
Clear Margins	33	24	13	3	-	-
Close Margins	14	10	8	2	-	-
Involved Margins	11	7	2	1	-	-

Figure 3. 3: Kaplan Meier Survival Curves for "Traditional" Pathological Factors in HPV Positive OPSCC

		Overall Survival			Disease Specific Survival		
		Unadjusted HR	**Adjusted HR**	**p-value**	**Unadjusted HR**	**Adjusted HR**	**p-value**
Tumour Grade	*Well/Moderately Differentiated*	1	1		1	1	
	Poorly Differentiated	0.65 (0.46-0.94)	0.88 (0.58-1.33)	0.54	0.80 (0.52-1.24)	0.99 (0.60-1.62)	0.96
Invasive Margin	*Cohesive*	1	1		1	1	
	Discohesive	2.02 (1.39-2.92)	1.99 (1.31-3.01)	0.001	2.31 (1.49-3.61)	2.33 (1.42-3.82)	0.001
Perineural Spread	*No*	1	1		1	1	
	Yes	0.54 (0.13-2.17)	0.79 (0.19-3.25)	0.75	0.79 (0.19-3.23)	1.07 (0.26-4.42)	0.93
Intravascular Spread	*No*	1	1		1	1	
	Yes	1.97 (1.02-3.80)	1.87 (0.93-3.79)	0.08	1.60 (0.69-3.71)	1.36 (0.54-3.44	0.52
Resection Margin	*Clear*	1	1		1	1	
	Close	0.67 (0.29-1.54)	0.70 (0.25-1.90)	0.48	0.81 (0.32-2.02)	0.78 (0.25-2.42)	0.67
	Involved	0.99 (0.48-2.05)	0.87 (0.40-1.89)	0.73	1.26 (0.57-2.80)	1.15 (0.49-2.72)	0.74
Dysplasia at Margin	*No*	1	1		1	1	
	Yes	1.26 (0.36-4.45)	0.99 (0.24-4.07)	0.98	1.28 (0.28-5.91)	0.86 (0.14-5.24)	0.87
ECS	*No*	1	1		1	1	
	Yes	2.20 (0.94-5.15)	1.84 (0.76-4.43)	0.17	2.25 (0.90-5.64)	1.82 (0.70-4.72)	0.22

Table 3. 9: Hazard ratios for Overall and Disease Specific Survival from OPSCC according to commonly reported histological features (multivariate analysis adjusted for age, T-stage, N-stage and smoking history)

		Overall Survival			Disease Specific Survival		
		Unadjusted HR	**Adjusted HR**	**p-value**	**Unadjusted HR**	**Adjusted HR**	**p-value**
Tumour Grade	*Well/Moderately Differentiated*	1	1		1	1	
	Poorly Differentiated	1.14 (0.72-1.82)	1.31 (0.78-2.19)	0.31	1.58 (0.92-2.72)	1.62 (0.89-2.94)	0.11
Invasive Margin	*Cohesive*	1	1		1	1	
	Discohesive	1.33 (0.83-2.13)	1.49 (0.88-2.51)	0.14	1.42 (0.81-2.49)	1.74 (0.94-3.25)	0.08
Perineural Spread	*No*	Unable to resolve HRs					
	Yes						
Intravascular Spread	*No*	1	1		1	1	
	Yes	1.40 (0.56-3.51)	1.43 (0.56-3.65)	0.45	0.70 (0.17-2.89)	0.74 (0.18-3.08)	0.67
Resection Margin	*Clear*	1	1		1	1	
	Close	0.97 (0.38-2.44)	0.74 (0.22-2.54)	0.64	1.15 (0.41-3.24)	0.79 (0.19-3.22)	0.74
	Involved	0.49 (0.17-1.44)	0.48 (0.16-1.47)	0.20	0.70 (0.23-2.17)	0.70 (0.21-2.29)	0.55
Dysplasia at Margin	*No*	1	1		1	1	
	Yes	0.84 (0.18-3.90)	0.60 (0.08-4.82)	0.63	1.28 (0.26-6.38)	0.78 (0.08-7.79)	0.83
ECS	*No*	1	1		1	1	
	Yes	2.11 (0.59-7.52)	1.86 (0.51-6.83)	0.35	1.92 (0.53-6.93)	1.73 (0.47-6.46)	0.41

Table 3. 10: Hazard ratios for Overall and Disease Specific Survival from HPV-negative OPSCC according to commonly reported histological features (multivariate analysis adjusted for age, T-stage, N-stage and smoking history)

		Overall Survival			Disease Specific Survival		
		Unadjusted HR	**Adjusted HR**	**p-value**	**Unadjusted HR**	**Adjusted HR**	**p-value**
Tumour Grade	*Well/Moderately Differentiated*	1	1		1	1	
	Poorly Differentiated	0.68 (0.36-1.29)	0.64 (0.31-1.29)	0.21	0.74 (0.32-1.69)	0.65 (0.27-1.60)	0.35
Invasive Margin	*Cohesive*	1	1		1	1	
	Discohesive	2.24 (1.20-4.17)	2.55 (1.29-5.04)	0.007	2.81 (1.32-5.99)	2.90 (1.27-6.65)	0.01
Perineural Spread	*No*	1	1		1	1	
	Yes	1.38 (0.33-5.77)	1.39 (0.32-5.94)	0.66	2.15 (0.50-9.19)	1.74 (0.39-7.87)	0.47
Intravascular Spread	*No*	1	1		1	1	
	Yes	2.87 (1.11-7.40)	3.34 (1.12-9.56)	0.03	3.58 (1.21-10.54)	3.15 (0.86-11.61)	0.08
Resection Margin	*Clear*	1	1		1	1	
	Close	0.39 (0.05-3.23)	0.51 (0.06-4.52)	0.54	0.58 (0.07-5.22)	0.76 (0.07-8.33)	0.83
	Involved	3.09 (1.00-9.59)	2.48 (0.67-9.15)	0.17	3.81 (1.02-14.19)	3.50 (0.72-17.10)	0.12
Dysplasia at Margin	*No*	1	1		Unable to resolve HRs		
	Yes	1.70 (0.19-15.35)	2.46 (0.10-60.27)	0.58			
ECS	*No*	1	1		1	1	
	Yes	2.28 (0.61-8.44)	2.08 (0.52-8.26)	0.30	2.71 (0.56-13.08)	2.24 (0.41-12.32)	0.36

Table 3. 11: Hazard ratios for Overall and Disease Specific Survival from HPV-positive OPSCC according to commonly reported histological features (multivariate analysis adjusted for age, T-stage, N-stage and smoking history)

3.5 Discussion

In spite of their advanced stage and poor differentiation, HPV-positive tumours appear to have improved survival compared to those that are HPV-negative, possibly related to lower rates of tumour recurrence (237). The biological reasons behind this are unclear, and may well be multifactorial. A major outcome of this project has been the identification of an important factor, namely tumour infiltrating lymphocytes (TILs), which may potentially underlie the improved survival rates seen in most HPV-positive tumours. To ensure the reliability of the various analyses relating to TILs, it was first vital to ensure that the patient cohort on which the results were based was behaving as predicted, in terms of presentation, risk factors, tumour biology, and outcomes.

3.5.1 Characteristics of the cohort

The characteristics of the patients included in this study are representative of a typical cohort of patients with oropharyngeal cancer. The study cohort consisted of 442 patients treated in two centres over a ten year period. The mean age at diagnosis was just under 60 years of age and there was a male:female ratio of approximately 3:1, as would be expected. Nearly half of patients in the study admitted to being current smokers, and over 60% currently consumed alcohol. Interestingly, approximately 80% of tumours presented at an advanced stage (stage III/IV), largely due to the presence of nodal metastases, which were seen in nearly three-quarters of patients. At first glance this perhaps appears high, as typical teaching would suggest that approximately two-thirds of tumours present at this disease stage (1). However it is important to remember that "historical teaching" does not apply in the era of HPV-positive oropharyngeal cancer, and that the advanced staging seen in this cohort can largely be explained by the presence of a large number of HPV-positive tumours (see below). Similarly, over half of the tumours in this cohort were poorly differentiated, again high compared to the accepted norm that the majority of HNSCCs are moderately differentiated (435). This too can be explained by the large number of HPV-positive tumours.

The range of treatments received represents a typical distribution according to accepted treatment guidelines. It could be argued that the heterogeneous nature of the treatment modalities is a negative factor, especially in a study such as this where the majority of the analyses are related to treatment outcomes. However, the inclusion of patients treated in a number of ways allows for the comparison of treatments for both HPV-negative and HPV-positive OPSCC, and should be viewed as a positive. Furthermore, although there are a number of studies suggesting that HPV-positive tumours have improved survival outcomes regardless of treatment type, very few of

these actually directly compare surgery, radiotherapy and chemoradiotherapy within the same patient cohort. In fact, Southampton is one of a few centres in the UK to have a large transoral surgery practise for OPSCC, and this allows comparison of outcomes following transoral resections with those following (chemo)radiotherapy (see chapter 5). The patient follow up is adequate, with a median of over 4 years, and outcome data is available for the vast majority of patients, with mortality information missing in only 2.5%.

Of particular relevance is the comparison between HPV-positive and HPV-negative tumours, in terms of demographics, risk factors and presentation, and this is discussed next.

3.5.2 Diagnosis of HPV and correlation with clinicopathological features

The diagnosis of HPV-positive tumours was based on a combination of p16 IHC and HPV *ISH*. This dual approach has been suggested in the literature to be the best technique for the diagnosis of HPV in the clinical setting, with a sensitivity and specificity approaching 100% (341, 347, 349). It was possible to successfully determine p16 status and HPV DNA status in 287 cases for each marker. The combination of the two was available in 286 tumours, and of these just over half were HPV-positive, in keeping with recent literature (20, 100, 297). There was a strong correlation between HPV ISH and p16 IHC, with nearly 90% of HPV ISH positive tumours also p16 positive, and nearly 95% of p16 positive tumours also staining positive for HPV DNA on ISH. This is in keeping with previous studies. For example, Lewis *et al* found that out of 239 oropharyngeal cancers, 187 were p16 positive, and of these 158 (84.4%) were positive for HPV DNA. Of the 163 tumours that were HPV DNA positive, only 5 (~3%) were p16 negative (346).

Patients with HPV-positive tumours were younger (mean age at diagnosis: HPV-positive 56.9 years; HPV-negative 61.1 years; $p=0.002$) and had lower rates of both heavy smoking (>10 pack years: HPV-positive 26.8%, HPV-negative 57.9%, $p<0.001$) and heavy drinking (>20 units per week: HPV-positive 15.0%, HPV-negative 40.6%, $p<0.001$). Unfortunately, due to the retrospective nature of this study, no information was available regarding exposure to high risk sexual practices. HPV-negative tumours were evenly spread throughout the oropharynx, while tumours that were HPV-positive were generally found in either the lingual or palatine tonsils (tonsil/base of tongue tumour: HPV-positive 92.8%, HPV-negative 70.7%, $p<0.001$). HPV-positive tumours were more likely to present with nodal metastases (N1-3: HPV-positive 89.5%, HPV-negative 59.4%, $p<0.001$) and as a result tended to present at a more advanced disease stage (stage III/IV: HPV-positive 92.8%, HPV-negative 67.7%, $p<0.001$). There

were no differences in T-stage at presentation according to HPV-status when the cohort was considered as a whole, but if analysis was limited to those patients with stage III or stage IV tumours, then those that were HPV-positive were significantly more likely to present at an earlier T-stage (T1/2: HPV-positive 68.8%, HPV-negative 41.6%, $p<0.001$). This important finding is discussed in more detail in chapter 5.

These findings are all in keeping with in the literature (312). By way of comparison, in a recent meta-analysis, HPV-positive tumours were more likely to: occur in younger patients (mean age at diagnosis: HPV-positive 55 years, HPV-negative 60 years); have lower exposure to tobacco (ever smoker: HPV-positive 55.9%, HPV-negative 74.5%); have lower exposure to alcohol (ever drinker: HPV-positive 66.8%, HPV-negative 75.4%); present at an advanced disease stage (stage III/IV: HPV-positive 73.3%, HPV-negative 57.2%) and have nodal metastases at the time of presentation (N1-3: HPV-positive 66.5%, HPV-negative 54.1%) (100). Similar to the results presented here, in the meta-analysis by Dayyani *et al*, the male:female ratio was 3:1 in both HPV-positive and HPV–negative tumours. The finding that HPV-positive tumours were predominantly located in the tonsils and base of tongue is also echoed by others, including Hong *et al* who also found that only 7% of HPV-positive tumours occurred outside of these sites (353). In addition, Hong *et al* also found that advanced stage tumours were more likely to present at an earlier T-stage if they were HPV-positive (T1/2: HPV-positive 53%, HPV-negative 33%, $p=0.03$).

In terms of tumour histology, HPV-positive tumours were more likely to be poorly differentiated (HPV-positive 76.5%, HPV-negative 43.6%, $p<0.001$) and to have a cohesive invasive margin (HPV-positive 69.9%, HPV-negative 45.9%, $p<0.001$). There was no difference in the rates of perineural or perivascular spread, or in the rates of involved resection margins, dysplasia at the resection margin, or extracapsular spread according to HPV-status. In keeping with the actions of the E6 oncoprotein, levels of p53 expression were significantly lower in tumours that were HPV-positive.

The correlation between HPV-status and tumour grade is well established, and HPV-positive tumours are often described as poorly differentiated (23). Recently this "poor differentiation" has been the subject of some debate, with some authors arguing that these tumours are not, in fact, poorly differentiated, but very well differentiated with respect to the tonsillar crypt epithelium from which they are thought to arise (185, 186, 312). HPV-positive tumours are commonly described as basaloid and non-keratinising, and both of these features were noted during the course of this study, though not specifically recorded (337-339). The nature of the invasive margin is known to be an important prognostic factor in head and neck tumours, however the association of a cohesive invasive margin with HPV-positive tumours is not widely

reported (436, 437). Similarly, other authors have also noted an absence of any difference in the rates of perineural and intravascular invasion between HPV-positive and HPV-negative tumours (434). The anti-p53 antibody used in this study detected both wild type and mutated p53, and so it is impossible to comment on whether or not HPV-positive tumours were associated with wild type p53, as has been reported in the literature (324). However, the finding that lower levels of p53 were detected in HPV-positive tumours is in keeping with previous reports.

3.5.3 The effect of HPV-status on survival and the importance of biologically active infection

Tumours that were positive for HPV ISH, p16 IHC or a combination of both, all had significantly improved overall and disease-specific survival, both on Kaplan Meier and Cox Regression analysis (all $p<0.001$). The greatest reduction in the risk of overall and disease specific death was seen when both p16 and HPV ISH were used in combination, with hazard ratios for OS and DSS of 0.37 and 0.33 respectively (both $p<0.001$). These hazard ratios represent a reduction in the risk of all-cause and disease specific death of 63% and 67% respectively. Importantly, these findings are in keeping with a recent meta-analysis of over 5600 cases of HNSCC, of which 925 were located within the oropharynx, which found that HPV-positivity reduced the risk of death from unselected HNSCC by 58%, and from oropharyngeal cancer by 60% (HRs: HNSCC 0.42, $p<0.0001$; OPSCC 0.40, $p<0.0001$) (100).

In patients who had tumours that were positive for HPV ISH, only those in which p16 was also overexpressed had improved survival compared to patients who were HPV ISH negative and p16 negative (DSS: HR relative to HPV ISH-negative/p16 negative: HPV ISH-positive/p16 negative 0.88, $p=0.76$; HPV ISH-positive/p16 positive 0.32, $p<0.001$). Similarly, patients who were p16 positive but HPV ISH negative did not have improved survival compared to those who were negative for both (DSS HR 0.73, $p=0.61$). Because the vast majority of HPV ISH positive tumours are also p16 positive, and vice versa, both markers demonstrate improved survival when considered alone, and only examining the two in combination identifies the small groups of patients that do not fit in with the general picture. Furthermore, only patients who were positive for both HPV ISH and p16 had a reduction in the risk of treatment failure, tumour recurrence or late distant metastasis (OR 0.22, $p<0.001$). This reflects the findings of other authors and highlights the importance a dual approach to the diagnosis of HPV-positive patients (322, 323).

It has been suggested that in tumours that are HPV DNA-positive but p16 negative, it is likely that HPV is merely acting as a bystander, and is not driving oncogenesis (322,

325). Interestingly, this group of tumours had similar levels of exposure to tobacco as tumours that were HPV ISH-negative/p16 negative (current smoker >10 pack years: HPV ISH-negative/p16 negative 68.2%, HPV ISH-positive/p16 negative 72.2%, p=1.00) and indeed had higher levels of p53 expression (p53-positive: HPV ISH-negative/p16 negative 62.0%, HPV ISH-positive/p16 negative 100%, p=0.002), further evidence that HPV was not biologically active in these tumours. Again this is in keeping with the findings of other authors (322). In the small number of tumours that were HPV ISH-negative but p16 positive, it is likely that p16 was elevated for other reasons, such as mutational inactivation of the retinoblastoma protein (5, 185). This is especially the case given that the HPV ISH probe used in this study was not directed solely against HPV-16, but instead against 13 different high risk HPV-types, thus would be expected to detect the 10% of HPV-positive oropharyngeal tumours that are not caused by HPV-16.

The relationships between HPV-status and clinicopathological variables, as well as the effect of HPV-status on survival and tumour recurrence, are in keeping with much of the literature already published regarding HPV and oropharyngeal cancer. Indeed, none of the results described above are in any way surprising or unexpected. This supports the notion that the patients included in this study represented a typical cohort in terms of both the prevalence of HPV and the relationships with other important clinicopathological variables, and adds strength to the reliability and validity of the other findings generated from this research project.

3.5.4 Histological predictors of survival in HPV-positive and HPV-negative OPSCC

There are a number of histological features that have, over time, been firmly established as markers of poor survival. Perhaps the most commonly utilized of these is tumour differentiation. Head and neck tumours are typically graded as well, moderately, poorly or undifferentiated. Essentially, the grading system refers to how much the cells within the tumour resemble the cells of origin. Poorly differentiated and undifferentiated tumours are the most “abnormal”, i.e. tumour cells do not resemble cells that they originated from, and such tumours are often clinically aggressive. In unstratified tumours in this study, the grade of differentiation did predict for survival, at least on univariate analysis. However, it was well and moderately differentiated tumours that were more likely to have poor outcomes. Whilst this may initially appear paradoxical, it was a reflection of the fact that the vast majority of HPV-positive tumours were graded as poorly differentiated. In this instance differentiation is behaving as a surrogate marker for improved survival seen with HPV-positive tumours.

The only histological marker that remained a statistically significant independent predictor of survival for unstratified and HPV-positive OPSCC was tumour cohesion. The cohesive nature of the invasive tumour margin is also well established as a predictor of survival in HNSCC, having first been described as such by Bryne and colleagues in 1992 (433, 437). Tumours can be graded on a 4 point scale, where grade 1 tumours comprise those with an well-defined invasive front composed of broad, bulbous bands of tumour cells, and grade 4 tumours have an ill-defined invasive margin with non-cohesive tumour cells and frequently have satellite islands far in advance of the main margin (433). The nature of the invasive front has been shown to correlate with a number of in-vitro markers in malignancy, such as the secretion of proteolytic enzymes, loss of contact inhibition, and tumour cell motility (438).

An association between HPV-16 and cohesive tumours has been described previously in cervical cancer (439). However, the association between HPV status and the nature of the invasive front is not widely reported in OPSCC. To our knowledge, this is the first study to demonstrate that tumour cohesion is an independent prognostic marker in HPV-positive OPSCC. It is possible that a discohesive invasive front in HPV-positive OPSCC is related to additional mutations as a result of alternate carcinogens, i.e. tobacco. However, there was no association between cohesion and smoking in HPV-positive tumours in this patient group. In patients with discohesive tumours, 39% were heavy smokers, compared to 26.9% of those with cohesive tumours ($p=0.22$). Furthermore, the independent predictive ability of cohesion in HPV-positive tumours was demonstrated in a multivariate model that included smoking. It is likely therefore that other factors underlie the development of discohesive tumours in some HPV-positive patients, and this warrants further investigation. It is interesting that this histological predictor of survival in HPV-positive OPSCC was only reported in 12 out of 152 routine clinical histology reports.

Rather unexpectedly, none of the typically utilized histological prognostic markers were significant predictors of survival in HPV-negative tumours, although discohesive tumours showed a trend towards reduced survival. Whilst this is not straightforward to explain, it is possible that relatively small numbers accounted for loss of statistical significance, for example there were only 7 HPV-negative tumours without ECS.

3.6 Conclusions

In general, the tumours in this study behaved as predicted, and the associations between HPV-status and various demographic, clinical and pathological features, and the effects of HPV-status on survival were in keeping with previously published studies. In HPV-positive tumours, tumour cohesion was the only significant predictor of survival on multivariate analysis. HPV-negative tumours, on the other hand, had no statistically significant associations between any established histological markers and survival, although the trends seen were as would be expected.

4 Prognostic factors in HPV-associated oropharyngeal cancer

4.1 Introduction

Human papillomavirus (HPV) is now the primary cause of oropharyngeal cancer (OPSCC), in the Western world, accounting for 40-80% of cases (100, 294, 302, 312). The incidence of HPV-associated OPSCC has increased significantly over the past 40 years, and continues to rise (20, 312).

HPV-associated OPSCC commonly presents at advanced stage, and is poorly differentiated (312, 440). Both factors are traditionally associated with poor outcomes. However, a number of studies have reported significantly better long-term survival in most HPV-positive OPSCC patients, compared to those with HPV-negative disease of matched stage (99, 100, 356, 358, 359, 362). The explanation for this remains unclear, and may be multifactorial. HPV-positive tumours have an improved response to chemotherapy/radiotherapy compared with HPV-negative tumours, possibly modulated through expression of wild-type p53 and Rb tumour suppressor genes; patients with HPV-positive cancers have a lower risk of developing secondary primary tumours, probably due to the absence of field cancerisation; additionally, HPV-status inversely correlates with biomarkers of poor prognosis such as EGFR (312, 326, 380).

A potential explanation for the survival benefit may be the presence of tumour-infiltrating T-lymphocytes (TILs) that permeate many HPV-positive oropharyngeal tumours, suggesting an adaptive host immune response directed against viral antigens. Indeed, an increased frequency of HPV16 E7-specific T-cells has been reported in OPSCC patients (418, 421, 423). Although TIL abundance is associated with improved clinical outcomes in several other tumour types, their role in head and neck malignancy is not well documented (401, 441).

Several studies have reported that the survival advantage of HPV-driven tumours is independent of treatment modality, whether surgery, radiation, or chemoradiotherapy (or combinations thereof), raising the possibility that some HPV-positive patients receive unnecessary treatment (99, 312, 353, 356, 358, 362). This has led to suggestions that treatment can safely be de-intensified to reduce therapy-related morbidity, and a number of clinical trials are testing this currently (312). However, within the HPV-positive OPSCC population, there remains a significant minority that respond poorly to treatment and consequently have a poor prognosis. Ang et al identified 3 different “at risk” groups for patients with oropharyngeal cancer, and found that HPV-positive patients who were heavy smokers and had advanced nodal disease (greater than 10 pack years smoking with N2b disease or above) were at an

"intermediate" risk of death. These factors have subsequently been validated independently and have been used as exclusion criteria in some de-escalation trials (99, 377). Other authors have also described an association between heavy smoking and poor outcome in patients with HPV-positive tumours, whilst there are also reports that EGFR expression can alter survival (312, 357, 373, 440). However there is still no widely-accepted strategy for identifying high risk HPV-positive patients.

4.2 Aims

The aims of this chapter are to:

- Determine whether there are differences in TIL-levels between HPV-positive and HPV-negative OPSCC.
- Determine whether tumour infiltrating lymphocytes predict for survival in both unstratified and HPV-positive OPSCC.
- Identify other factors that influence survival in HPV-positive OPSCC.
- Create a prognostic model for HPV-positive OPSCC.

4.3 Results

4.3.1 Patient Demographics

As previously described, data were collected for 442 patients with oropharyngeal cancer (UHS n=316, PFT/BLT n=126). Patients were excluded from further analysis if they had incomplete survival data, follow up of less than 6 months, had received either no treatment or treatment without curative intent, or had an unknown cause of death. A total of 402 patients remained after these exclusions and, of these, archival pathology material was available for 274 (UHS n=178, PFT/BLT n=96). Only these 274 patients were used subsequently as all analyses required archival tissues, however there was no difference in overall or disease specific survival between those patients who did and did not have archival material available (DSS p=0.83, appendix 4). Clinical, pathological, and immunochemical characteristics at the time of diagnosis are shown in Table 4.1. Due to low numbers of stage I tumours, for all subsequent analyses stage was defined as either early (stage I/II) or late (stage III/IV). Data on smoking and alcohol consumption can be difficult to accurately obtain in retrospective studies, often due to poor documentation. In this study, smoking data was more accurately recorded in the medical records than alcohol consumption, with reliable data available in 87% and 74% of cases, respectively. Smoking was categorized as either greater than or less than a 10 pack year history, in keeping with the findings of two recent papers describing this level as being a significant prognostic marker in both HPV-positive and HPV-negative oropharyngeal cancer (99, 357).

		All OPSCC n=274 Number (%)	**HPV-Positive OPSCC N=149** Number (%)	**HPV-Negative OPSCC n=121** Number (%)
Gender	*Male*	200 (73.0)	111 (74.5)	86 (71.1)
	Female	74 (27.0)	38 (25.5)	35 (28.9)
Age at Diagnosis	*<50*	58 (21.2)	40 (26.8)	18 (14.9)
	50-69	168 (61.3)	91 (61.1)	74 (61.2)
	70+	48 (17.5)	18 (12.1)	29 (24.0)
	Mean (SD)	58.2 (11.2)	56.4 (10.8)	60.4 (11.4)
Smoking	*Non/Ex-Smoker*	107 (39.1)	82 (55.0)	23 (19.0)
	Current <10 pack years	18 (6.6)	10 (6.7)	8 (6.6)
	Current >10 pack years	114 (41.6)	41 (27.5)	72 (59.5)
	Not Known	35 (12.8)	16 (10.7)	18 (14.9)
Alcohol	*Non/Ex Drinker*	34 (12.4)	20 (13.4)	14 (11.6)
	Current Drinker	167 (60.9)	94 (63.1)	72 (59.5)
	Not Known	73 (26.6)	35 (23.5)	35 (28.9)
Tumour Site	*Tonsil*	158 (57.7)	99 (66.4)	57 (47.1)
	Tongue Base	70 (25.5)	40 (26.8)	28 (23.1)
	Other Oropharynx	46 (16.8)	10 (6.7)	36 (29.8)
Median Length of Follow Up in Years (Range)		58.0 (8-137)	58.5 (8-137)	57 (8-128)
Disease Stage	*I/II*	53 (19.3)	10 (6.7)	42 (34.7)
	III/IV	219 (79.9)	138 (92.6)	78 (64.5)
	Not Known	2 (0.7)	1 (0.7)	1 (0.8)
T-Stage	*T1/2*	187 (68.2)	107 (71.8)	77 (63.6)
	T3/4	82 (29.9)	41 (27.5)	41 (33.9)
	Not Known	5 (1.8)	1 (0.7)	3 (2.5)
Nodal Metastases	*No*	68 (24.8)	15 (10.1)	52 (43.0)
	Yes	204 (74.5)	133 (89.3)	68 (56.2)
	Not Known	2 (0.7)	1 (0.7)	1 (0.8)
N-Stage	*N0-N2a*	142 (51.8)	68 (45.6)	73 (60.3)
	N2b-N3	130 (47.4)	80 (53.7)	47 (38.8)
	Not Known	2 (0.7)	1 (0.7)	1 (0.8)
Grade	*Well/Moderately Differentiated*	103 (37.6)	34 (22.8)	68 (56.2)
	Poorly Differentiated	171 (62.4)	115 (77.2)	53 (43.8)
Treatment	*Surgery+/- Radiotherapy*	117 (42.7)	60 (40.3)	55 (45.5)
	Radiotherapy	59 (21.5)	24 (16.1)	34 (28.1)
	Chemoradiotherapy	98 (35.8)	65 (43.6)	32 (26.4)
TIL Status	*Low*	79 (28.8)	22 (14.8)	56 (46.3)
	Moderate	101 (36.9)	53 (35.6)	46 (38.0)
	High	92 (33.6)	72 (48.3)	19 (15.7)
	Not Known	2 (0.7)	2 (1.3)	0 (0.0)
HPV Status	*Negative*	121 (44.2)	-	-
	Positive	149 (54.4)	-	-
	Not Known	4 (1.5)	-	-
EGFR	*Negative*	108 (39.4)	77 (51.7)	29 (24.0)
	Positive	166 (60.6)	72 (48.3)	92 (76.0)

Table 4. 1: Demographics of the dataset both unstratified and according to HPV-status

HPV-positive tumours were defined as those positive for both p16 IHC and HPV ISH (349). HPV status was successfully determined in 270 patients. HPV-status could not be established for 4 patients in whom there was insufficient tissue remaining in the archival block (2 from UHS and 2 from PFT/BLT). As described in chapter 2, a prominent lymphocytic infiltrate (TIL) was scored under low-power magnification (x2·5 objective) as high (diffuse; present in >80% of tumour/stroma), low (weak/absent; present in <20% of tumour/stroma), or moderate (patchy; present in 20-80% of tumour/stroma). TIL status could not be established in two patients due to poor quality H&E slides and insufficient tissue to create a new slide (both from UHS). Examples of TIL^{high} and TIL^{low} tumours are shown in figure 4.1, while p16 immunostaining and HPV-ISH are shown in figure 4.2. Similar to previous studies, 93.9% of p16-positive cases were HPV ISH-positive; 89% of HPV ISH-positive cases were p16-positive (discussed in more detail in chapter 3)(349). The association of HPV with various demographic factors has been discussed in chapter 3.

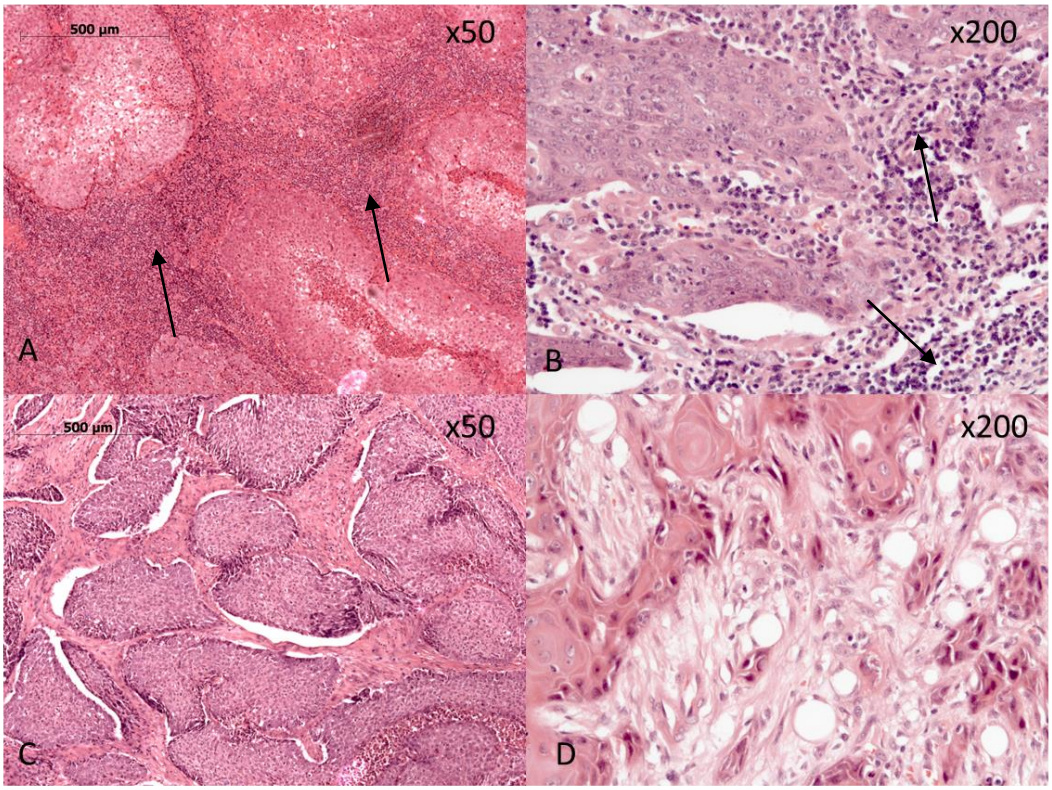


Figure 4. 1: Examples of TIL^{high} (A/B) and TIL^{low} (C/D) tumours (H&E stain, areas of lymphocyte infiltration indicated with arrows)

4.3.2 Predictors of Survival – Unstratified Disease

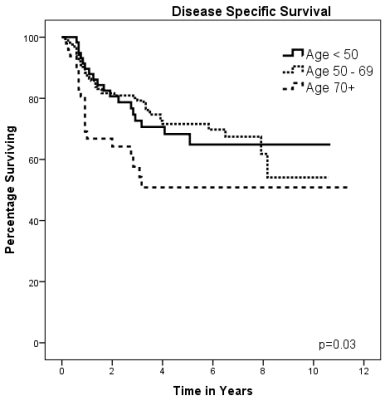
Initial analysis was aimed at establishing predictors of survival in unstratified OPSCC. The entire combined cohort was used to maximize the numbers available for analysis and thus minimize the chances of missing important predictive variables. The combined cohort consisted of 274 oropharyngeal tumours with a median follow up of 58 months, and a minimum of 8 months (table 4.1). Of the 274 tumours, a total of 149 (54.4%) were classified as HPV-positive based on the criteria described previously. There were 119 deaths (43.4% of total cohort) recorded over the study period, of which 82 were categorized as tumour related (68.9% of all deaths, 29.9% of total cohort). Therefore the outcome measures of overall and disease specific survival are based on 119 and 82 deaths respectively. The effect of TNM staging is discussed in detail in chapter 5, and will not be described here.

On Kaplan Meier analysis; age ($p<0.001$), smoking status ($p<0.001$), tumour grade ($p=0.02$), treatment received ($p=0.02$), HPV-status ($p<0.001$), TIL-status ($p<0.001$), and EGFR ($p=0.03$) were all significant predictors of overall survival (appendix 4). However, only age ($p=0.03$), smoking ($p<0.001$), TILs ($p<0.001$), HPV ($p<0.001$) and EGFR ($p=0.05$) significantly predicted for disease specific survival (figure 4.3).

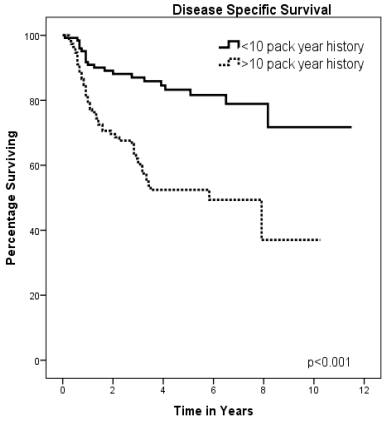
To enable a more detailed analysis of predictive factors in unstratified OPSCC, and to take into account relationships between individual variables, both univariate and multivariate cox regression was performed. On univariate analysis, a reduction in the risk of all-cause death was significantly associated with tumours that were HPV-positive, poorly differentiated, or had a high level of tumour-infiltrating lymphocytes (TIL; assessed on an H+E-stained section); while tumours that were: EGFR-positive, patients who had an older age at diagnosis, had a greater than 10 pack year smoking history, or were treated with radiotherapy, had an increased risk of all-cause death. Increasing age, heavy smoking, and EGFR-positive tumours were all associated with a significantly increased risk of disease specific death, while HPV-positive tumours and those that were TIL^{high} had a significant reduction in the risk of death from oropharyngeal cancer. Hazard ratios for disease specific survival are shown in table 4.2, while those for overall survival are shown in appendix 4.

A multivariate model was constructed adjusting for age, T-stage, N-stage and smoking status, for both overall and disease specific survival. Only age, smoking, HPV-status and high TILs remained as independent predictors of both overall and disease specific death. Interestingly, the strongest predictor of improved survival was the presence of high TIL levels, which outperformed HPV-status as a prognostic marker.

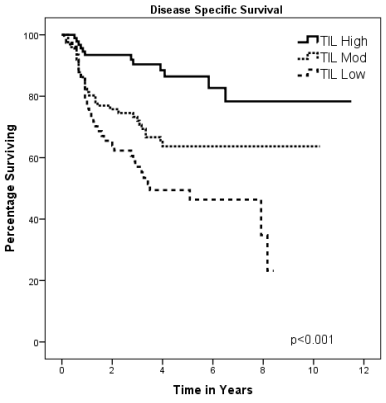
TIL^{high} tumours had a 72% reduction in the risk of tumour specific death when compared to TIL^{low} tumours, whereas for HPV-positive tumours this reduction in risk was 55% (HPV-positive HRs: DSS 0.45, p=0.005. TIL^{high} HR: DSS 0.28, p=0.002; table 4.2).



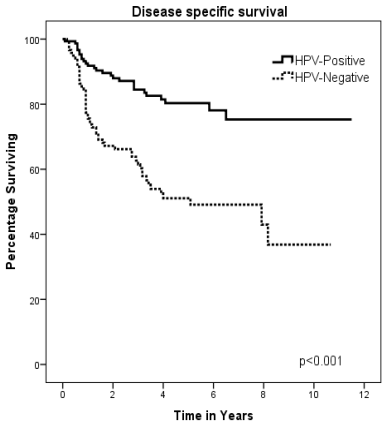
Time	0	2	4	6	8	10
<50	58	43	30	12	4	2
50-69	168	110	70	34	11	1
70+	48	26	10	6	4	1



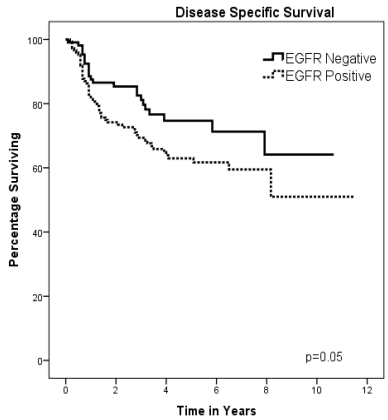
Time	0	2	4	6	8	10
<10 pack years	125	90	64	33	14	3
>10 pack years	114	70	36	16	3	1



Time	0	2	4	6	8	10
TILhigh	92	72	45	21	7	3
TILmod	101	65	44	18	9	1
TILlow	79	41	21	13	3	-



Time	0	2	4	6	8	10
HPV-positive	149	110	73	31	12	2
HPV-negative	121	67	36	20	7	2



Time	0	2	4	6	8	10
EGFR Negative	108	71	38	20	9	3
EGFR Positive	148	99	68	30	10	1

Figure 4. 3: Kaplan Meier Survival Curves for Disease Specific Survival from OPSCC according to age, smoking, TIL-levels, HPV-status, and EGFR in unstratified tumours

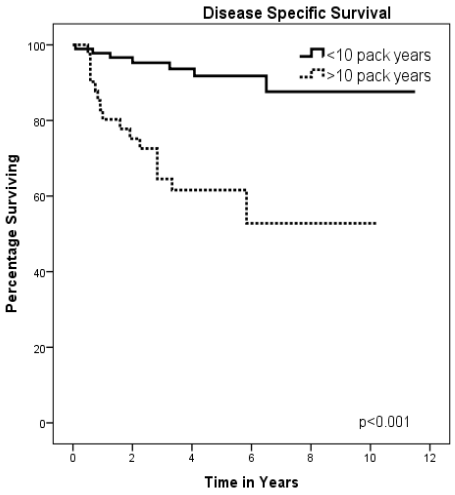
Multivariate analysis adjusted for age, T-stage, N-stage and smoking status. Patients with no treatment excluded from analysis.		All OPSCC (n=274)			
		Univariate HR (95% CI)	**p-value**	**Multivariate HR (95% CI)**	**p-value**
Age	*For each additional year*	1.02 (1.00-1.04)	0.04	1.03 (1.01-1.05)	0.01
Gender	*Female*	1		1	
	Male	1.49 (0.89-2.48)	0.13	1.10 (0.61-1.96)	0.75
Smoking	*<10 pack years*	1		1	
	>10 pack years	3.24 (1.94-5.42)	<0.001	3.38 (2.01-5.70)	<0.001
Drinking	*Non/Ex Drinker*	1		1	
	Current Drinker	0.67 (0.36-1.24)	0.20	0.44 (0.22-0.88)	0.02
Stage	*Early (I/II)*	1		-	-
	Late (III/IV)	1.01 (0.58-1.74)	0.98	-	-
T-Stage	*T1/2*	1		1	
	T3/4	1.89 (1.21-2.94)	0.005	1.56 (0.95-2.57)	0.08
Nodal Metastases	*No*	1		-	-
	Yes	1.11 (0.67-1.84)	0.68	-	-
N-Stage	*N0-N2a*	1		1	
	N2b-N3	1.68 (1.08-2.60)	0.02	1.75 (1.07-2.86)	0.03
Tumour Grade	*Well/Moderately Differentiated*	1		1	
	Poorly Differentiated	0.80 (0.52-1.24)	0.31	0.97 (0.59-1.60)	0.90
Treatment	*Surgery*	1		1	
	Radiotherapy	1.53 (0.90-2.61)	0.12	1.06 (0.56-2.03)	0.85
	Chemoradiotherapy	0.95 (0.57-1.58)	0.83	0.91 (0.51-1.63)	0.74
HPV Status	*Negative*	1		1	
	Positive	0.33 (0.21-0.53)	<0.001	0.45 (0.26-0.79)	0.005
TIL Status	*TIL Low*	1		1	
	TIL Moderate	0.59 (0.37-0.95)	0.03	0.66 (0.39-1.13)	0.13
	TIL High	0.21 (0.11-0.41)	<0.001	0.28 (0.13-0.62)	0.002
HPV/TIL combined	*HPV-*	1		1	
	HPV+/TIL Low	0.83 (0.40-1.75)	0.63	1.01 (0.45-2.24)	0.98
	HPV+/TIL Mod	0.41 (0.22-0.78)	0.006	0.49 (0.24-1.01)	0.054
	HPV+/TIL High	0.14 (0.06-0.33)	<0.001	0.17 (0.06-0.49)	0.001
EGFR	*Negative*	1		1	
	Positive	1.62 (1.00-2.63)	0.05	1.37 (0.81-2.34)	0.25
HPV/Smoking Combined	*HPV-*	1		1	
	HPV+/>10 pack years	0.78 (0.44-1.37)	0.38	0.86 (0.47-1.58)	0.62
	HPV+/<10 pack years	0.13 (0.06-0.30)	<0.001	0.14 (0.06-0.32)	<0.001

Table 4. 2: Univariate and multivariate hazard ratios for disease specific death from OPSCC in unstratified tumours

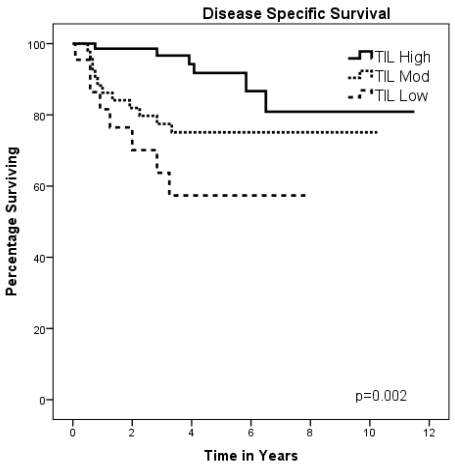
4.3.3 Predictors of Survival – HPV Positive Disease

Although HPV-positive tumours are known to have improved survival outcomes compared to those that are HPV-negative, there remains a small proportion that do not respond well to treatment. In an attempt to identify factors that may impact on survival in HPV-positive tumours, the analysis above was repeated utilizing only tumours that were known to be HPV-positive. In all survival tables, hazard ratios for HPV-negative tumours are also presented for comparison, though will not be discussed in detail.

The HPV-positive cohort consisted of 149 patients with a median follow up of 58.5 months and a minimum of 8 months. There were 43 deaths (28.9%) over the study period, of which 27 were deemed to be tumour related (63% of deaths, 18.1% of HPV-positive patients).



Time	0	2	4	6	8	10
<10 pack years	92	70	50	24	9	1
>10 pack years	41	29	17	6	2	1



Time	0	2	4	6	8	10
TILhigh	72	59	39	16	5	1
TILmod	53	38	28	11	7	1
TILlow	22	12	6	4	-	-

Figure 4. 4: Kaplan Meier Survival Curves for Disease Specific Survival from HPV-positive OPSCC according to smoking status and TIL-levels

On Kaplan Meier analysis, TIL-levels and smoking status were the only significant predictors of both overall and disease specific survival (OS: $p<0.001$, $p=0.001$; DSS: $p=0.002$, $p<0.001$) (appendix 4 and figure 4.4). This was reflected on univariate cox regression, where heavy smoking was significantly associated with an increased risk of all-cause death, while only high TIL-levels were associated with a reduction in the risk of all-cause mortality (appendix 4). Heavy smokers had an increased risk of disease specific death, while again only TIL^{high} tumours had a significant reduction in the risk of death

from oropharyngeal cancer (table 4.3). Interestingly, EGFR expression did not alter outcomes in HPV-positive disease.

A multivariate model was constructed to take into account interactions between variables, with the same adjustments as unstratified tumours, namely age, T-stage, N-stage and smoking status. In this model, increasing age and heavy smoking were independent predictors of an increased risk of all-cause mortality, with moderate and high TIL-levels predicting a reduction in the risk of all-cause death (appendix 4). Heavy smoking was also an independent predictor for an increased risk of tumour specific death on multivariate analysis: However, only high TIL-levels significantly predicted for a reduction in risk (table 4.3).

4.3.4 Correlation between HPV-status and TIL-levels

There was a highly significant association between HPV-status and TIL-levels. In HPV-positive tumours, 15% were TIL^{low}, 36% were TIL^{mod} and 49% were TIL^{high}. The corresponding levels for HPV-negative tumours were 46.3%, 38% and 15.7% ($p<0.001$).

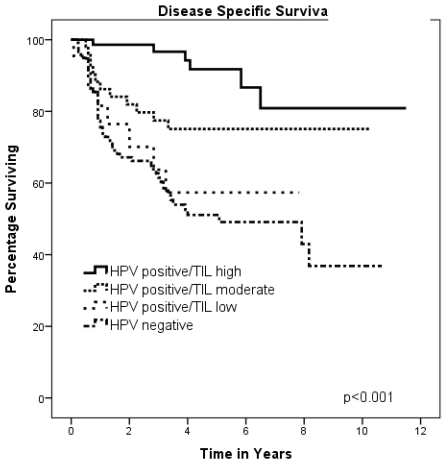
To establish whether the combination of HPV-status and TIL-levels added any further prognostic value, the two variables were combined. TIL-levels were predictive of survival in HPV-positive tumours (see above) though not in those that were HPV-negative (table 4.3). Therefore, all HPV-negative tumours were considered as one group, whilst HPV-positive tumours were stratified according to TIL-level. Demographics of these groups are shown in the appendix 4. There was a highly significant difference between the Kaplan–Meier curves for OPSCC for both overall and disease-specific survival according to this combination (both $p<0.001$, appendix 4 and figure 4.5). Importantly, there was no significant difference in the risk of all-cause or disease specific death between patients with HPV-positive/TIL^{low} tumours and those with HPV-negative tumours (adjusted HRs: OS 1.36, $p=0.37$; DSS 1.01, $p=0.98$; appendix 4 and table 4.2). The hazard ratios (adjusted for age, T-stage, N-stage and smoking) for HPV-positive tumours with moderate and high TIL-levels indicated a 38% and an 81% reduction in the risk of all-cause death, and a 51% and 83% reduction in the risk of death from OPSCC, compared with HPV-negative tumours (adjusted HRs: OS 0.62 and 0.19, respectively, $p=0.12$ and $p<0.001$; DSS 0.49 and 0.17, respectively, $p=0.05$ and $p=0.001$; appendix 4 and table 4.2). TIL levels predicted similarly for progression-free survival (PFS; log rank $p<0.001$; data not shown); patients with HPV-positive/TIL^{low} tumours also showed similar PFS to HPV-negative patients with an adjusted hazard ratio of 0.64 ($p=0.23$, data not shown).

Multivariate analysis adjusted for age, T-stage, N-stage and smoking status. Patients with no treatment excluded from analysis.		HPV-positive OPSCC n=149			HPV-negative OPSCC n=121		
		Unadjusted HR (95% CI)	**Adjusted HR (95% CI)**	**p value**	**Unadjusted HR (95% CI)**	**Adjusted HR (95% CI)**	**p value**
Age	*For each additional year*	1.00 (0.97-1.04)	1.02 (0.98-1.06)	0.28	1.02 (0.99-1.04)	1.02 (0.99-1.05)	0.19
Gender	*Female*	1	1		1	1	
	Male	1.19 (0.50-2.81)	0.74 (0.26-2.11)	0.58	2.06 (1.06-4.02)	1.70 (0.79-3.64)	0.17
Smoking	*Non/Ex-Smoker/Current Smoker <10 pack year*	1	1		1	1	
	Current Smoker >10 pack year	5.81 (2.38-14.16)	5.80 (2.31-14.54)	<0.001	1.37 (0.71-2.64)	1.61 (0.82-3.16)	0.17
Drinking	*Non/Ex Drinker*	1	1		1	1	
	Current Drinker	0.63 (0.23-1.71)	0.29 (0.08-1.04)	0.06	0.68 (0.31-1.49)	0.54 (0.22-1.28)	0.16
Stage	*Early (I/II)*	1	-	-	1	-	-
	Late (III/IV)	1.93 (0.26-14.27)	-	-	1.83 (0.99-3.37)	-	-
T-Stage	*T1/2*	1	1		1	1	
	T3/4	3.42 (1.61-7.28)	3.04 (1.28-7.22)	0.01	1.24 (0.71-2.17)	0.98 (0.52-1.84)	0.94
Nodal Metastases	*No*	1	-	-	1	-	-
	Yes	1.65 (0.39-7.00)	-	-	2.13 (1.19-3.79)	-	-
N-Stage	*N0-N2a*	1	1		1	1	
	N2b-N3	2.29 (1.00-5.24)	1.67 (0.67-4.14)	0.27	2.06 (1.20-3.53)	2.15 (1.14-4.06)	0.02
Tumour Grade	*Well/Moderately Differentiated*	1	1		1	1	
	Poorly Differentiated	0.74 (0.32-1.69)	0.74 (0.30-1.84)	0.52	1.58 (0.92-2.72)	1.50 (0.81-2.81)	0.20
Treatment	*Surgery*	1	1		1	1	
	Radiotherapy	1.51 (0.55-4.15)	1.01 (0.30-3.36)	0.99	1.64 (0.87-3.12)	1.50 (0.70-3.22)	0.30
	Chemoradiotherapy	0.98 (0.42-2.31)	1.02 (0.39-2.70)	0.96	1.48 (0.76-2.88)	1.18 (0.55-2.57)	0.67
TIL Status	*TIL High*	1	1		1	1	
	TIL Moderate	2.96 (1.11-7.89)	2.47 (0.72-8.50)	0.15	1.39 (0.55-3.48)	1.59 (0.54-4.71)	0.40
	TIL Low	5.67 (1.96-16.38)	4.86 (1.34-17.60)	0.02	1.71 (0.71-4.13)	2.11 (0.73-6.16)	0.17
EGFR	*Negative*	1	1		1	1	
	Positive	1.65 (0.76-3.62)	1.54 (0.63-3.74)	0.34	0.99 (0.53-1.87)	1.31 (0.61-2.83)	0.49

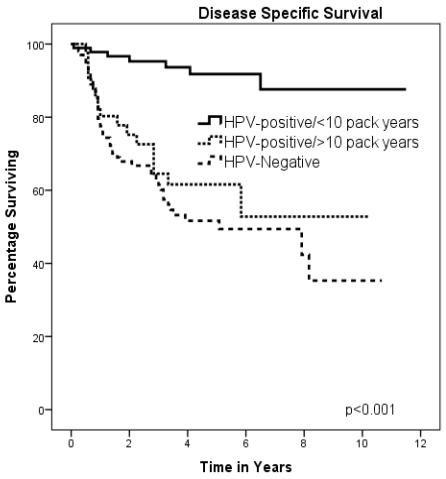
Table 4. 3: Univariate and Multivariate hazard ratios for disease specific survival from OPSCC stratified according to HPV-status

4.3.5 Correlations between HPV-status and smoking

There was a highly significant correlation between HPV-status and smoking. In HPV-positive patients, 69.2% were either non, ex or light smokers (i.e. less than 10 cigarettes per day) while in HPV-negative patients only 30% fell into this category. To assess the effect of HPV-status and smoking together, a combined variable was again created, in a similar manner to HPV and TILs. As with TILs, smoking predicted survival in HPV-positive tumours (see above) but not in HPV-negative tumours. All HPV-negative tumours were therefore considered as one group while HPV-positive tumours were stratified according to smoking status. Again there was a highly significant difference between the Kaplan–Meier curves for OPSCC according to these groupings, for both overall and disease-specific survival (both p<0.001, appendix 4 and figure 4.5). Patients with HPV-positive tumours who were heavy smokers had no difference in the risk of all-cause or disease specific death compared to those who had HPV-negative tumours (HRs: OS 1.08, p=0.81; DSS 0.96, p=0.90; appendix 4 and table 4.2), while those who were light/non/ex-smokers had a 64% reduction in the risk of all-cause, and an 83% reduction in the risk of disease specific death (HRs: OS 0.36, p=0.001; DSS 0.17, p<0.001; appendix 4 and table 4.2), after adjustment for age, T-stage and N-stage.



Time	0	2	4	6	8	10
HPV-positive/TILhigh	72	59	39	16	5	1
HPV-positive/TILmod	53	38	28	11	7	1
HPV-positive/TILlow	22	12	6	4	-	-
HPV-negative	121	67	36	20	7	2



Time	0	2	4	6	8	10
HPV-positive/<10 pack years	92	70	50	24	9	1
HPV-positive/>10 pack years	41	29	17	6	2	1
HPV-negative	103	60	32	18	6	2

Figure 4. 5: Kaplan Meier Survival Curves for Disease Specific Survival from OPSCC according to the combination of HPV-status and TIL-levels, and HPV-status and smoking history

4.3.6 Correlations between TIL-status and Smoking

To ensure that TIL status and smoking were not merely acting as surrogate markers for each other in HPV-positive tumours, correlations between the two variables were assessed. Although TIL^{low} tumours had a tendency to occur more in heavy smokers (the proportion of heavy smokers for TIL high, moderate, and low tumours was 22.7%, 36.4% and 40.9% respectively), these findings were not statistically significant ($p=0.15$). When T-cell subset counts were compared between HPV-positive heavy and light smokers, only the proportion of $CD8^+$ cells differed, and was significantly lower in the heavy smoking group (<10 pack year 0.51, >10 pack year 0.43, $p=0.03$). This absence of surrogacy is also indicated by the independent prognostic power of each marker on the multivariate analysis shown in table 4.3.

4.3.7 Detailed TIL analysis

A more complex analysis of the immune infiltrate was performed, quantifying total T-lymphocyte number (CD4+CD8) and T-cell subsets (CD4, CD8, Foxp3) and ratios (CD4:CD8 and CD8:Foxp3). The average T-cell counts and ratios are shown in table 4.4 and are stratified according to HPV-status. HPV-positive tumours had higher mean counts for all T-cell subsets, and lower CD4:CD8 and Foxp3:CD8 ratios, than tumours that were HPV-negative (table 4.4).

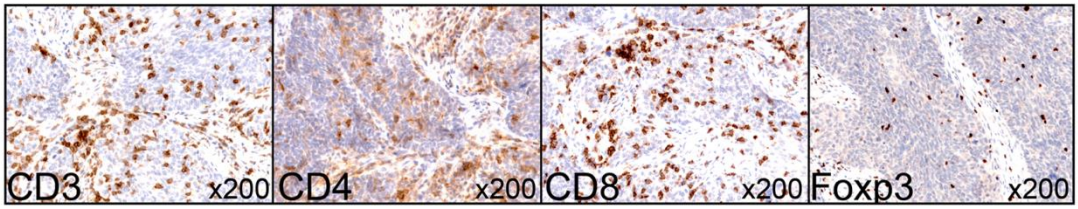
	HPV-positive N=149	HPV-negative N=121	p-value
Total T-Cell (CD4+CD8)	111.7	53.7	<0.001
$CD8^+$	57.1	24.0	<0.001
$CD4^+$	54.0	29.9	<0.001
$Foxp3^+$	32.1	16.7	<0.001
Proportion $CD8^+$	51.1%	44.7%	<0.001
Proportion $CD4^+$	48.9%	54.0%	<0.001
Proportion $Foxp3^+$	31.2%	36.8%	0.08
CD4:CD8 Ratio	0.96:1	1.21:1	<0.001
Foxp3:CD8 Ratio	0.78:1	0.69:1	<0.001

Table 4. 4: T-cell subset counts and ratios according to HPV status

To establish whether detailed T-cell counts provided more prognostic information than H&E graded TIL-levels alone in HPV-positive patients, ROC analysis was performed using death from OPSCC at 3 years as the dependent variable. When TIL-levels alone were used as the predictor variable, the area under the curve was 76%. None of the

individual T-cell counts or ratios outperformed TIL-levels as a predictive marker, with the highest AUC of 70% seen for the total T-cell count (figure 4.6).

A



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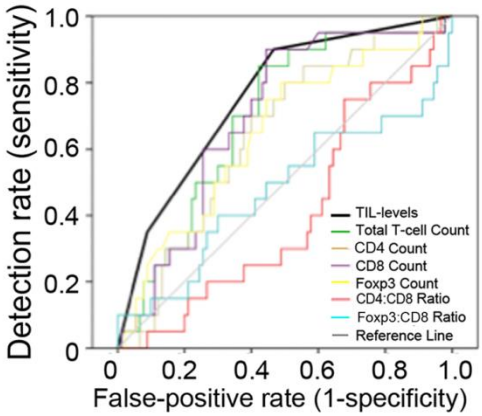


	AUROC
TIL-levels	0.76
Total T-cell Count	0.70
CD4+	0.64
CD8+	0.70
Foxp3+	0.65
CD4:CD8 ratio	0.41
Foxp3:CD8 ratio	0.48

Figure 4. 6 : A. Typical immunohistochemical staining for CD3, CD4, CD8 and Foxp3. B. ROC analysis for prediction of 3-year disease specific survival from OPSCC according to TIL-levels (graded on H&E sections) and T-cell subsets/ratios

4.3.8 Why do TIL-levels vary in HPV-positive OPSCC?

Antigen presentation is a central process in the adaptive immune system. Foreign antigens are presented to the T-cell receptor (TCR) on the surface membrane of T-lymphocytes, and this facilitates their recognition and subsequent activation of the T-lymphocyte. In the absence of antigen presentation, T-lymphocytes fail to recognize antigens. The TCR is only able to recognize antigenic peptides when they are bound to cell surface molecules known as MHC (major histocompatibility complex). MHC-class I is present of the cell surface of all nucleated cells, whilst MHC-class II is only expressed by "professional" antigen presenting cells such as dendritic cells. CD8-positive cytotoxic T-lymphocytes respond to antigens presented on MHC-class I, while MHC-class II is required for the activation of CD4-positive helper T-lymphocytes (442). HPV is known to alter MHC-class I expression (443), and it is possible that either differential levels of dendritic cells, or altered expression of MHC class I are responsible for differences in the levels of TILs seen in HPV-positive OPSCC. Another explanation for reduced TIL-levels in some tumours may relate to the surrounding stroma, rather than the tumour cells, and specifically stromal myofibroblasts. Our

group has previously shown that the presence of high levels of smooth muscle actin (SMA; a myofibroblast marker) is an independent negative prognostic marker in a large cohort of oral cancers (156). In reviewing the H&E histology slides in this current study, it became clear that in a number of cases, myofibroblastic stroma was often associated with low levels of TILs (figure 4.7), and there is some evidence that myofibroblasts may be immunosuppressive (444). It is therefore possible that variations in the levels of myofibroblasts might account for altered TIL levels in some tumours. In an attempt to establish why TIL-levels vary in HPV-positive OPSCC, correlations were thus established between TIL-levels and CD1a (a dendritic cell marker), MHC class I and SMA.

Correlations between TIL-levels, CD1a, MHC Class I and SMA are shown in table 4.5. There was no significant difference in TIL-levels according to either CD1a or MHC-1 status (p=0.54 and p=0.84). In contrast to this, SMA expression strongly correlated with TIL-levels. Two thirds of TIL^{high} tumours were SMA^{low}, while there were no tumours that were both TIL^{high} and SMA^{high} (p<0.001, table 4.5). When TIL levels were summarised as low or moderate/high, increasing levels of positive SMA staining were associated with a significantly increased number of TIL^{low} tumours (percentage TIL^{low}: SMA^{low} 8.8%, SMA^{mod} 18.9%, SMA^{high} 35.7%, p=0.03).

		HPV-Positive OPSCC n=147*			
		Number (%)			
		TIL^{low} n=22	**TIL^{mod} n=53**	**TIL^{high} n=72**	**p-value**
CD1a	*Low*	12 (54.5)	31 (58.5)	36 (50.0)	
	Moderate	3 (13.6)	14 (26.4)	19 (26.4)	0.54
	High	5 (22.7)	7 (13.2)	16 (22.2)	
	Not Known	2 (9.0)	1 (1.9)	1 (1.4)	
MHC-1	*Low*	8 (36.4)	28 (52.8)	35 (48.6)	
	Moderate	9 (40.9)	22 (41.5)	29 (40.3)	0.84
	High	2 (9.0)	3 (5.7)	3 (4.2)	
	Not Known	3 (13.6)	0 (0.0)	5 (6.9)	
SMA	*Low*	6 (27.3)	22 (41.5)	48 (66.7)	
	Moderate	10 (45.5)	22 (41.5)	21 (29.2)	<0.001
	High	5 (22.7)	9 (17.0)	0 (0.0)	
	Not Known	1 (4.5)	0 (0.0)	3 (4.2)	

*n=147 as TIL-levels unknown in 2 HPV-positive tumours

Table 4. 5: Relationships between TIL levels and Dendritic Cells (CD1a), MHC Class 1, and Myofibroblasts (SMA) in HPV-positive OPSCC.

The mean T-cell counts and ratios for the individual T-cell subsets are shown in the appendix 4 for the three markers studied. There were no significant differences in any of the T-cell subtypes or ratios according to MHC class I level, while the only significant difference seen for CD1a was in the mean Foxp3^{+} T-cell count, between CD1a^{low} and CD1a^{high} tumours (mean FOXp3^{+} count: CD1a^{low} 27.72, CD1a^{high} 43.95, p=0.04). With the exception of the proportion of CD4^{+} and CD8^{+} T-lymphocytes, significant differences were seen for all T-cell counts and ratios according to SMA-status, with SMA^{high} tumours having lower mean counts for all the T-cell subsets, and higher CD4:CD8 and Foxp3:CD8 ratios.

4.3.9 Prediction of 3-year disease specific survival

To further clarify the important prognostic factors in HPV-positive and HPV-negative OPSCC, likelihood ratios (LR, i.e. detection rate divided by false positive rate) were calculated for 3-year disease specific death. Table 4.6 shows the prognostic performance of each factor (using DR and FPR) for predicting 3-year OPSCC mortality in HPV-positive and HPV-negative patients. Commonly reported prognostic factors are included. In HPV-positive OPSCC, TIL-levels were the best performing factor, with a likelihood ratio for TIL^{low} tumours of 3.3, followed by heavy smoking and advanced T-stage (LR 2.78 and 2.44, respectively). In HPV-negative OPSCC, advancing age, advanced stage, and poor differentiation were the predictive factors with the highest likelihood ratios (Table 4.6).

4.3.10 Development of a prognostic model for HPV-positive OPSCC

The results above demonstrate that the combination of HPV-status and TIL-levels, or HPV-status and smoking-status, provided improved prognostic capabilities above and beyond either variable in isolation. In an attempt to maximize prognostication in HPV-positive tumours, logistic regression was used to create a predictive model. Using the UHS cohort as a training set, logistic regression was used to develop a predictive model for survival from HPV-positive OPSCC (at 3-years). The initial variables entered into the model were: age, T-stage, N-stage, grade, HPV-status, TIL-level, alcohol history, EGFR and smoking history. The final model included TIL-levels (low versus moderate or high), T-stage (T1/2 vs. T3/4), and smoking status (heavy versus light-, ex-, or non-smoker). The regression parameter estimates were used to create a prognostic score [equation: -4.948 + 2·768 (if a current heavy smoker) + 2·310 (if TILlow) + 2.928 (if T3/4)]. ROC analysis was performed and produced an area under the curve of 0·87 (Figure 4.8A). A cut off score of -0·945 was selected based on this ROC curve (DR 72.7%, FPR 10.2%; high risk n=14) and applied to the PFT/BLT (test; high risk n=7) cohort. This score was highly predictive of 3-year mortality, with 66.7%

of deceased patients testing positive (DR=67%) compared to just 5.6% of those alive (FPR=5.6%). The resultant likelihood ratio for prediction of 3-year mortality was 11.9 (PFT/BLT AUROC 0·82; Figure 4.8B). Replacing TIL-levels with counts or ratios of T-lymphocyte subsets did not improve this model.

	Prognostic Values for prediction of Death from OPSCC at 3 years					
	HPV-Positive OPSCC			**HPV-negative OPSCC**		
	DR % (21 deaths)	**FPR %** (95 alive)	**LR**	**DR %** (41 deaths)	**FPR %** (53 alive)	**LR**
Age, years						
≥50	57.1	72.6	0.79	85.4	81.1	1.05
≥55	47.6	51.6	0.92	73.2	58.5	1.25
≥60	47.6	30.5	1.56	61.0	35.8	1.70
Gender						
Male	81.0	66.3	1.22	85.4	62.3	1.37
Smoking						
Smoker >10/day	77.8	27.9	2.78	71.4	66.7	1.07
Alcohol						
Drinker	72.2	86.1	0.84	75.9	87.5	0.86
Stage						
≥II, III, IV	100.0	97.9	1.02	97.6	73.6	1.33
≥III, IV	95.2	93.7	1.02	80.5	56.6	1.42
IV	90.5	76.8	1.18	68.3	41.5	1.65
T-Stage						
>T3, T4	57.1	23.4	2.44	43.9	28.8	1.52
Nodal Metastases						
Yes	95.2	88.4	1.08	78.0	45.3	1.72
N-Stage						
>N2b	76.2	49.5	1.54	58.5	30.2	1.94
Differentiation						
Moderate, Poor	95.2	98.9	0.96	100.0	98.1	1.02
Poor	66.7	74.7	0.89	53.7	34.0	1.58
EGFR						
Positive	66.7	52.6	1.27	78.0	75.5	1.03
TILs						
Low, Moderate	90.0	46.8	1.92	85.4	84.9	1.01
Low	35.0	10.6	3.30	53.7	41.5	1.29

Table 4. 6: Likelihood ratios for 3 year survival from HPV-positive and HPV-negative OPSCC

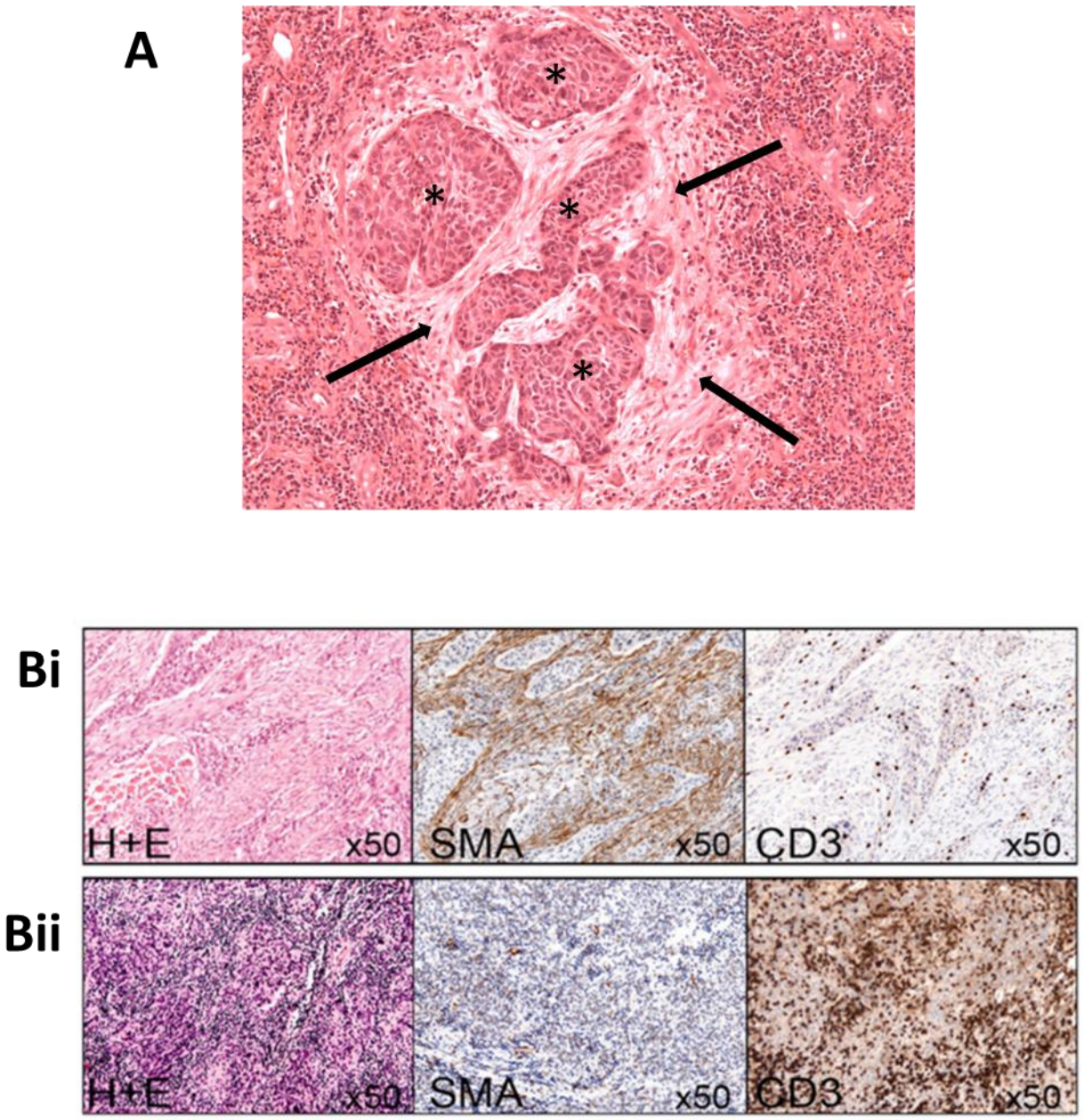


Figure 4. 7: Figure 4. 7: A: H&E section (x50) showing tumour islands (starred) surrounded by a myofibroblastic stroma (arrows). There is a dense stromal inflammatory response but little by way of intra-tumoral inflammation. Bi: Serial sections of a tumour with a prominent myofibroblastic response (strong SMA staining) and a very poor immune response (weak CD3 staining). Bii: Serial sections of a tumour with a dense inflammatory response (high CD3) and a very low number of myofibroblasts (weak SMA).

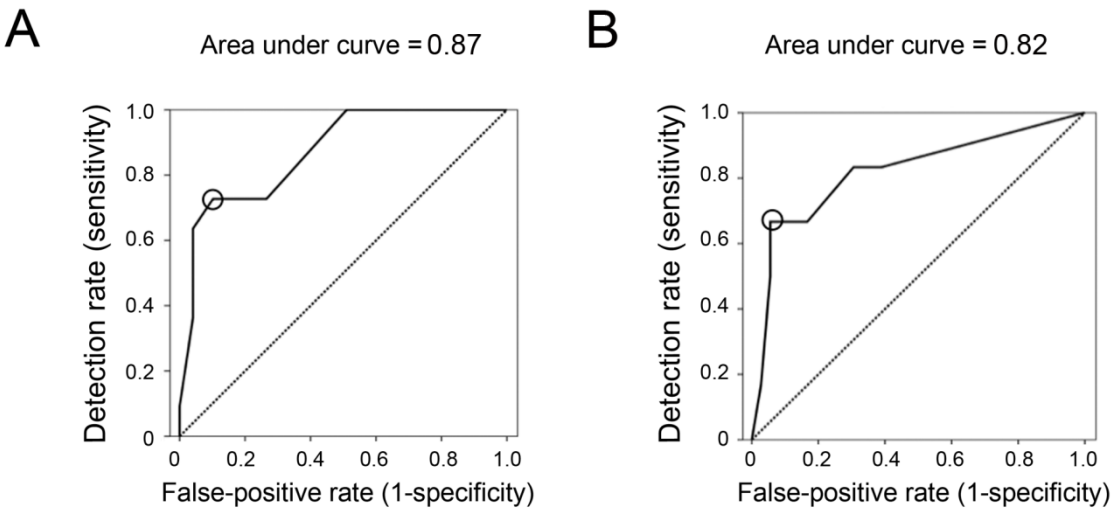


Figure 4. 8: Receiver operating characteristic (ROC) curves for 3-year HPV-positive OPSCC mortality. A: Training Set (UHS). B: Test Set (PFT/BLT)

4.4 Discussion

In recent years there has been a considerable increase in the prevalence of oropharyngeal cancer as a result of an epidemic of HPV-positive tumours. HPV-positive OPSCC typically present at an advanced stage and is poorly differentiated. Despite this, there is an increasing body of evidence demonstrating that patients with HPV-positive OPSCC have significantly improved survival, although the biological reasons for this remain unclear (312). This survival benefit is seemingly independent of treatment modality, and this has led to calls for de-escalation of treatment in an attempt to reduce therapy-associated morbidity (99, 100, 312, 353, 356, 358, 359, 362). Indeed, there are currently several treatment de-escalation trials underway on both sides of the Atlantic (91, 386, 387). One important caveat of the improved outcomes seen in HPV-positive tumours is the fact that a significant minority of HPV-positive OPSCC patients experience treatment failure and have poor prognosis. Consequently it has been suggested that future clinical investigations concentrate on this group in order to improve outcome, and also to avoid treatment de-escalation in these patients (312). There is growing evidence that smoking can alter survival in HPV-positive patients, and conflicting evidence regarding interactions between HPV and EGFR expression as a prognostic indicator (99, 357, 365, 377, 445). However, currently there is no single clinical, pathological, or molecular feature that can be used to identify poor prognosis in HPV-positive patients prior to therapy.

A possible explanation for improved survival of HPV-positive patients is that virally-driven tumours provoke an adaptive immune response directed against tumour-expressed viral antigens (oncoproteins). Cytotoxic $CD8^+$ T-cells are the principal anti-tumour effector cells, and their abundance has been shown to be a predictor of positive outcome in several tumour types, particularly colorectal cancer, suggesting that the adaptive immune system plays a role in suppressing tumour progression (401, 403). Quantification of densities of various T-cell subsets, including $CD8^+$, $CD4^+$ and T_{reg} cells ($FoxP3^+CD4^+$), or subset ratios, has been suggested to improve predictive power over that of absolute T-cell number (391). There is conflicting evidence for a role of TILs in head and neck malignancy, and few studies specifically investigating HPV-associated oropharyngeal cancer (441).

A number of recent findings have implicated a role for the immune system in HPV-positive OPSCC. HPV-16 E7 specific CD8-positive T-cells have been detected in higher levels in the blood of patients with HPV-positive OPSCC than those with HPV-negative disease. Furthermore, these T-cells have been shown to proliferate in response to viral peptides, and to recognize HPV16 E7-positive cells in vitro (418, 446). More recently,

T-cells have been isolated from HPV-positive oropharyngeal tumours and draining lymph nodes, and shown to proliferate and produce cytokines in response to HPV-16 E6 and E7 antigens, implicating a role in the anti-tumour response (422). Liang and colleagues have shown a stronger survival advantage in HPV-positive patients with seropositivity to E6/E7 (447), while infiltration of HPV-positive HNSCC by PD-1-expressing T-lymphocytes has been shown to be a favourable prognostic factor (448). Finally, animal models have demonstrated that an intact immune system is vital in the clearance of HPV-positive tumours (369).

This study was conducted to examine the effects of HPV-status, TIL-levels and smoking on patient survival using two cohorts of OPSCC patients. Similar to other large studies, HPV-positive tumours were associated with significantly improved survival (3-year survival: HPV-positive 82% versus HPV-negative 56%, $p<0.001$); pathological and clinical features of HPV-positive tumours were consistent with these series. HPV-positive OPSCC were generally late stage and poorly differentiated (both $p<0{\cdot}001$). HPV-positivity inversely correlated with smoking, and with EGFR expression (both $p<0{\cdot}005$).

4.4.1 TILs as a predictive marker in OPSCC

TIL-levels (based on H&E grading) correlated significantly with HPV-status ($p<0.001$), with around 85% of HPV-positive OPSCC tumours containing high or moderate levels of TIL. This may indicate an adaptive anti-tumour response, and suggests a possible mechanism for improved outcome in these patients. TIL-levels stratified HPV-positive OPSCC patients into those with good and poor prognosis. The 3-year survival for HPV-positive/TIL^{high} tumours was 96% compared to 76% for HPV-positive/TIL^{mod}, and 59% for HPV-positive/TIL^{low}. The HPV-positive/TIL^{low} tumours had similar survival to HPV-negative tumours (3-year survival 56%). There is some literature reporting that TILs can predict survival in head and neck cancer. Previous work by our group using the same grading system as for this current study, demonstrated that TIL^{high} oral cavity SCC had improved disease specific survival compared to TIL^{low} tumours on univariate, though not multivariate, analysis (156). Brandwein-Gensler et al, using a semi-quantitative grading system of lymphocytic infiltrate at the invasive margin of 292 oral cavity tumours, found that a poor lymphocytic response was associated with a significantly increased risk of local recurrence and a reduction in overall survival (404). However, this is the first study to report an association between a simple semi-quantitative grading system for TILs and survival in HPV-associated oropharyngeal cancer.

As mentioned previously, it has been suggested that quantification of T-cell subsets can increase the prognostic ability of TILs (391). Therefore densities of CD4-, CD8-, and Foxp3-positive T-cells were also quantified and ROC analysis carried out to determine the predictive value of cell numbers or subset ratios. These more complex analyses did not outperform the simpler scoring method performed on H+E-stained sections. This is an important point in terms of the applicability of TIL grading in routine clinical practice. The semi-quantitative grading system utilized in this study is straightforward to learn, quick, and is performed on a routine H&E stained slide. In contrast, quantification of TIL subsets requires additional immunohistochemistry, with its associated costs, and is incredibly time consuming, thus rendering it less than ideal in a busy clinical setting. Furthermore, the use of individual cell counts as a prognostic marker ideally requires the setting of arbitrary cut-off points to divide patients into high and low risk groups. This raises the question of where these cut-off points should be set. Other authors have used the median cell count as a cut-off point, or divided patients up according to quartiles. The finding that individual cell counts do not outperform the simple semi-quantitative grading system on ROC analysis renders this point moot, in a clinical setting at least. It is possible that lymphocyte counts performed on randomly selected TMA cores may not be representative of the tumour as a whole. However, TMA cell counts significantly correlated with whole section TIL levels, arguing against this (e.g. total [CD3+] T-lymphocyte count: TIL^{low} 42.9, TIL^{mod} 60.4, TIL^{high} 96.3, $p<0.001$).

4.4.2 Differences in T-cell counts between HPV-positive and HPV-negative tumours

All T-cell subsets were found at higher levels in HPV-positive tumours compared to those that were HPV-negative, reflecting the generally increased levels of TILs seen on H&E examination. Most significant, perhaps, was the finding that the CD4:CD8 and Foxp3:CD8 ratios were significantly lower in HPV-positive than in HPV-negative tumours. Indeed, in HPV-positive tumours $CD8^+$ T-cells made up a significantly higher proportion of the total TIL infiltrate than in HPV-negative tumours (0.49 vs. 0.39, $p<0.001$). It has been shown that $CD8^+$ T-lymphocytes are the major effector in the anti-tumour T-cell response (403), and thus the finding that they are present in higher numbers (both absolute and as a proportion of the total infiltrate) suggests a significant anti-tumour activity in HPV-positive tumours. In addition, the lower levels of "pro-tumour" regulatory T-cells seen in HPV-positive tumours may also contribute to a more anti-tumour microenvironment.

Other authors have also suggested an important role for $CD8^+$ T-cells in the improved prognosis of HPV-positive OPSCC. Jung *et al* recently examined 144 oropharyngeal

tumours for differences in the expression of immune related genes, and 17 of these (7 HPV-negative and 10 HPV-positive) for the presence of $CD4^+$ and $CD8^+$ T-cells using a semi-quantitative scoring system based on immunohistochemical staining of tumour tissues. They preferentially selected tumours with high levels of TILs, so do not comment on the relative overall TIL levels between HPV-positive and HPV-negative tumours: However, similar to the findings presented here, they found a higher proportion of $CD8^+$ T-lymphocytes in HPV-positive tumours. Furthermore, they found that several immune related genes, notably CD3ζ, CD8α and CD4, had a significant upregulation in mRNA expression in HPV-positive tumours (e.g. CD8α was upregulated in 42% of HPV-positive tumours vs. 10% of HPV-negative). Interestingly they found that patients (unstratified by HPV-type) with upregulated CD8 mRNA expression had significantly improved 5-year survival, and that HPV-positive patients who did not have upregulated CD8 mRNA expression had significantly poorer survival than those who did (449). The same authors have previously identified 850 genes that were differentially expressed in HPV-positive and HPV-negative tumours. The majority of these encode proteins involved in immune signaling pathways, for example cytokines involved in T-cell recruitment, components of MHC-class 1 and subunits of the T-cell receptor, further highlighting the importance of the immune system in HPV-positive OPSCC (330).

Wansom *et al* examined levels of $CD4^+$ and $CD8^+$ T-lymphocytes in peripheral blood and found that patients with HPV-positive tumours had a higher proportion of $CD8^+$ lymphocytes (HPV-positive 20% vs. HPV-negative 15%, $p=0.04$), and a lower CD4:CD8 ratio (HPV-positive 2.5:1 vs. HPV-negative 3.5:1, $p=0.02$) than patients with HPV-negative tumours. They also found that a lower CD4:CD8 ratio, and a higher percentage of $CD8^+$ cells, were associated with improved survival ($p=0.01$ and $p=0.04$) (421). In a more recent study by the same group, higher total t-cell count (CD4+CD8) and a lower CD4:CD8 ratio within tumour biopsies were found to be significantly associated with improved overall and disease specific survival, and to correlate with peripheral blood levels, in 46 patients with advanced OPSCC. They interestingly found no significant differences in T-cell subsets and ratios between HPV-positive and HPV-negative tumours, though did report a trend towards a lower CD4:CD8, and Foxp3:CD8 ratio in HPV-positive tumours ($p=$not reported and $p=0.099$, respectively), mirroring the findings of this current study. Despite finding no significant differences in T-cell counts according to HPV-status, the survival benefit of increased TIL levels remained after adjustment for HPV-status (423).

In keeping with previously published studies (411, 450), a finding of this study was that infiltration with FoxP3-positive regulatory T-lymphocytes was associated with improved survival (figure 4.6B). However, it is likely that the increased numbers seen in

HPV-positive tumours simply reflect an increase in overall T-lymphocyte numbers in these tumours. Indeed, the proportion of Foxp3-positive cells was in fact reduced in HPV-positive tumours, compared to those that were HPV-negative (table 4.4). Interestingly, TIL-levels did not predict for survival in HPV-negative tumours.

One interesting finding of the current study was that TIL-levels outperformed HPV-status as a prognostic marker, both in terms of predicting survival and also predicting death. The hazard ratios for overall and disease specific survival indicated a 72% and a 76% reduction in the risk of death for TIL^{high} tumours, compared to a 47% and 60% reduction for HPV-positive tumours. The likelihood ratio for 3-year disease specific death for TIL^{low} tumours outperformed that of HPV-negative tumours, being 2.21 and 1.85 respectively. Interestingly, this is not the first report of the immune response outperforming HPV-status as a prognostic marker in OPSCC. In the study by Jung *et al* discussed above, the p-values for improved survival were higher for CD8 mRNA upregulation than they were for HPV (log rank test, HPV p=0.012, CD8 p=0.005)(449). Wansom *et al* found that peripheral CD8 levels were more predictive of response to neoadjuvant chemotherapy than HPV-status (p=0.01 and p=0.04, respectively) (421). Thurlow *et al* found that HPV-positive tumours expressed adaptive immune response genes, and that this gene signature was a stronger predictor of survival that HPV-status in unstratified disease (414). Although these findings can potentially be explained, in part, by the strong correlation seen between HPV-positive tumours and a prominent immune response, this would not account for the outperformance as a prognostic marker of HPV-status by immune response.

4.4.3 Smoking and HPV-positive OPSCC

Several studies have reported that smoking is an important prognostic indicator in HPV-positive OPSCC, with heavy smoking reducing the survival benefit of HPV-positivity (99, 334, 357, 365, 440). In particular, Ang *et al* describe 3 levels of risk in OPSCC based on a combination of HPV-status, smoking (less than or greater than 10 pack years) and nodal status. HPV-positive patients with a heavy smoking history fell into an intermediate risk category, with a 71% 3-year survival compared to 93% in those with a lower smoking history (99). The findings of this study reflected this, and HPV-positive current heavy-smokers had reduced survival benefit compared with non-, ex-, or light-smokers (3 year survival; HPV-positive/current-heavy smoker 63% versus HPV-positive/non-, ex-, current-light smoker 94%; p<0.001). Furthermore, HPV-positive heavy smokers had no difference in the risk of all-cause or disease specific death than patients with HPV-negative tumours (HRs: OS 1.08, p=0.81; DSS 0.86, p=0.62), while those who were light/non/ex-smokers had a 64% reduction in the risk of all-cause, and an 86% reduction in the risk of disease specific death (HRs: OS

0.36, p=0.001; DSS 0.14, p<0.001). One of the proposed theories for the improved survival seen in HPV-positive OPSCC is the presence of wild type p53 and the absence of field cancerisation (5, 40). It has been suggested that heavy smoking could potentially modify these factors, through the development of genetic abnormalities typically associated with tobacco exposure, leading to increased disease recurrence and subsequently reduced survival (357, 365, 377). In support of this suggestion, Maxwell *et al* found that HPV-positive current smokers were at a 5-fold increased risk of tumour recurrence compared to HPV-positive never smokers (375). In the current study, 31.6% of HPV-positive heavy smokers had either treatment failure, late recurrence or late distant metastases, compared to only 9.2% of HPV-positive light, ex or non-smokers (p=0.003), supporting these suggestions.

4.4.4 Why does the immune response to HPV-positive OPSCC vary?

The findings of this study, and others, suggest a strong role for the immune system for improved survival of patients with HPV-positive tumours. However, approximately 15% of HPV-positive patients had a poor immune response (TIL^{low}, n=22) and these patients had similar survival to those who were HPV-negative. Smoking has been shown to have a number of immunosuppressive effects and one potential explanation for the differences observed in TIL-levels among HPV-positive tumours might be differing rates of smoking (451). However, although there was a tendency towards increased rates of heavy smoking in patients with TIL^{low} tumours, this was not statistically significant. The only difference seen in T-cell subsets between HPV-positive heavy and light smokers was in the proportion of $CD8^+$ T-cells, which were significantly higher in patients with less than a 10 pack year smoking history. It is possibly that poor documentation in retrospective records may be responsible for this lack of correlation, and this should be examined prospectively. However, these findings echo those of Wansom *et al*, who found no differences in the levels of peripheral T-lymphocytes of HPV-positive smokers and non-smokers, but higher total T-cell infiltrates (i.e. CD4+CD8) in the tumours of non-smokers (421, 423). Further studies are required to further explore the relationship between smoking and TILs in HPV-associated tumours, and to try to establish why some patients raise a prominent immune response against their tumour whilst others do not.

MHC class I expression was lower in HPV-positive tumours compared with HPV-negative tumours (MHC-1 low/moderate: HPV-positive 94.4%, HPV-negative 77.4%, p<0.001) in keeping with the suppression of MHC class I expression by the HPV E5 gene product (452). Despite this, HPV-positive tumours had significantly higher TIL levels than HPV-negative tumours. However, there were no significant differences in MHC class I expression between HPV-positive tumours that were TIL^{low} and those that

were $TIL^{mod/high}$. There was also no significant difference in the expression of CD1a according to TIL-levels for HPV-negative tumours. These findings are perhaps surprising, especially given that both MHC class I and CD1a have been shown to correlate with TIL levels in a number of other tumour types (453-455). Although there were no significant differences in MHC-expression according to TIL-levels, there was a trend towards increased MHC class I expression in TIL^{low} tumours. This raises the possibility that $TIL^{mod/high}$ tumours are able to upregulate MHC class I expression and thus the presentation of HPV antigens in response to treatment, whilst TIL^{low} tumours may potentially already be expressing maximal levels of MHC class I. If this were the case, then upregulation of MHC class I in response to therapy might be expected to results in further $CD8^+$ CTL recruitment and activation, and destruction of any remaining tumour cells. This is speculative, and would require further investigation. Of course, CD1a is just one of a number of dendritic cell markers, and MHC class I is just one of a number of factors involved in antigen presentation and T-cell recruitment. It is therefore possible that other factors not investigated in this study are altered according to TIL levels in HPV-positive OPSCC, and this requires further investigation.

Any solid tumour contains both tumour cells and "normal" stromal cells. There is now evidence that tumour development and progression depends not only on the tumour cells themselves, but also the stromal cells (456). One of the most important groups of "normal" cells in terms of tumour progression are the myofibroblasts, also known as "cancer associated fibroblasts". These are activated fibroblasts that are characterised by the expression of smooth muscle actin (SMA) (457). Myofibroblasts are capable of secreting several soluble factors, such as growth factors, cytokines, and pro-angiogenic factors, that aid the proliferation and survival of the tumour cells (458, 459).There is also some evidence that SMA-positive cells are able to alter immune infiltrates (458, 459). Previous work by our group has demonstrated that myofibroblasts are an independent negative prognostic marker in oral cancer, and in that study SMA expression was inversely correlated with TILs (156). In this current study, the only factor that significantly correlated with TIL-levels in HPV-positive OPSCC was the expression of SMA. In tumours that were TIL^{low}, 68.2% had either moderate or high SMA expression, compared to only 41.6% of those that were TIL^{mod} or TIL^{high}. Furthermore, of the HPV-positive tumours with high SMA expression, none were graded as TIL^{high}. When analysing individual T-cell subsets, SMA^{high} tumours had significantly lower mean counts than SMA^{low} tumours for all four T-cell counts analysed (Total T-cell: SMA^{low} 141.9, SMA^{high} 42.3, $p<0.001$; $CD4^+$: SMA^{low} 67.3, SMA^{high} 24.9, $p=0.001$; $CD8^+$: SMA^{low} 73.7, SMA^{high} 17.3, $p<0.001$; $Foxp3^+$: SMA^{low} 37.9, SMA^{high} 12.5, $p=0.008$).

Other authors have also shown that increased levels of SMA-positive cells (myofibroblasts) are associated with alterations in the levels of tumour infiltrating lymphocytes in different tumour types. In a mouse model of colorectal carcinoma, tumours that developed a prominent myofibroblastic response were found to have a distinct pattern of T-cell infiltration, with TILs separated from tumour islands by large myofibroblast sheets (460). Interestingly, this pattern was also noted in a number of tumours in this current study (figure 4.7). Lieubeau *et al* also found that the presence of myofibroblasts in a collagen gel was shown to reduce T-cell penetration of the gel, suggesting a possible mechanical mechanism by which T-cells may be separated from tumour islands by SMA-positive myofibroblasts (460). Another potential mechanism by which myofibroblasts might alter TIL levels is through the promotion of apoptosis. T-lymphocytes express a surface receptor known as PD1 (programmed death 1) which, on interacting with its ligand, PD-L1 (PD-ligand 1), stimulates apoptosis or anergy of the T-cell (461, 462). PD-L1 is normally expressed by a number of cells, including antigen presenting cells and activated T-cells, where it acts to regulate T-cell activation and is thought to be particularly important in preventing autoimmunity (463). Myofibroblasts have also been shown to express PD-L1, and it might be expected that high PD-L1 expression by stromal myofibroblasts would be associated with lower TIL levels (456). Recently, Cho *et al* investigated the relationship between PD-L1 expression and TIL levels in oral cancer, and found that both tumour cells and stromal myofibroblasts expressed PD-L1. High expression of PD-L1 by tumour cells was associated with reduced TIL-levels, though there was no association between TIL-levels and PD-L1 expression by myofibroblasts (464). It would be interesting to compare expression of PD-L1 by stromal and tumour cells in the HPV-positive/TIL^{low} tumours in this study with that of HPV-positive/TIL$^{mod/high}$ tumours.

In support of an interaction between myofibroblasts and TILs in HPV-associated tumours, Gorter *et al* recently showed that in cervical carcinoma, another HPV-related malignancy, increased expression of the extracellular matrix component Versican was significantly associated with reduced levels of TILs, and in particular CD8$^+$ CTLs. In this study, double immunofluorescence demonstrated that Versican was secreted predominantly by SMA-positive myofibroblasts (465). Interestingly, versican has been shown to bind to several T-cell chemo attractants, such as CCL5, which is a potential explanation as to why it might alter T-cell infiltration into tumours (466). To date this remains the only other description of an associated between myofibroblasts and reduced TIL levels in an HPV-associated cancer .High expression of versican has also been shown to be an independent predictor of poor outcomes in oral cancer, highlighting the important role of stromal features in HNSCC (467).

In addition to having lower counts for all the T-cell subsets, SMA^{high} tumours also had higher CD4:CD8 and, perhaps more importantly, Foxp3:CD8 ratios. Regulatory T-cells, characterised by the expression of Foxp3, have been shown to regulate the anti-tumour activity of $CD8^+$ cytotoxic T-lymphocytes and promote a more pro-tumour microenvironment (394, 395). Interestingly, there is evidence that myofibroblasts are capable of switching naïve $CD4^+$ T-lymphocytes towards a regulatory, $Foxp3^+$ phenotype in the colon (468). This raises the possibility that SMA-positive myofibroblasts are not only capable of reducing the intra-tumoral T-cell infiltrate, but also modulating it towards a more pro-invasive phenotype.

Building on the findings here, work by Dr Catherine Riley, a postdoctoral research fellow in our lab, has demonstrated that SMA-positive myofibroblasts are capable of reducing both the proliferation and migration of T-lymphocytes in vitro (Riley C, unpublished data). This work is ongoing and the results will hopefully clarify further the relationships between myofibroblasts and TILs.

4.4.5 A prognostic model for HPV-positive OPSCC

Likelihood ratios were calculated to identify the best markers for predicting high risk patients, i.e. those who would die by 3-years post treatment.. These indicate the strength of a marker by maximizing the detection rate whilst minimizing the false-positive rate. In HPV-positive patients low TIL-levels and smoking-status were the best predictors of mortality at 3 years (likelihood ratios 3.3 and 2.8 respectively). Using these markers, 36.8% of heavy smokers, and 41.2% of TIL^{low} patients were dead at 3-years.

This was reflected in our prognostic model where, in the 'training' set (UHS patients) only TIL-levels, smoking, and T-stage were significant (AUROC analysis 0.87). When this model was applied to the 'validation set' (PFT/BLT cohort) it was found to be highly predictive of 3-year survival (DR 66.7%; FPR 5.6%; LR for 3-year mortality 11.9). This LR is better than other currently used predictive tests. For example, the unrehydrated guaiac faecal occult blood test used in screening for colorectal carcinoma has a LR of 6.4 (DR 64%, FPR 10%) (469). The 3-yr survival of low risk HPV-positive patients (score < -0.945) was 94% compared with 36.8% for those that were classified as high risk (score > -0.945, $p<0.001$).

Ang et al have suggested a predictive model for HPV-positive OPSCC where patients who are heavy smokers (> 10 pack year history) and have advanced nodal disease (>N2b) are at increased risk of death. We compared this model to our own, and calculated both LRs and 3-year survival rates. The LR for the Ang model in our

combined cohort was 4.8 (DR 61.1%, FPR 12.8%; compared with LR=8.6 [DR=70.6% FPR=8.2%] using our model) and the 3-year survival was 91.5% for low risk patients and 50% for high-risk patients (compared with 94% and 36.8% respectively using our model). Clearly both models have significant predictive power. Interestingly, we found no association between TIL-levels and nodal status in HPV-positive OPSCC ($p=0.43$).

4.5 Conclusions

HPV-status is an important predictor of survival in oropharyngeal cancer. HPV-positive tumours are frequently associated with a prominent lymphocytic infiltrate, and this infiltrate predicts for survival in both unstratified and HPV-positive OPSCC, suggesting that the reason for improved survival in most HPV-positive OPSCC is the presence of an adaptive host anti-tumour immune response. In fact, TIL levels are arguably a better predictor of survival than HPV-status. Detailed analysis of the TIL infiltrate identifies differences between HPV-positive and HPV-negative tumours, with HPV-positive tumours having a more "anti-tumour" phenotype. However, this quantification is time consuming and does not add any further prognostic power over simple H&E evaluation alone. HPV-positive tumours without a prominent immune response are at a high risk of death, with survival outcomes comparable to HPV-negative tumours. Smoking also predicts for survival in HPV-positive OPSCC, and HPV-positive heavy smokers are at increased risk of death and tumour recurrence.

When smoking and TIL levels are combined, prognostic power improves further, and a prognostic model based on TIL-levels, heavy-smoking, and T-stage is extremely effective at identifying a group of HPV-positive patients with poor survival. TIL-evaluation can be performed quickly on diagnostic biopsy, and more complex analysis of the T-cell infiltrate does not improve on the predictive power of the simpler scoring method. This is an effective tool for identifying HPV-positive OPSCC patients who are likely to respond poorly to treatment. These results are highly relevant for the clinical evaluation and treatment of OPSCC patients, particularly when enrolling patients into clinical trials seeking to de-intensify treatment.

5 Staging and Treatment of Oropharyngeal Cancer in the HPV Era

5.1 Introduction

The TNM classification proposed by the AJCC is the most commonly used staging system in head and neck malignancy (39, 41)(39, 41)(39, 41)(39, 41)(39, 41)(39, 41)(39, 41)(39, 41)(39, 41)(39, 41)(39, 41). This system follows the TNM (tumour, node, metastasis) format, with the individual components combined to give an overall disease stage from I to IV which helps guide treatment and predict prognosis. The American Cancer Society quotes 5-year survival rates for oropharyngeal cancer from 56% for stage I disease to 43% for stage IV disease (http://www.cancer.org/cancer/oralcavityandoropharyngealcancer/detailedguide/oral-cavity-and-oropharyngeal-cancer-survival-rates accessed 19/01/2014). Historically, the most important component of the TNM stage, in terms of prognosis, is the presence of nodal metastases at diagnosis, which are reported to reduce 5-year survival by 50% (1).

The incidence of oropharyngeal squamous cell carcinoma (OPSCC) has risen over the past 20 years, and is predicted to continue to rise (312). Traditional risk factors include both tobacco and alcohol consumption, both of which are reducing in the Western world (26). Virally-induced OPSCCs as a result of oncogenic human papillomavirus (HPV), specifically HPV-16, are thought to be responsible for doubling the number of OPSCC cases in the past 10 years. Early stage oropharyngeal disease, i.e. stage I or stage II, is typically treated with either surgery or radiotherapy. In contrast, stage III or IV disease is usually treated with combined modalities, i.e. surgery and post-operative radiotherapy or combined chemoradiotherapy, with or without neo-adjuvant chemotherapy, in an attempt to preserve organ function (1). Chemoradiotherapy is not without its adverse effects, and a significant proportion of patients require temporary or permanent assistance with feeding following treatment, in the form of nasogastric tubes (NG) or percutaneous gastrostomies (PEG), as a result of treatment associated dysphagia (46, 47).

The current staging system was established before HPV-driven OPSCC was recognised as a disease entity. HPV-positive OPSCC typically presents at an advanced stage (i.e. stage III or IV) and therefore might be expected to respond poorly to treatment. However, a number of studies have reported survival rates for advanced stage HPV-positive tumours that far exceed the 30-50% range that is currently accepted (470). Furthermore, the survival advantage of HPV-positive tumours has been suggested to be independent of treatment modality (99, 353, 356, 362). This has led to calls for trials of de-escalation of treatment for HPV-positive tumours, in an attempt to minimise treatment-associated morbidity whilst maintaining positive outcomes. Studies that have previously investigated the effect of HPV on survival from OPSCC are

typically single modality studies, and evidence is lacking for direct comparisons of different treatment types within the same patient cohort. Similarly, there are few published examples of whether current TNM staging accurately predicts for survival in HPV-positive tumours.

5.2 Aims

The aims of this part of the study were:

- to investigate the prognostic value of TNM staging system in the era of HPV-associated OPSCC
- to directly compare surgery, radiotherapy and chemoradiotherapy as treatments for HPV-positive OPSCC, both in terms of outcome and dependence on post-treatment assisted feeding

5.3 Results

5.3.1 Differences in TNM staging according to HPV-status

Clinical and pathological demographics are shown in Table 5.1. HPV-status was determined in 266 patients using a combination of p16 immunohistochemistry and HPV *in-situ* hybridisation, and was positive in 54.6% of tumours (table 5.1). As described in chapters 3 and 4, survival was significantly better in patients with HPV-positive disease (Log rank test $p<0.001$, HR HPV-positive vs. HPV-negative 0.33, $p<0.001$). Patients with HPV-positive tumours were more likely to present with advanced stage tumours (stage III/IV: HPV-positive 93.2%, HPV-negative 64.7%, $p<0.001$). The advanced stage in HPV-positive tumours was predominantly a result of nodal metastases, with 89.8% of HPV-positive patients presenting with nodal involvement, compared to 56.3% in HPV-negative patients ($p<0.001$). Patients with HPV-positive tumours were also significantly more likely to present with advanced nodal disease (N2b or above: HPV-positive 54.4%, HPV-negative 39.5%, $p=0.019$).

There was no difference in T-stage between HPV-positive and negative patients when all disease stages were considered (HPV-positive T1/T2 72.1% verses HPV-negative 64.7%, $p=0.29$). However, advanced stage, HPV-positive tumours were more likely to be either T1 or T2 than those that were HPV-negative (Stage III/IV, HPV-positive T1/T2 70.1% verses HPV-negative 46.8%, $p=0.002$). TNM staging stratified according to HPV-status is shown in table 5.2.

5.3.2 TNM staging as a prognostic tool in HPV-negative and HPV-positive disease

Hazard ratios (DSS) for individual components of the TNM staging system in HPV-positive and HPV-negative tumours are shown in table 5.3, while Kaplan Meier curves are shown in figure 5.1. On Kaplan Meier analysis, both overall disease stage and nodal status were significant predictors of death from OPSCC in HPV-negative tumours (Log rank tests: Stage I/II vs. Stage III/IV, $p=0.04$; N^+ vs. N^-, $p=0.007$; N2b or greater vs. N2a or less, $p=0.008$). When examining HPV-positive tumours, T-stage was strongly predictive of disease-specific survival (T1/2 vs. T3/4, $p=0.001$) but neither overall disease stage nor the presence of nodal metastases were predictive. However, in keeping with the findings of Ang et al, the presence of advanced nodal disease, i.e. N2b or greater, was a significant predictor of DSS ($p=0.05$).

Characteristic		Entire Cohort n=395 Number (%)	FFPE Tissue Unavailable n=126 Number (%)	FFPE Tissue Available n=269 Number (%)	
Gender	Male	289 (73.2)	92 (73.0)	197 (73.2)	p=1.00
	Female	106 (26.8)	34 (27.0)	72 (26.8)	
Age	<50	79 (20.0)	23 (18.3)	56 (20.8)	p=0.76
	50-69	244 (61.8)	78 (61.9)	166 (61.7)	
	70+	72 (18.2)	25 (19.8)	47 (17.5)	
	Mean (SD)	58.55 (10.82)	59.21 (10.14)	58.24 (11.13)	p=0.41
Smoking	Never Smoked	78 (19.7)	24 (19.0)	54 (20.1)	p=0.85
	Current Smoker <10/day	21 (5.3)	5 (4.0)	16 (5.9)	
	Current Smoker >10/day	168 (42.5)	55 (43.7)	113 (42.0)	
	Ex-Smoker	78 (19.7)	26 (20.6)	52 (19.3)	
	Not Known	50 (12.7)	16 (12.7)	34 (12.6)	
Alcohol	Non/Ex Drinker	51 (12.9)	18 (14.3)	33 (12.3)	p=0.82
	Current Drinker	247 (62.5)	83 (65.9)	164 (61.0)	
	Not Known	97 (24.6)	25 (19.8)	72 (26.8)	
Tumour Site	Tonsil	218 (55.2)	62 (49.2)	156 (58.0)	p=0.20
	Tongue Base	103 (26.1)	35 (27.8)	68 (25.3)	
	Other Oropharynx	74 (18.7)	29 (23.0)	45 (16.7)	
Median Length of Follow Up in Months (Range)		50 (6-137)	35 (6-130)	58 (8-137)	p=0.002
Disease Stage	I	30 (7.6)	8 (6.3)	22 (8.2)	p=0.45
	II	40 (10.1)	9 (7.1)	31 (11.5)	
	III	55 (13.9)	20 (15.9)	35 (13.0)	
	IV	270 (68.4)	89 (70.6)	181 (67.3)	
Tumour Stage	T1	105 (26.6)	30 (23.8)	75 (27.9)	P<0.001
	T2	142 (35.9)	30 (23.8)	112 (41.6)	
	T3	49 (12.4)	22 (17.5)	27 (10.0)	
	T4	99 (25.1)	44 (34.9)	55 (20.4)	
Nodal Stage	N0	98 (24.8)	30 (23.8)	68 (25.3)	p=0.80
	N1	56 (14.2)	21 (16.7)	35 (13.0)	
	N2	223 (56.5)	69 (54.8)	154 (57.2)	
	N3	18 (4.6)	6 (4.8)	12 (4.5)	
Grade	Well/Moderately Differentiated	152 (38.5)	50 (39.7)	102 (37.9)	p=0.16
	Poorly Differentiated	225 (57.0)	58 (46.0)	167 (62.1)	
	Not Known	18 (4.6)	18 (14.3)	0 (0.0)	
HPV Status	Negative	-	-	119 (44.2)	
	Positive	-	-	147 (54.6)	
	Not Known	-	-	3 (1.1)	

Table 5. 1: Demographics of patients used for TNM and treatment analysis

Characteristic		HPV-Negative (n=119) Number (%)	HPV-Positive (n=147) Number (%)	
Stage	I	20 (16.8)	2 (1.4)	
	II	22 (18.5)	8 (5.4)	
	III	16 (13.4)	19 (12.9)	
	IV	61 (51.3)	118 (80.3)	p<0.001
Stage Groups	I/II	42 (35.3)	10 (6.8)	
	III/IV	77 (64.7)	137 (93.2)	p<0.001
T-Stage	T1	33 (27.7)	42 (28.6)	
	T2	45 (37.8)	64 (43.5)	
	T3	15 (12.6)	12 (8.2)	
	T4	26 (21.8)	29 (19.7)	p=0.58
T-Stage Groups	T1/T2	78 (64.7)	106 (72.1)	
	T3/T4	41 (34.5)	41 (27.9)	p=0.29
N-Stage	N0	52 (43.7)	15 (10.2)	
	N1	14 (11.8)	21 (14.3)	
	N2	48 (40.3)	104 (70.7)	
	N3	5 (4.2)	7 (4.8)	p<0.001
Nodal Metastasis	No	52 (43.7)	15 (10.2)	
	Yes	67 (56.3)	132 (89.8)	p<0.001
Advanced N-Stage	N0-N2a	72 (60.5)	67 (45.6)	
	N2b-N3	47 (39.5)	80 (54.4)	p=0.02

Table 5. 2: TNM Staging according to HPV Status

Similar to previous studies, heavy smoking (i.e. >10 pack year history) adversely affected outcomes in HPV-positive OPSCC (log rank test p<0.001, chapter 4) (99, 357, 377). Therefore, a multivariate analysis was performed, adjusting for patient age and smoking status. In HPV-negative tumours, both overall disease stage and nodal status were significant predictors of an increased risk of death from OPSCC. Patients with stage III/IV disease had double the risk of disease-specific death compared to those with stage I/II tumours (DSS: Stage III/IV HR 2.00, p=0.05, table 5.3). The greatest increase in risk was seen for stage IV tumours, where the risk of death from OPSCC was increased more than 4-fold compared to stage I disease (DSS: Stage IV HR 4.27, p=0.02, table 5.3). Patients with HPV-negative tumours who had nodal metastases at the time of presentation had more than double the risk of death from OPSCC than those who were node negative (DSS: N^+ HR 2.19, p=0.02). T-stage did not significantly increase the risk of disease specific death for patients with HPV-negative tumours (DSS: T3/4 HR 1.23, p=0.49).

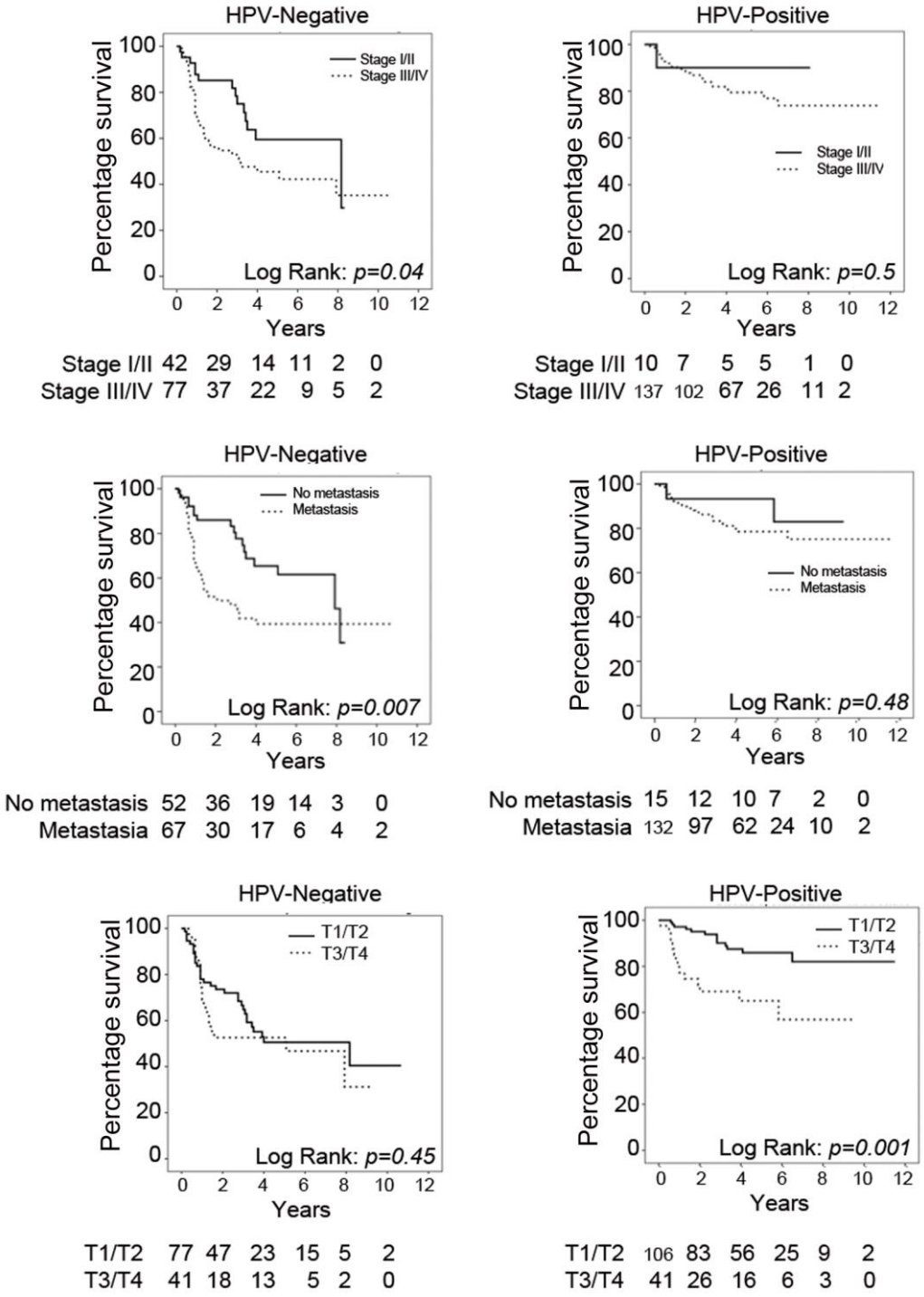


Figure 5. 1: Kaplan Meier Survival Curves for Disease Specific Survival from OPSCC according to components of the TNM staging system (stratified by HPV-status)

		HPV-positive OPSCC (n=149)			HPV-negative OPSCC (n=125)		
		Univariate HR	Multivariate HR	P	Univariate HR	Multivariate HR	P
Stage Groups	*I/II*	1	1		1	1	
	III/IV	1.95 (0.26-14.39)	1.50 (0.20-11.29)	0.69	1.85 (1.00-3.40)	2.00 (1.01-3.98)	0.05
Stage	*I*	-	-	-	1	1	
	II	-	-	-	2.92 (0.91-9.35)	2.94 (0.78-11.09)	0.11
	III	-	-	-	2.63 (0.77-8.98)	2.86 (0.73-11.13)	0.13
	IV	-	-	-	3.76 (1.32-10.61)	4.27 (1.28-14.24	0.02
T-stage Groups	*T1/2*	1	1		1	1	
	T3/4	3.40 (1.60-7.25)	3.31 (1.47-7.72)	0.006	1.24 (0.71-2.17)	1.23 (0.68-2.25)	0.49
T-Stage	*T1*	1	1		1	1	
	T2	3.86 (0.86-17.42)	3.61 (0.79-16.52)	0.10	2.00 (0.95-4.20)	1.80 (0.79-4.11)	0.16
	T3	3.63 (0.51-25.79)	1.53 (0.13-17.43)	0.73	1.51 (0.55-4.17)	1.27 (0.41-3.90)	0.68
	T4	12.26 (2.74-54.89)	13.28 (2.89-61.05)	0.001	2.09 (0.93-4.70)	2.12 (0.88-5.14)	0.10
Nodal metastases	*No*	1	1		1	1	
	Yes	1.67 (0.39-7.06)	1.30 (0.30-5.67)	0.73	2.15 (1.20-3.83)	2.19 (1.16-4.11)	0.02
N-Stage	*N0*	1	1		1	1	
	N1	1.56 (0.29-8.53)	2.42 (0.39-15.06)	0.34	1.82 (0.76-4.40)	1.89 (0.75-4.78)	0.18
	N2	1.69 (0.39-7.29)	1.18 (0.27-5.24)	0.83	2.19 (1.18-4.04)	2.25 (1.15-4.42)	0.02
	N3	1.57 (0.14-17.42)	1.14 (0.10-12.88)	0.92	2.93 (0.85-10.07)	2.81 (0.63-12.60)	0.18
Advanced N-Stage	*N0-N2a*	1	1		1	1	
	N2b-N3	2.26 (0.99-5.16)	1.98 (0.81-4.84)	0.14	2.04 (1.19-3.50)	2.11 (1.16-3.83)	0.02

Table 5. 3: Hazard ratios for Disease specific survival for HPV-positive and HPV-negative OPSCC according to the components of the TNM staging system

Similarly analysing HPV-positive OPSCC (age and smoking adjusted), neither overall disease stage nor nodal status (no metastasis vs. metastasis; N0-2a vs. N2b-3) predicted for survival (DSS: Stage III/IV HR 1.50, p=0.69; N^+ HR 1.30, p=0.73; N2b or greater HR 1.98, p=0.14, table 5.3). In contrast, advanced T-stage remained highly predictive of reduced survival. Patients with T3 /T4 tumours had more than a 3-fold increase in the risk of death from OPSCC (DSS: T3/T4 HR 3.31, p=0.006) while those with T4 tumours had a greater than 13-fold increase in the risk of disease specific death compared to those with small, T1, tumours (DSS: T4 HR 13.28, p=0.001).

5.3.3 The effect of treatment modality on survival of HPV-positive patients

Patients in the study received a range of different treatments: surgery alone (n=26; 9.7%), surgery + adjuvant therapy (n=89; 33.1%), radiotherapy alone (n=56; 20.8%); and chemoradiotherapy (n=98; 36.4%). Of the 115 surgically treated patients, 60 (52%) underwent transoral resections with the remainder undergoing open surgical procedures involving mandibulotomy. There was no difference in survival of surgically treated patients according to whether they received transoral or open resection, nor whether they had received adjuvant therapy. Therefore, all surgically treated patients were analysed as one group. Treatment breakdown is shown in table 5.4.

	HPV-Positive OPSCC N=147	HPV-Negative OPSCC N=119
	Number (%)	*Number (%)*
Transoral Surgery (+/- adjuvant therapy)	38 (25.9)	22 (18.5)
Open Surgery (+/- adjuvant therapy)	20 (13.6)	32 (26.9)
Radiotherapy Alone	23 (15.6)	33 (27.7)
Concurrent Chemoradiotherapy	65 (44.2)	32 (26.9)
Not Known	1 (0.7)	-

Table 5. 4: Treatments received by patients with HPV-positive and HPV-negative tumours

Initially, the effect of HPV-status on patient survival was examined, stratified by the treatment received. On Kaplan Meier analysis, patients with HPV-positive tumours had significantly improved disease specific survival, regardless of treatment modality (DSS,

Log Rank test HPV-positive vs. HPV-negative: surgery p=0.01, RT p=0.04, CRT p<0.001; figure 5.2). This was reflected on univariate Cox Regression analysis, where HPV-positive patients had a greater than 60% reduction in the risk of disease-specific death with all forms of treatment (DSS, HPV-positive vs. HPV-negative: Surgery HR 0.39, p=0.01; RT HR 0.40, p=0.06; CRT HR 0.27, p=0.001).

5.3.4 The relationship between TNM stage, treatment, and survival in HPV-positive patients

Next, an attempt was made to establish whether any treatment modality improved survival outcomes in HPV-positive tumours, when stratified according to the components of the TNM system. On Kaplan Meier analysis (figure 5.3), there was no difference in survival according to treatment received when tumours were separated by either T-stage (Log rank tests: T1/2, p=0.25; T3/4, p=0.91) or the presence of nodal metastases (N^-, p=0.71; N^+, p=0.48). There was also no difference in age-adjusted survival according to treatment received when patients were stratified by T-stage (HR relative to surgery: T1/2 - RT 1.23, p=0.77; CRT 0.51, p=0.33; T3/4 – RT 1.10, p=0.91; CRT 1.01, p=0.99; table 5.5) or in patients who presented with nodal metastases (HR relative to surgery: N^+ - RT 1.73, p=0.33; CRT 0.91, p=0.83; table 5.4).

When stratified by nodal status using an N2b cut-off, Kaplan Meier analysis showed that HPV-positive patients with advanced nodal disease had significantly poorer survival if treated with radiotherapy alone (log rank test p=0.02, figure 5.3). Patients who were in the lower nodal stage group (i.e. N2a and below) showed no differences in survival according to treatment modality (p=0.75, figure 5.3). Age-adjusted, HPV-positive patients with advanced nodal disease who received radiotherapy alone had a 4-fold increase in the risk of disease-related death, compared to those treated with surgery +/- adjuvant therapy (HR 3.96, p=0.03, table 5.5) or chemoradiotherapy (HR 3.84, p=0.03). There was no difference in survival for patients who received chemoradiotherapy compared to those undergoing surgery +/- adjuvant therapy (HR 1.03, p=0.95, table 5.5). For patients in whom the nodal stage was N2a or less, multivariate analysis demonstrated no difference in survival according to treatment modality (table 5.5).

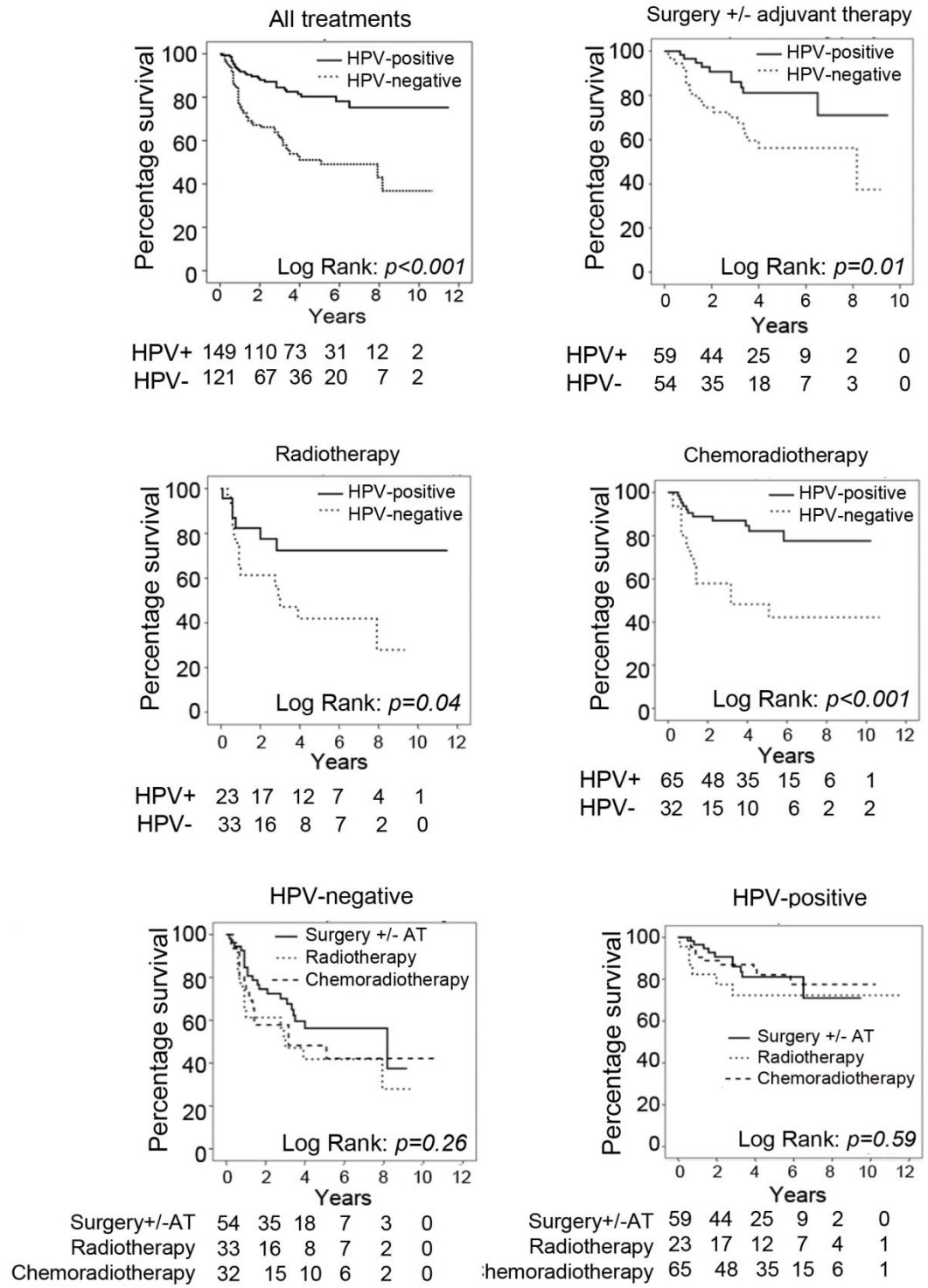


Figure 5. 2 : Kaplan Meier Curves for disease specific survival from OPSCC according to HPV-status (stratified by treatment received) and treatment received (stratified by HPV status)

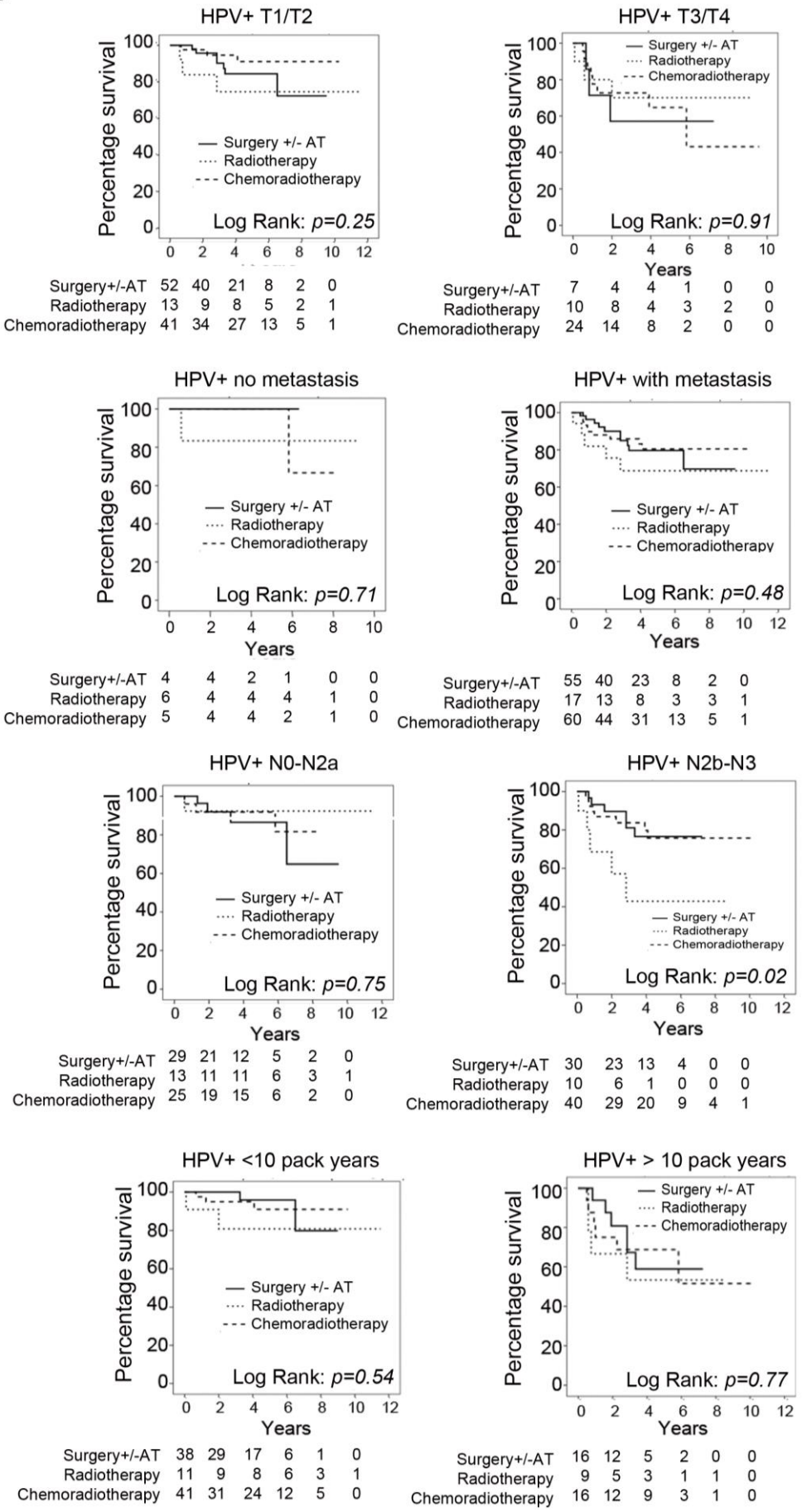


Figure 5. 3: Kaplan Meier Curves for disease specific survival from HPV-positive OPSCC according to treatment received (stratified by components of the TNM staging system, and by smoking status)

5.3.5 The relationship between smoking, treatment and survival in HPV-positive patients

Heavy smoking has been reported to reduce the survival benefit of HPV-positive tumours, and may potentially influence treatment choice. We therefore analysed HPV-positive heavy and light/non-smokers separately to see whether any treatment modality gave more favourable outcomes. There was no significant difference in survival according to treatment received (figure 5.3, table 5.5).

5.3.6 The effect of treatment on feeding tube dependency

Rates of feeding tube dependency (i.e. nasogastric tube or percutaneous gastrostomy; FTD) were determined according to treatment type. Of the entire cohort of 395 patients, 120 (30.4%) were feeding tube dependent at the end of treatment. Of these, 83 patients (69%) were able to have the tube removed after a median of 3 months (IQR 2-4, range 1-24). Patients who had HPV-positive tumours had the same rate of FTD as those who were HPV-negative (HPV-positive 31.4%, HPV-negative 31.6%, $p=1.00$). The entire cohort ($n=395$) was therefore used to analyse the effects of treatment type on FTD. There was not a significant difference in rates of FTD in patients treated with chemoradiotherapy (40%), radiotherapy (27%) or surgery (30%). Similarly there was no difference in 3- or 6- month FTD rates according to treatment modality (3 month FTD: Surgery 21%, RT 16%, CRT 24%, $p=0.34$; 6 month FTD: Surgery 12%, RT 8%, CRT 11%, $p=0.74$).

When surgically treated patients were stratified according to whether they underwent open or transoral resections, a different picture emerged. Rates of FTD significantly differed across the treatment groups (TOR 18%, open surgery 54%, RT 27%, CRT 40%, $p<0.001$) and this was maintained at both 3- and 6-months (3-months: TOR 10%, open surgery 41%, RT 16%, CRT 24%, $p=0.001$; 6-months: TOR 6%, open surgery 24%, RT 8%, CRT 11%, $p=0.02$).

	Univariate HR (95% CI)	Multivariate HR (95% CI)	p	Univariate HR (95% CI)	Multivariate HR (95% CI)	p
	HPV-positive light/<10 pack years			**HPV-positive/>10 pack years**		
Surgery	1	1		1	1	
Radiotherapy	2.78 (0.38-20.12)	2.00 (0.25-15.91)	0.51	1.54 (0.43-5.47)	1.17 (0.27-5.02)	0.83
Chemoradiotherapy	1.27 (0.21-7.63)	1.39 (0.23-8.34)	0.72	1.04 (0.33-3.24)	1.03 (0.33-3.25)	0.94
	HPV-positive/Early T-Stage (T1/2)			**HPV-positive/Late T-stage (T3/4)**		
Surgery	1	1		1	1	
Radiotherapy	1.66 (0.43-6.46)	1.23 (0.30-5.03)	0.77	0.71 (0.14-3.52)	1.10 (0.21-5.74)	0.91
Chemoradiotherapy	0.46 (0.12-1.78)	0.51 (0.13-1.99)	0.33	0.89 (0.23-3.37)	1.01 (0.27-3.83)	0.99
	HPV-positive/Nodes Absent			**HPV-positive/Nodes Present**		
Surgery	1	1		1	1	
Radiotherapy	Unable to resolve HR due to small numbers			1.71 (0.58-5.01)	1.73 (0.57-5.27)	0.33
Chemoradiotherapy				0.91 (0.38-2.19)	0.91 (0.37-2.19)	0.83
	HPV-positive/Early Nodal (N0-N2a)			**HPV-positive/Late Nodal (N2b-N3)**		
Surgery	1	1		1	1	
Radiotherapy	0.42 (0.05-3.84)	0.46 (0.05-4.40)	0.50	3.88 (1.17-12.82)	3.96 (1.15-13.62)	0.03
Chemoradiotherapy	0.76 (0.17-3.42)	0.74 (0.16-3.37)	0.70	1.04 (0.36-2.99)	1.03 (0.36-2.99)	0.95

Table 5. 5: Disease specific survival according to treatment received for HPV-positive OPSCC, stratified by components of the TNM system and by smoking status

To assess the impact of other clinicopathological factors on FTD, univariate and multivariate logistic regression were performed, and odds ratios for FTD calculated (table 5.6). When surgically treated patients were stratified by either open or transoral surgery (TOR), using TOR as the reference category, there was a significant increase in the risk of FTD in patients treated with open surgery and chemoradiotherapy, but not for those who only received radiotherapy (ORs: open surgery 5.47, $p<0.001$; radiotherapy 1.74, $p=0.16$; chemoradiotherapy 3.13, $p=0.001$; table 5.6). Other clinicopathological factors that influenced FTD rate were tumour site and TNM stage (table 5.6). A multivariate model was constructed to examine for the effect of treatment on FTD after adjustment for these factors. In this model, open surgery and chemoradiotherapy remained significant predictors of an increased risk of FTD (ORs: open surgery 6.75, $p<0.001$; chemoradiotherapy 2.53, $p=0.01$). These differences in risk were maintained at 3- (ORs: open surgery 7.96, $p<0.001$; chemoradiotherapy 2.40, $p=0.05$) but not 6-months.

To establish whether the addition of chemotherapy increased the risk of FTD, ORs were calculated according to whether or not patients received chemotherapy as part of their treatment, regardless of the timing (i.e. neoadjuvant or concurrent) or the primary treatment modality (i.e. surgery or radiotherapy). On univariate analysis, patients who received chemotherapy had more than twice the risk of FTD than those who did not (OR 2.10, $p<0.001$, table 5.6). The increased risk of FTD in patients receiving chemotherapy was maintained on multivariate analysis, again adjusting for tumour site and TNM stage (OR 1.72, $p=0.03$, table 5.6).

		Unadjusted OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Gender	*Female*	1		-	-
	Male	1.10 (0.67-1.81)	0.71	-	-
Age	*Each additional year*	1.01 (0.99-1.03)	0.21	-	-
Tumour Site	*Tonsil*	1		1	
	Base of Tongue	2.52 (1.50-4.23)	<0.001	2.60 (1.51-4.47)	0.001§
	Other oropharynx	1.43 (0.80-2.58)	0.23	1.89 (1.00-3.57)	0.05§
Disease stage	*I/II*	1		1	
	III/IV	2.53 (1.23-5.24)	0.01	2.84 (1.26-6.37)	0.01§
T-Stage	*I/II*	1		-	-
	III/IV	1.78 (1.13-2.78)	0.01	-	-
Nodal Metastases	*No*	1		-	-
	Yes	1.99 (1.13-3.51)	0.02	-	-
Tumour grade	*Well/moderately differentiated*	1		-	-
	Poorly differentiated	1.52 (0.95-2.44)	0.08	-	-
HPV-status	*Negative*	1		-	-
	Positive	0.99 (0.57-1.73)	0.97	-	-
Smoking	*<10 pack years*	1		-	-
	>10 pack years	1.06 (0.66-1.70)	0.81	-	-
Treatment	*Trans-oral surgery*	1		1	
	Open Surgery	5.47 (2.30-13.05)	<0.001	6.85 (2.76-16.99)	<0.001§
	Radiotherapy	1.74 (0.81-3.75)	0.16	1.76 (0.80-3.92)	0.16§
	Chemoradiotherapy	3.13 (1.59-6.17)	0.001	2.62 (1.30-5.27)	0.007§
Chemotherapy	*No*	1		1	
	Yes	2.10 (1.33-3.30)	0.001	1.72 (1.06-2.78)	0.03§

Table 5. 6: Odds ratios for the requirement of a post-treatment feeding tube

5.4 Discussion

Accurate tumour staging plays a vital role in cancer management. It is central to both treatment planning and prognosis and, importantly, allows comparison of treatment and outcomes between centres (471). The AJCC staging for oropharyngeal cancers uses the TNM system to give an overall disease stage (39, 41). It is generally accepted that patients with advanced stage disease (i.e. stage III/IV) have a worse prognosis than those with early stage disease. However, the current staging system does not take into account important biological and molecular features within the tumour which may influence outcome (471). This is particularly relevant in the era of HPV-positive OPSCC, as these tumours have improved survival compared to HPV-negative tumours, despite tending to present at a more advanced stage (472). The improved prognosis in HPV-positive tumours is seemingly independent of treatment modality, and studies in which patients have been treated with surgery, radiotherapy and chemoradiotherapy (or combinations thereof) have all shown a survival benefit for HPV-positive tumours (99, 356, 358, 362, 470). However, until now, there have been few large studies that have compared different treatment modalities within the same patient cohort.

5.4.1 Differences in TNM staging between HPV-negative and HPV-positive tumours

Within this patient cohort, 93% of HPV-positive OPSCC patients presented with advanced stage disease (stage III or IV), compared with only 65% of those that were HPV-negative ($p<0.001$). In the HPV-positive group, nodal status predominantly accounted for the advanced stage, with nearly 90% of HPV-positive patients having nodal metastasis at presentation, compared with 56% in HPV-negative tumours ($p<0.001$). Furthermore, HPV-positive patients presented with more advanced nodal disease, with around 55% presenting at stage N2b or above, compared to under 40% of HPV-negative tumours ($p=0.02$). This is in keeping with published data describing the majority of HPV-positive tumours as presenting with advanced nodal disease (356, 358, 434, 473).

There is some evidence that HPV-positive OPSCC presents at an earlier T-stage than HPV-negative tumours (99, 353). In this cohort there was no significant difference in the T-stage at presentation when tumours were stratified by HPV-status, (T1/T2: HPV-positive 72% verses HPV-negative 65% $p=0.29$, table 5.2). However, in advanced stage tumours, 70% of HPV-positive tumours were T1 or T2 compared with only 47% of HPV-negative tumours ($p=0.002$). Taken together, these results suggest that the reasons for advanced tumour staging differ between HPV-positive and HPV-negative tumours. Advanced stage HPV-positive tumours tend to have small primaries with advanced

nodal disease, while advanced stage HPV-negative tumours have a lower rate of nodal disease, and an even spread of T-stages, with over 50% of these tumours presenting as T3/T4 (99, 353, 472, 474). It is not clear whether the neck disease seen with HPV-driven tumours is related to earlier metastasis or more pronounced 'cystic' nodal disease being detected earlier (335). However, there is evidence that patients with HPV-positive tumours are more likely to cite a 'lump in the neck' as the first reason for seeking medical attention (334, 474).

5.4.2 The effect of TNM status on survival

Overall disease stage was predictive of an increased risk of disease-specific death in HPV-negative tumours (HR for stage IV 4.27 p=0.02) while in HPV-positive disease there were too few stage I tumours (n=2) to resolve a HR. To overcome this problem, tumours were grouped into early and late stage (i.e. I/II verses III/IV). When analysing DSS using these groups, a significantly increased risk of disease specific death was seen in advanced stage, HPV-negative tumours (HR 2.00, p=0.05) while there was no difference in survival for HPV-positive patients. It is generally accepted that the most important predictor of poor survival from HNSCC is the presence of involved regional lymph nodes at presentation, which have been shown to reduce 5-year survival by 50% (1, 43). In recent years there has been some controversy in the literature as to whether nodal status is a useful prognostic marker in OPSCC. There is supporting evidence that the presence of metastatic nodes is only predictive of survival in HPV-negative tumours (471, 472). In keeping with this, in HPV-negative OPSCC, the presence of nodal metastases more than doubled the risk of disease specific death (HR 2.19, p=0.06) and more than halved the 5-year survival (N^- 54.8% vs. N^+ 20.0%, p=0.003). By contrast, in HPV-positive tumours, there have been conflicting reports regarding the impact of nodal status on survival. In this study, neither overall, nor disease-specific survival, were affected by the presence of nodal metastases. On univariate analysis, HPV-positive patients with advanced nodal disease (i.e. N2b or greater) showed reduced survival compared with those staged as N0-N2a (HR 2.26). However, this difference was not observed on multivariate analysis, adjusting for age and smoking status (HR 1.98, p=0.14), suggesting that these factors may be linked.

While nodal status did not predict for survival in HPV-positive tumours in this study, increasing T-stage was highly predictive of both overall, and disease specific survival (e.g. DSS HR T4 tumour 13.28, p=0.001). There was no statistically significant association between T-stage and survival for HPV-negative OPSCC. This reflects the findings of Hong *et al,* who found that the risk of all cause and disease-specific death, and the risk of recurrence, increased with advancing T-stage in HPV-positive, but not HPV-negative tumours (472). Furthermore, several other studies have shown that T-

stage, but not N-stage, is an independent predictor of disease specific survival after adjustment for HPV-status in OPSCC (471, 475, 476). On the basis of these findings the current TNM staging system would appear inadequate for HPV-positive tumours. This same conclusion has been reached by other authors (471, 472).

5.4.3 Does the survival benefit of HPV-positive tumours depend on treatment?

A large number of studies have suggested that HPV-positive oropharyngeal tumours have improved survival outcomes compared to those that are HPV-negative. This survival benefit appears independent of treatment-type. However, there are few studies directly comparing different treatment modalities within the same patient cohort. In this study, just over a third of patients received surgery (+/- adjuvant therapy) or chemoradiotherapy, and the remainder (~20%) underwent primary radiotherapy. This enabled us to directly compare the effect of HPV on survival across the full range of treatment modalities. On univariate analysis, the survival benefit for HPV-positive OPSCC was seen regardless of treatment modality, with reductions in the risk of death of approximately 60% irrespective of treatment received.

Hong *et al* assessed the effect of HPV-status on survival in 195 patients treated for oropharyngeal cancer by the 3 treatment types. Their data support these findings and show that HPV-positive tumours had improved overall and event free survival, and reduced tumour recurrence, after adjustment for age, grade, gender, stage and primary site; regardless of treatment modality(353). Fischer *et al* examined 76 oropharyngeal tumours treated with either surgery and adjuvant radiotherapy or chemoradiotherapy, and found that HPV-positive tumours had a 53% reduction in the risk of death, even after adjusting for treatment received, suggesting that the survival benefit of HPV-positive tumours was independent of treatment (354).

A number of clinical trials are currently underway seeking to de-escalate treatments for HPV-positive OPSCC, in an attempt to reduce morbidity while maintaining the excellent survival outcomes generally associated with this group of tumours. Therefore, an attempt was made to establish whether components of the TNM system, or heavy smoking influenced the response of HPV-positive OPSCC to differing treatment modalities. There was no difference in survival according to treatment received when tumours were stratified by T-stage, the presence or absence of nodal metastases, or heavy smoking. In contrast, patients who presented with advanced nodal disease, namely N2b or above, had a significant reduction in survival if treated solely with radiotherapy, as compared to surgery +/- adjuvant therapy or chemoradiotherapy. This would suggest that patients who present at this nodal stage, more than half of HPV-positive tumours in this cohort, should probably not be

included in de-escalation trials. Indeed, the De-ESCALaTE HPV trial counts N2b disease or above as one of its exclusion criteria (387).

5.4.4 The effect of treatment modality on swallowing

As a surrogate marker of treatment associated dysphagia, the requirement for either NG or PEG feeding after treatment was assessed. Just under a third of patients required assisted feeding following treatment, and the rates of feeding tube dependency (FTD) did not differ according to HPV status. When treatment was subdivided into the 3 treatment modalities, there was no significant difference in the rate of FTD or the likelihood of requiring a tube, although there was a trend towards increased rates of FTD in patients receiving chemoradiotherapy. There was also no difference in the rates of FTD at 3- or 6-months according to treatment modality. Interestingly, the inclusion of chemotherapy as part of the treatment regimen significantly increased the risk FTD, regardless of the primary treatment modality (adjusted OR 1.72). This is in keeping with a previous study demonstrating that chemoradiotherapy is associated with increased rates of dysphagia compared against radiotherapy alone (477).

Of the surgically treated patients in this cohort, 57% underwent transoral resections (TOR), as opposed to open procedures involving mandibulotomy. Relative to patients who had undergone TOR, those who had received either open surgery or chemoradiotherapy had a significantly increased risk of requiring assisted feeding after treatment (OR 5.47 and 3.13, $p<0.001$ and $p=0.001$ respectively), while patients treated with radiotherapy alone were not at increased risk. These odds ratios were calculated after adjustment for tumour site and TNM stage, both of which also increased the risk of FTD on univariate analysis, and suggest that patients treated with TOR or radiotherapy have improved swallowing function compared to those treated with open surgery or chemoradiotherapy. Importantly, treatment modality did not affect the percentage of patients who had their feeding tube removed, nor the median length of time that patients' required assisted feeding. It may well be that, once a feeding tube is inserted and a patient becomes reliant on it, then it is difficult to remove regardless of the reason for initial insertion.

Other authors have also demonstrated that patients undergoing TOR for oropharyngeal cancer have excellent swallowing outcomes. Rich *et al* examined 118 patients who had undergone TOR for advanced stage oropharyngeal tumours (T1/T2 72%) and found that 82% had good swallowing at one month post-operatively, based on the Functional Outcome Swallowing Scale (478), a figure that increased with time and was 93% by 5-years (479). They also found that advanced T-stage (T4) was an independent predictor of poor swallowing, especially in base of tongue tumours. Our data reflect these

findings (table 5). Of 66 TORs for OPSCC reported by Moore *et al*, only 4.5% required a long term PEG (480).

Clearly, the central consideration in treatment choice is DSS. However, long-term treatment associated morbidity are likely to become a significant concern as more HPV-driven OPSCC are treated, with their improved survival. In this cohort, the majority of HPV-positive tumours presented at an early T-stage (T1/2 72%) and with advanced nodal disease (N2b or above 54%). Early T-stage tumours are those most amenable to TOR, and patients with advanced nodal disease had no difference in survival if treated with either surgery or chemoradiotherapy. However, those undergoing chemoradiotherapy had significantly higher rates of FTD than those treated with TOR and adjuvant RT. If this finding holds true on prospective investigation, then it could be argued that the optimum treatment for these "early T and late N-stage" tumours is TOR and neck dissection with adjunctive RT, preserving positive survival outcomes while minimising the morbidity associated with chemotherapy. Clearly this requires validation in a prospective clinical trial.

5.5 Conclusions

These data suggest that the current staging system for head and neck cancers is inadequate as a prognostic tool in the era of HPV-OPSCC. While overall TNM stage and nodal metastases are predictors of survival in HPV-negative tumours, only T-stage is a useful prognostic marker in HPV-positive disease, and the predictive value of the N2b stage is lost when age and smoking are also considered. Patients with HPV-positive tumours show improved survival regardless of treatment received, with the caveat that those with N2b or N3 disease show significantly reduced survival if treated with radiotherapy alone. There are no significant survival differences in HPV-positive patients treated by surgery +/- radiotherapy compared with chemoradiotherapy. However, patients in receipt of chemoradiotherapy show significantly increased morbidity. This suggests that, small T1/T2 HPV-tumours (which comprise ~40% of all cases in this study) might be better treated initially with surgery, and adjunctive radiotherapy if required.

6 Conclusions

6.1 Summary of Findings

Oropharyngeal carcinoma represents a significant burden to healthcare systems throughout the western world, and one that is gradually growing due to the impact of HPV-driven disease. The emergence of HPV-positive disease has led to a rapid increase in the incidence of OPSCC, especially amongst the younger population. Evidence suggests that patients with HPV-positive OPSCC have improved survival outcomes compared to those whose tumours are HPV-negative. This has resulted in calls for "treatment de-escalation" in patients with HPV-positive tumours, in an attempt to reduce treatment related morbidity. At present there is insufficient data to confidently alter treatment modalities for these patients outside of clinical trials. In particular, there is no accepted method of identifying the small, but significant, proportion of HPV-positive patients who are at high risk of poor outcomes. Although there is increasing evidence that heavy smoking is a negative prognostic marker, further studies are necessary to identify other prognostic factors that impact on survival. It is vital to find ways to stratify HPV-positive patients in terms of risk, and thus to avoid recruiting "high risk" patients into de-escalation trials.

The work presented in this thesis aimed to establish rates of HPV-positive and HPV-negative OPSCC in our local population, to identify key differences between these tumours, and to investigate factors that influence survival.

A large tumour database was established containing details of over 400 tumours treated at three sites over a ten year period from 2000-2010. Detailed analysis was subsequently performed on nearly 300 tumours for which pathological material was available. In keeping with previously published studies, over 50% of tumours were classified as HPV-positive using a combination of p16 immunhistochemistry and HPV *in-situ* hybridisation. Patients with HPV-positive tumours were younger, and had less exposure to tobacco and alcohol. HPV-positive tumours were predominantly found in the tonsil and base of tongue, and were more likely to be poorly differentiated and to present at an advanced disease stage. The advanced disease staging seen in HPV-positive tumours was primarily as a result of advanced nodal disease. The demographic and pathological features of the tumours included in this study were similar to those of other large published series, and patients with HPV-positive tumours had significantly improved survival. All of these "expected" findings suggest that this dataset is reliable, and lends support to the rest of the study's findings.

A major feature of this study was an attempt to establish factors that influence survival in HPV-positive OPSCC. Similar to the findings of others (in particular Ang *et al*), smoking was a negative prognostic factor in patients with HPV-positive tumours,

and indeed was a significant predictor of 3-year mortality in these patients. Other authors have described EGFR expression as being a marker of poor outcome in HPV-positive OPSCC. While HPV-positive tumours were less likely to express EGFR in this study, a finding echoed by others, EGFR expression did not significantly predict for survival.

The strongest predictive marker in HPV-positive OPSCC was an intrinsic feature of the tumours themselves, namely the presence or absence of an immune response. Over 80% of HPV-positive tumours included in this study were associated with a prominent (mainly T-lymphocyte, TILs) inflammatory infiltrate. In approximately 15% of HPV-positive patients however, no immune response was seen in the tumours. Importantly, these patients had no difference in survival when compared to those with HPV-negative disease, on either Kaplan Meier or Cox Regression analysis. Furthermore, a weak immune response was the strongest predictor of 3-year mortality in patients with HPV-positive OPSCC. Taking this one step further, a predictive model based on TILs, smoking and T-stage was developed. This was highly predictive of 3-year mortality in HPV-positive OPSCC and accurately stratified patients with HPV-positive tumours into high and low risk groups, outperforming the model proposed by Ang *et al* (based on smoking and advanced N-stage).

These findings are based on an assessment of the immune infiltrate in a routine H&E stained diagnostic biopsy specimen. This is a quick and simple technique for an established histopathologist and does not add significant time to the routine reporting of these tumours. Further categorisation of the immune infiltrate using immunohistochemistry and counting individual cells creates a significant workload, takes time, and does not appear to improve the predictive power of the model compared to TIL levels alone. The simplicity of assessing TIL levels is attractive in terms of routine clinical assessment of HPV-positive tumours. On the basis of this work, we hope that assessment of TIL status might be incorporated into the Royal College of Pathologists reporting guidelines for head and neck cancer.

At present one of the key factors influencing treatment decisions in OPSCC, and in fact most tumours, is the TNM stage. Furthermore, classical teaching suggests that the strongest marker of poor outcome in head and neck cancer is the presence of nodal metastases at the time of diagnosis, said to reduce survival by 50%. While overall TNM stage and nodal status were accurate predictors of survival in HPV-negative tumours in this study, only T-stage was a significant prognostic marker in tumours that were HPV-positive. This is an important finding, also noted by other authors, and suggests that the current TNM staging system for OPSCC is potentially inadequate and may need refinement, with consideration of the inclusion of initial stratification by HPV status.

A number of studies have suggested that the survival benefit of HPV-positive tumours is independent of treatment modality. These are typically single modality studies and there is little data directly comparing different modalities in the same patient cohort. In this study, HPV-positive tumours had improved survival regardless of treatment modality, with the exception of those with N2b disease or greater, where treatment with radiotherapy alone resulted in poorer outcomes. Given these findings, which fit with the literature, functional outcome becomes an increasingly important factor in treatment choice. Patients in this study had a significantly increased risk of long term feeding dependency if treated with chemoradiotherapy as opposed to transoral surgery and post-operative radiotherapy (PORT). Indeed, the addition of chemotherapy doubled the risk of long term feeding tube dependency. Whilst feeding tube dependency is only a surrogate marker of functional outcome, and further studies are required to confirm this finding, these results would suggest that transoral surgery and PORT may represent an attractive treatment regimen in appropriate patients.

6.2 Limitations of this work

One of the intrinsic problems with retrospective studies is that of missing data. A significant number of the variables recorded in this study were obtained from the patient notes, and were thus reliant on the accuracy of these notes. This led to wide variations in the rates of missing data. For example, in nearly a quarter of patients there was no record of alcohol consumption, potentially resulting in a loss of statistical significance in any analyses utilising this variable. In contrast, tumour location was accurately recorded in over 98% of cases.

Approximately a third of the patients in this study had been treated at UHS or PFT, but had undergone diagnostic biopsies at peripheral sites such as Salisbury or the Isle of Wight. As these sites were not covered by the ethical approval for this study, it was impossible to access these tissues. The generalisation of many findings of this study to those without available tissues must therefore be treated with caution, and requires validation. Reassuringly there was no difference in survival between patients with and without archival tissues available, suggesting that the findings derived from those with available tissues are likely to be applicable to the cohort as a whole.

Most patients in this study came from the same geographical area of England, and thus it is not possible to assess the effect of geographical, genetic, or ethnic influences on these findings. Patients treated with transoral resections were generally diagnosed more recently than those who received open surgery, though this represents a change in practice generally observed in the treatment of head and neck malignancy and is not specific to this study.

Despite these limitations, a large number of the findings in this study echo those of others, suggesting that this cohort is typical of similarly sized series in the literature. Although this lends credence to the newer findings, these require prospective validation prior to inclusion in clinical treatment protocols.

6.3 Future Work

The most important findings of the work presented here are the identification of a group of TIL^{low} HPV-positive tumours with poor survival, and the development of a highly predictive model for "high risk" tumours. However, the major limitation is the retrospective nature of the dataset. Before any potential alteration in treatment strategies as a result of these findings can be considered, validation in other cohorts is essential. To that end, the effect of TIL-levels on survival is currently being assessed in a number of other patient cohorts, both prospectively and retrospectively. These include patients recruited to the "PET Neck" study, and a retrospective cohort examined by Dr Michelle Reitenberg at the University of Amsterdam.

If the poor outcomes seen here for TIL^{low} tumours are validated in other cohorts, then this would provide strong evidence that TIL^{low} tumours are indeed high risk, and should not be included in treatment de-escalation studies. Furthermore, validation of the prognostic model would potentially allow far more accurate prediction of outcome in patients with HPV-positive tumours. It can be envisaged that, at diagnosis, a patient's smoking status, T-stage and TIL-level could be combined to calculate their score, which could not only predict outcome, but also potentially help guide treatment.

TIL^{low} tumours appear to have poor prognosis, and one important consideration is whether a TIL^{low} tumour can be converted into a TIL^{high} tumour. One potential method for achieving this might be by using immunotherapy, likely in the form of therapeutic vaccines. If this were feasible, then an obvious subsequent question would be whether this could also improve survival outcomes. HPV vaccines have been shown to induce tumour regression in animal models, and there is some evidence from clinical trials that immunotherapy has a role to play in advanced cervical carcinoma, another HPV mediated disease (481). This is clearly an exciting area of research and one that will no doubt rapidly expand in the near future.

One other possible use for therapeutic vaccines might be as a method of de-escalating treatment. For example, tumours that are HPV-positive and TIL^{high}, i.e. those with the best prognosis, could potentially be managed using immunotherapy in addition to surgery or radiotherapy, thus sparing the additional side effects of traditional chemotherapy. This would obviously require prospective validation in a clinical trial.

A number of questions remain unanswered regarding interactions between HPV and the immune system. Firstly, there is, as yet, no consensus opinion on why the vast majority of patients who are infected with HPV clear the virus, yet a small minority go on to develop long standing infections and subsequently malignancy. Furthermore, of

those that develop tumours, it is not clear why some do not mount an immune response. Although myofibroblast levels inversely correlate with TILs in this study, we are far from unraveling the complex interactions between HPV-positive tumours and the immune response. What appears increasingly clear, however, is the critical role of the immune system in the behaviour and treatment response of HPV-positive tumours.

APPENDIX 1

VARIABLES RECORDED IN TUMOUR DATABASE

Variable	Categories	Comments
Study No		
Hospital No		
Hospital	0=Southampton	
	1=Poole	
	3=Bart's/London	
Date of Birth		
Sex	0=Male	
	1=Female	
Smoker	0=Non-smoker	Later summarised as < or > 10 pack years
	1=<10 pack years	
	2=10-20 pack years	
	3=>20 pack years	
	4=<10/day	
	5=10-20/day	
	6=>20/day	
	7=Ex-smoker	
Alcohol	0=Non-drinker	
	1=<10u/week	
	2=10-20u/week	
	3=>20u/week	
Referral date		Taken from referral letter
Date first contact		Date first seen by any member of the MDT
Time to contact		Time in days from referral date to date first seen
Date of Diagnosis		Taken from pathology report
Time to diagnosis		Time in days from date first seen to date of diagnosis
Date first treatment		Date of first definitive treatment e.g. resection/first dose of chemotherapy/first radiotherapy fraction
Time to treatment		Time in days from date first seen to first definitive treatment
Age at Diagnosis		
Life status	0=Dead	
	1=Alive	
Date of Death		
Age at death		
Survival		Time in months from date of diagnosis until date of death or last recorded follow up

Primary Site	0=Unknown	Later summarised as: Tonsil, Tongue Base or Other Oropharynx
	9=Tongue Base	
	10=Soft Palate	
	11=Tonsil	
	12=Lateral Pharyngeal Wall	
	13=Posterior Pharyngeal Wall	
	14=Tonsillolingual Sulcus	
	22=Oropharynx (Unspecified)	
Primary side	0=Unknown	
	1=Right	
	2=Left	
	3=Midline/Bilateral	
Synchronous primary	0=No	
	1=Yes	
Synchronous primary site	As above plus 21=Lung	
Synchronous primary side	As above	
Primary CT stage	0=T0	T-stage according to imaging (CT/MRI)
	1=T1	
	2=T2	
	3=T3	
	4=T4	
	5=Tx	
	6=Tis	
	7=T1a	
	8=T1b	
	9=T4a	
	10=T4b	
Primary Histological Stage	As above	T-stage according to histology report (surgically treated tumours only)
Primary Clinical Stage	As above	T-stage according to clinical assessment
Final T Stage	As above	T-stage used for final staging (Pathological>Imaging>Clinical, see chapter 1)

Clinical Nodes	0=N0	N-stage according to clinical assessment
	1=N1	
	2=N2a	
	3=N2b	
	4=N2c	
	5=N3	
CT Nodes	As above	N-stage according to imagine (CT/MRI)
Final N Stage	As above	N-stage used for final staging
Stage	1=I	Disease stage
	2=II	
	3=III	
	4=Iva	
	5=IVb	
	6=IVc	
USS Nodes	0=not performed	Ultrasound assessment of cervical nodes (if applicable)
	1=equivocal	
	2=normal	
	3=reactive	
	4=suspicious SCC	
	5=suspicious lymphoma	
	6=suspicious	
	7=malignant	
	8=branchial cyst	
	9=other	
FNA	0=not performed	FNA result from cervical nodes (if applicable)
	1=inconclusive	
	2=normal	
	3=reactive	
	4=SCC	
	5=carcinoma	
	6=lymphoma	
	7=suspicious	
	8=malignant cells	
	9=branchial cyst	
	10=warthins tumour	
Distant metastases at presentation	0=No	
	1=Yes	
Metastasis site	1=lung	
	2=liver	
	3=bone	
	4=brain	
	5=other	

Primary treatment	0=surgery	Treatment received
	1=radiotherapy	
	2=chemoradiotherapy	
	3=none/palliative	
	4=neoadjuvant chemo/surgery	
	5=neoadjuvant/CRT	
	6=chemotherapy	
	7=neoadjuvant chemo/radiotherapy	
Primary surgery	1=open	Type of surgery (if applicable)
	2=transoral	
Laser	0=No	Laser surgery (if trans-oral resection)
	1=Yes	
Clinical tumour diameter		In millimetres
Imaging tumour diameter		
Histological tumour diameter		
Depth of invasion		
Grade	0=CIS/severe dysplasia	
	1=well differentiated	
	2=moderately differentiated	
	3=poorly differentiated	
	4=undifferentiated	
Cohesion	0=cohesive	Nature of the invasive margin
	1=discohesive	
	2=mixed	
Margin status	0=clear	
	1=close	
	2=involved	
Dysplasia at margin	0=No	
	1=Yes	
Perineural spread	0=No	
	1=Yes	
Intravascular spread	0=No	
	1=Yes	
Lymphatic spread	0=No	
	1=Yes	

Inflammation	1=none/little	AKA TIL-status
	2=patchy/focal	
	3=diffuse	
Frozen sections	0=not taken	
	1=negative	
	2=dysplasia	
	3=positive	
Fixed frozen	0=agree	Correlation of frozen and embedded sections
	1=disagree	
Neck Dissection	0=No	
	1=Yes	
Neck dissection side	0=none	
	1=right	
	2=left	
	3=bilateral	
Neck dissection type	1=Radical	Recorded for left and/or right as appropriate
	2=MRND	
	3=Selective	
	4=WLE	
ND timing	1 - Primary	Recorded for left and/or right as appropriate
	2 - Failure	
	3 - Recurrence	
ND nodes		Number of nodes in specimen. Recorded for left and/or right as appropriate
ND positive nodes		Number of positive nodes. Recorded for left and/or right as appropriate
Positive node size		Largest (in mm). Recorded for left and/or right as appropriate
ND ECS	0=No	Extra-capsular spread. Recorded for left and/or right as appropriate
	1=Yes	
Levels involved	1=level 1	Recorded for left and/or right as appropriate
	2=level 2	
	3=level 3	
	4=level 4	
	5=level 5	
	6=level 6	
	7=>1 level involved	
Radiotherapy	0=none	
	1=primary	
	2=post-operative	
Radiotherapy side	1=right	
	2=left	
	3=bilateral/midline	

Radiotherapy dose total		Dose in Gy
Radiotherapy fractions		Number of fractions
Chemotherapy	0=none	
	1=concurrent	
	2=neoadjuvant	
	3=neoadjuvant and concurrent	
	4=sole treatment	
Neoadjuvant Chemo cycles		
Neoadjuvant Chemo regime		
Concurrent chemo regime		
Concurrent chemo cycles		
Date treatment finished		
Treatment failure	0=No	
	1=Yes	
Date treatment failure		
Failure site	0=local	
	1=regional	
	2=locoregional	
Nodal failure USS	0=equivocal	
	1=normal	
	2=reactive	
	3=suspicious SCC	
	4=suspicious lymphoma	
	5=not performed	
Nodal failure FNA	0=not performed	
	1=inconclusive	
	2=normal	
	3=reactive	
	4=SCC	
	5=carcinoma	
	6=lymphoma	
	7=suspicious	
Salvage surgery	0=No	
	1=Yes	

Salvage surgery site	0=primary	
	1=neck	
	2=both	
Salvage surgery date		
Late recurrence	0=No	
	1=Yes	
Recurrence site	1=local	
	2=regional	
	3=locoregional	
Recurrence date		
Time to recurrence (months)		Taken from date completed treatment
Nodal recurrence side	0=right	
	1=left	
	2=bilateral	
Nodal recurrence USS	As above	
Nodal recurrence FNA	As above	
Recurrence Treatment	0=none	
	1=surgery	
	2=radiotherapy	
	3=chemotherapy	
	4=chemoradiotherapy	
	5=surgery/chemotherapy	
	6=surgery/radiotherapy	
	7=surgery/CRT	
Second primary	0=No	
	1=Yes	
Second primary date		
Time to second primary (months)		Taken from date treatment completed

Second primary site	0=unknown	
	1=lip	
	2=upper alveolus	
	3=lower alveolus	
	4=hard palate	
	5=buccal mucosa	
	6=floor of mouth	
	7=anterior tongue	
	8=retromolar trigone	
	9=tongue base	
	10=soft palate	
	11=tonsil	
	12=lateral pharyngeal wall	
	13=posterior pharyngeal wall	
	14=tonsillolingual sulcus	
	15=pyriform fossa	
	16=post cricoid	
	17=supraglottis	
	18=glottis	
	19=subglottis	
	20=lung	
	21=other	
Second primary side	0=right	
	1=left	
	2=bilateral	
	3=unknown	
Late distant metastases	0=No	
	1=Yes	
Date distant metastases		
Time to distant mets (months)		Taken from date treatment finished
Late metastasis site	1=lung	
	2=liver	
	3=bone	
	4=brain	
	5=other	

Palliative treatment	0=none/not required	
	1=surgery	
	2=radiotherapy	
	3=chemotherapy	
	4=chemoradiotherapy	
Cause of death	0=still alive/other cause	
	1=Died of Disease	
Nutrition post treatment	0=swallow	
	1=NG	
	2=PEG/RIG	
NG/PEG removed	0=No	
	1=Yes	
Time with PEG/NG (months)		
Late Swallowing Problems (1yr>Rx)	0=none	
	1=NG	
	2=PEG	
	3=TOF tube	
Tracheostomy on discharge	0=No	
	1=Yes	
Last contact date		
Length Follow Up		

APPENDIX 2

DETAILED TREATMENT BREAKDOWN

Detailed treatment breakdown

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Surgery Alone	38	8.6	8.7	8.7
	Surgery/RT	94	21.3	21.5	30.1
	Surgery/CRT	9	2.0	2.1	32.2
	RT Alone	99	22.4	22.6	54.8
	CRT	73	16.5	16.7	71.5
	NeoCRT	81	18.3	18.5	90.0
	NeoRT	12	2.7	2.7	92.7
	None/Palliative Chemo	26	5.9	5.9	98.6
	Neo/Surgery/RT	6	1.4	1.4	100.0
	Total	438	99.1	100.0	
Missing	System	4	.9		
Total		442	100.0		

APPENDIX 3

Additional material for chapter 3

		UHS Cohort n=187 Number (%)	**PFT/BLT Cohort n=103** Number (%)
Gender	*Male*	137 (73.3)	74 (71.8)
	Female	50 (26.7)	29 (28.2)
Age at Diagnosis	*<50*	39 (20.9)	19 (18.4)
	50-69	112 (59.9)	64 (62.1)
	70+	36 (19.3)	20 (19.4)
	Mean (SD)	59.11 (11.73)	58.62 (11.53)
Smoking	*Non-Smoker*	33 (17.6)	22 (21.4)
	Current Smoker <10 pack year	8 (4.3)	10 (9.7)
	Current Smoker >10 pack year	78 (41.7)	41 (39.8)
	Ex-Smoker	44 (23.5)	16 (15.5)
	Not Known	24 (12.8)	14 (13.6)
Alcohol	*Non/Ex Drinker*	26 (13.9)	11 (10.7)
	Current Drinker	123 (65.8)	53 (51.5)
	Not Known	38 (20.3)	39 (37.9)
Tumour Site	*Tonsil*	104 (55.6)	58 (56.3)
	Tongue Base	53 (28.3)	25 (24.3)
	Other Oropharynx	30 (16.0)	20 (19.4)
Median Length of Follow Up in Years (Range)		3.67 (0.67-9.58)	5.83 (0.67-11.42)
Disease Stage	*I*	10 (5.3)	11 (10.7)
	II	18 (9.6)	13 (12.6)
	III	17 (9.1)	20 (19.4)
	IV	141 (75.4)	58 (56.3)
	Not Known	1 (0.5)	1 (1.0)
Grade	*Well/Moderately Differentiated*	64 (34.2)	46 (44.7)
	Poorly Differentiated	123 (65.8)	57 (55.3)
Treatment	*Surgery+/- Radiotherapy*	75 (40.1)	43 (41.8)
	Radiotherapy	45 (24.1)	14 (13.6)
	Chemoradiotherapy	59 (31.6)	39 (37.9)
	None/Palliative	8 (4.3)	7 (6.8)
TIL Status	*Low*	59 (31.6)	28 (27.2)
	Moderate	60 (32.1)	46 (44.7)
	High	66 (35.3)	29 (28.2)
	Not Known	2 (1.1)	0 (0)
HPV Status	*Negative*	82 (43.9)	51 (49.5)
	Positive	103 (55.1)	50 (48.5)
	Not Known	2 (1.1)	2 (1.9)
EGFR	*Negative*	84 (44.9)	31 (30.1)
	Positive	85 (45.5)	71 (68.9)
	Not Known	18 (9.6)	1 (1.0)

Baseline characteristics of patients included in the study stratified by cohort

Characteristic		Entire Cohort n=442 Number (%)	FFPE Tissue Available n=290 Number (%)	FFPE Tissue Unavailable n=152 Number (%)
Gender	*Male*	325 (73.5)	211 (72.8)	114 (75)
	Female	117 (26.5)	79 (27.2)	38 (25)
Age	*<50*	86 (19.5)	58 (20)	28 (18.4)
	50-69	268 (60.6)	176 (60.7)	92 (60.5)
	70+	88 (19.9)	56 (19.3)	32 (21.1)
	Mean (SD)	59.2 (11.3)	59.6 (11.6)	58.9 (10.7)
Smoking	*Non Smoker*	81 (18.3)	55 (19.0)	26 (17.1)
	Current Smoker <10/day	23 (5.2)	18 (6.2)	5 (3.3)
	Current Smoker >10/day	184 (41.6)	119 (41.0)	65 (42.8)
	Ex-Smoker	95 (21.5)	60 (20.7)	35 (23.0)
	Not Known	59 (13.3)	38 (13.1)	21 (13.8)
Alcohol	*Non/Ex Drinker*	61 (13.8)	37 (12.8)	24 (15.8)
	Current Drinker	271 (61.3)	176 (60.7)	95 (62.5)
	Not Known	110 (24.9)	77 (26.6)	33 (21.7)
Tumour Site	*Tonsil*	236 (53.4)	162 (55.9)	74 (48.7)
	Tongue Base	118 (26.7)	78 (26.9)	40 (26.3)
	Other Oropharynx	88 (19.9)	50 (17.2)	38 (25)
Median Length of Follow Up in Months (Range)		49 (2-137)	58 (8-137)	33 (2-137)
Disease Stage	*I*	30 (6.8)	21 (7.2)	9 (5.9)
	II	42 (9.5)	31 (10.7)	11 (7.2)
	III	60 (13.6)	37 (12.8)	23 (15.1)
	IV	306 (69.2)	199 (68.6)	107 (70.4)
	Not Known	4 (0.9)	2 (0.7)	2 (1.3)
Tumour Stage	*T1*	109 (24.6)	76 (26.2)	33 (21.7)
	T2	148 (33.5)	114 (39.3)	34 (22.4)
	T3	56 (12.7)	29 (10.0)	27 (17.8)
	T4	120 (27.1)	67 (23.1)	53 (34.9)
	Tx	4 (0.9)	3 (1.0)	1 (0.7)
	Not Known	5 (1.1)	1 (0.3)	4 (2.6)
Nodal Stage	*N0*	103 (23.3)	69 (23.8)	34 (22.4)
	N1	61 (13.8)	39 (13.4)	22 (14.5)
	N2	252 (57.0)	166 (57.2)	86 (56.6)
	N3	22 (5.0)	14 (4.8)	8 (5.3)
	Not Known	4 (0.9)	2 (0.7)	2 (1.3)
Grade	*Well/Moderately Differentiated*	168 (38.0)	110 (37.9)	58 (38.2)
	Poorly Differentiated	250 (56.6)	180 (62.1)	70 (46.1)
	Not Known	24 (5.4)	-	24 (15.8)

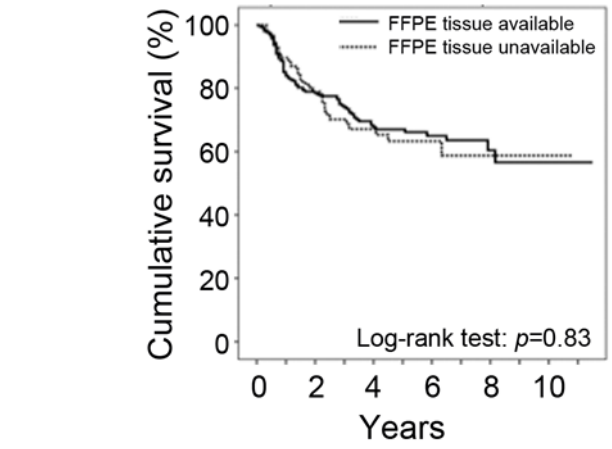
Demographics of patients with and without archival pathology samples available

Subsite	Number (%)
Soft Palate	51 (58.0)
Lateral Pharyngeal Wall	13 (14.8)
Posterior Pharyngeal Wall	18 (20.5)
Oropharynx Unspecified	6 (6.8)

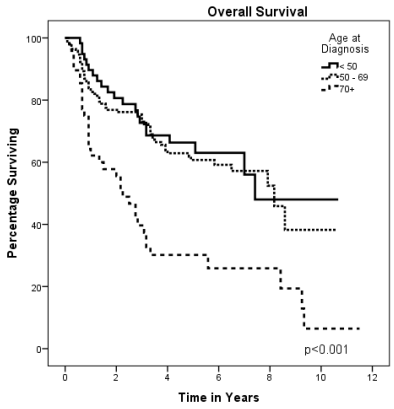
Subsite location of “other oropharyngeal” tumours (n=88)

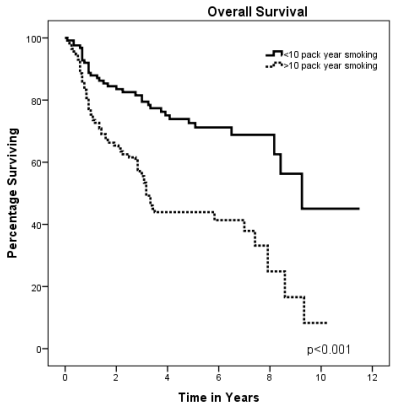
APPENDIX 4

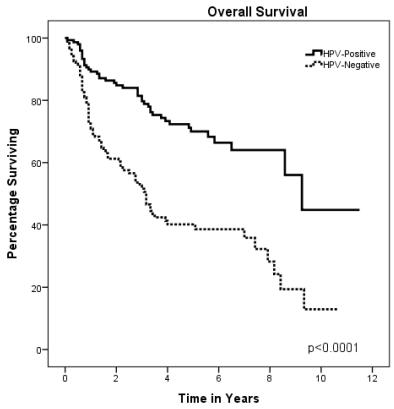
Additional material for chapter 4

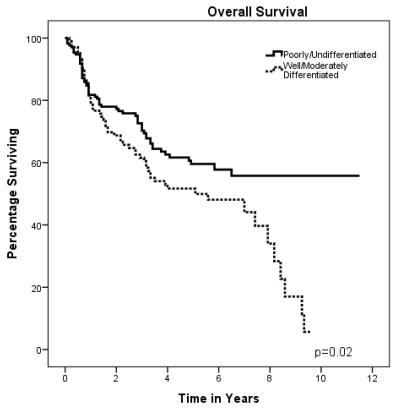


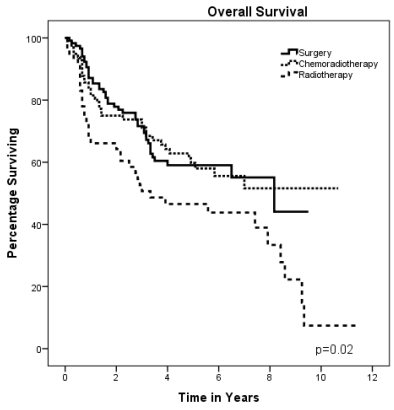
Disease specific survival for patients with and without archival pathology material available. The latter were excluded from the majority of analyses (see text)

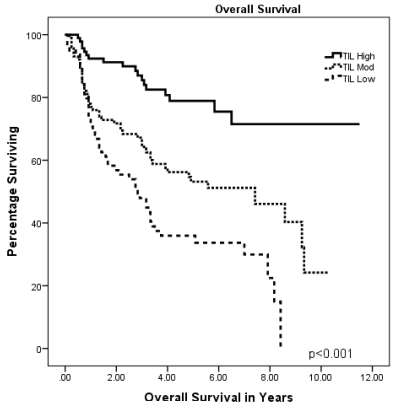


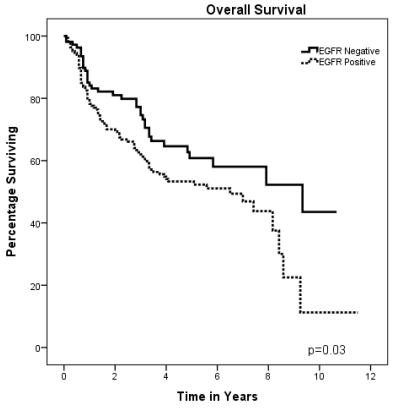








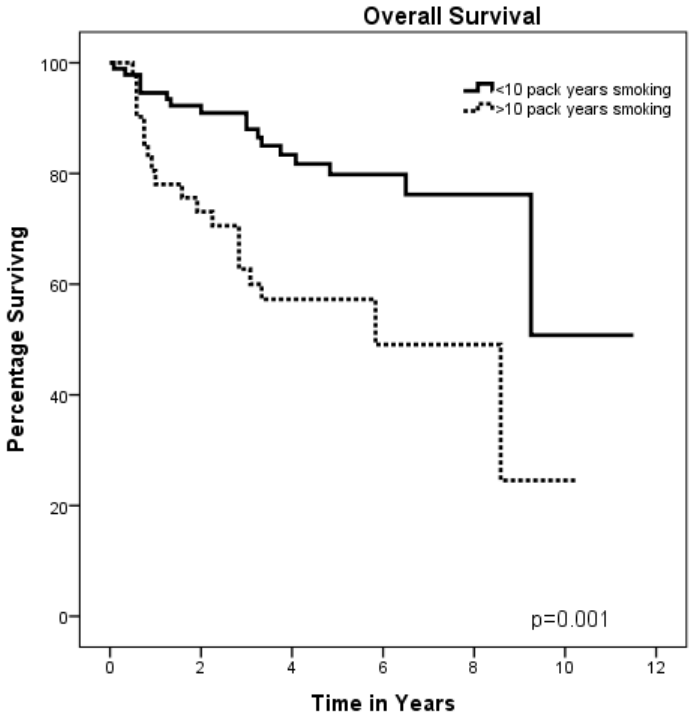


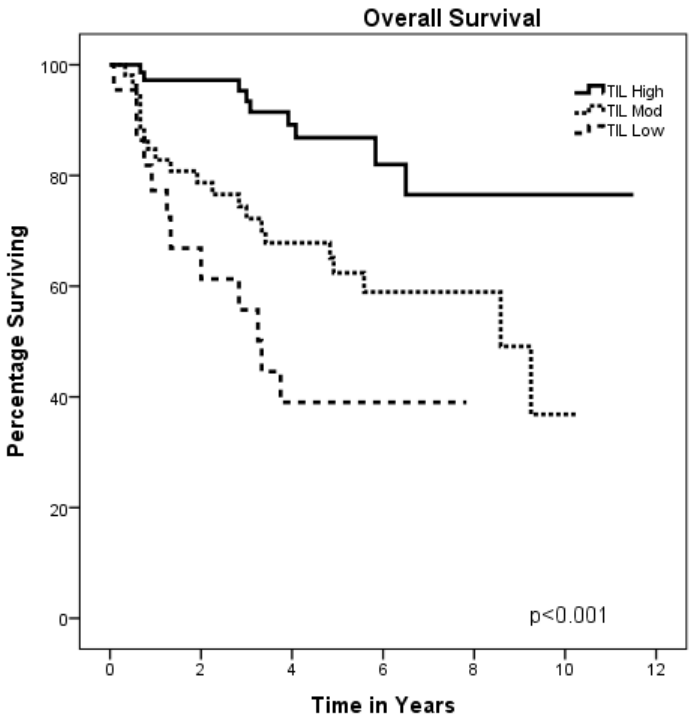


Kaplan Meier curves for overall survival for unstratified OPSCC

Multivariate analysis adjusted for age, T-stage, N-stage and smoking status.		All OPSCC (n=274)		
		Overall Survival 119 deaths		
		Unadjusted HR (95% CI)	**Adjusted HR (95% CI)**	**p value**
Age	*For each additional year*	1.04 (1.02-1.05)	1.04 (1.03-1.06)	<0.001
Gender	*Male*	1	1	
	Female	0.73 (0.48-1.10)	0.78 (0.50-1.23)	0.28
Smoking	*Non/Ex-Smoker/Current Smoker <10/day*	1	1	
	Current Smoker >10/day	2.64 (1.75-3.99)	2.93 (1.92-4.45)	<0.001
Drinking	*Non/Ex Drinker*	1	1	
	Current Drinker	0.81 (0.48-1.38)	0.89 (0.49-1.62)	0.69
Tumour Stage	*Early (I/II)*	1	1	
	Late (III/IV)	0.75 (0.49-1.16)	0.91 (0.57-1.45)	0.69
Tumour Grade	*Well/Moderately Differentiated*	1	1	
	Poorly Differentiated	0.65 (0.46-.094)	0.88 (0.58-1.33)	0.54
Tumour Site	*Tonsil*	1	1	
	Base of Tongue	1.00 (0.63-1.58)	0.73 (0.44-1.20)	0.21
	Other Oropharynx	1.88 (1.21-2.91)	0.90 (0.52-1.54)	0.70
Treatment	*Surgery*	1	1	
	Radiotherapy	1.74 (1.12-2.70)	1.10 (0.66-1.83)	0.72
	Chemoradiotherapy	1.00 (0.64-1.54)	1.09 (0.66-1.79)	0.74
HPV Status	*Negative*	1	1	
	Positive	0.37 (0.26-0.54)	0.53 (0.33-0.85)	0.008
TIL Status	*TIL Low*	1	1	
	TIL Moderate	0.58 (0.39-0.87)	0.64 (0.41-0.99)	0.05
	TIL High	0.22 (0.13-0.37)	0.28 (0.15-0.52)	<0.001
EGFR	*Negative*	1	1	
	Positive	1.55 (1.04-2.31)	1.55 (0.99-2.42)	0.06
p53	*Negative*	1	1	
	Positive	1.71 (1.15-2.53)	1.31 (0.84-2.03)	0.23

Hazard Ratios for overall survival from unstratified OPSCC





Kaplan Meier curves for overall survival in HPV-positive OPSCC

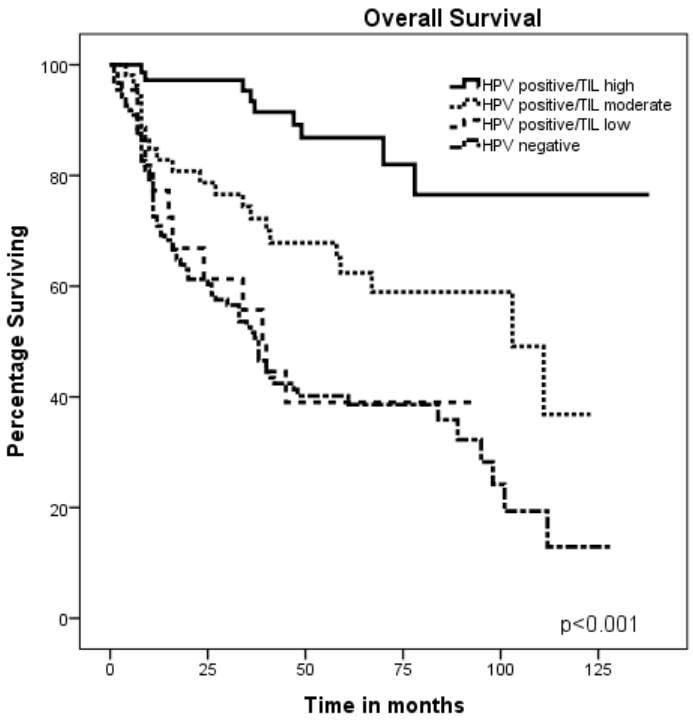
Multivariate analysis adjusted for age, Stage and smoking status.

		HPV-Positive OPSCC N=149			HPV-Negative OPSCC N=121		
		Unadjusted HR (95% CI)	**Adjusted HR (95% CI)**	**p value**	**Unadjusted HR (95% CI)**	**Adjusted HR (95% CI)**	**p value**
Age	*For each additional year*	1.03 (1.00-1.06)	1.05 (1.02-1.08)	0.004	1.03 (1.01-1.05)	1.03 (1.01-1.06)	0.007
Gender	*Male*	1	1		1	1	
	Female	0.71 (0.35-1.45)	0.62 (0.28-1.39)	0.25	0.61 (0.36-1.03)	0.70 (0.38-1.28)	0.24
Smoking	*<10 pack years*	1	1		1	1	
	>10 pack years	2.84 (1.47-5.46)	3.90 (1.94-7.82)	<0.001	1.57 (0.88-2.79)	1.65 (0.93-2.92)	0.09
Drinking	*Non/Ex Drinker*	1	1		1	1	
	Current Drinker	0.94 (0.39-2.27)	1.13 (0.40-3.19)	0.81	0.74 (0.38-1.45)	0.81 (0.39-1.69)	0.57
Tumour Stage	*Early (I/II)*	1	1		1	1	
	Late (III/IV)	0.96 (0.30-3.11)	0.78 (0.24-2.57)	0.68	1.30 (0.79-2.12)	1.25 (0.72-2.16)	0.43
Tumour Grade	*Well/Moderately Differentiated*	1	1		1	1	
	Poorly Differentiated	0.68 (0.36-1.29)	0.64 (0.31-1.29)	0.21	1.14 (0.72-1.82)	1.31 (0.78-2.19)	0.31
Tumour Site	*Tonsil*	1	1		1	1	
	Base of Tongue	0.99 (0.48-2.05)	0.57 (0.24-1.34)	0.21	1.05 (0.58-1.90)	0.95 (0.49-1.83)	0.87
	Other Oropharynx	3.23 (1.38-7.56)	1.82 (0.70-4.76)	0.22	1.06 (0.62-1.80)	0.75 (0.40-1.40)	0.36
Treatment	*Surgery*	1	1		1	1	
	Radiotherapy	1.89 (0.84-4.26)	1.25 (0.47-3.31)	0.66	1.75 (1.022.98)	1.21 (0.66-2.23)	0.54
	Chemoradiotherapy	1.26 (0.62-2.56)	1.26 (0.58-2.76)	0.56	1.30 (0.73-2.34)	1.13 (0.56-2.25)	0.74
TIL Status	*TIL Low*	1	1		1	1	
	TIL Moderate	0.53 (0.26-1.08)	0.46 (0.21-1.00)	0.05	0.78 (0.47-1.28)	0.80 (0.45-1.40)	0.43
	TIL High	0.17 (0.07-0.40)	0.16 (0.06-0.40)	<0.001	0.57 (0.27-1.19)	0.67 (0.29-1.57)	0.36
EGFR	*Negative*	1	1		1	1	
	Positive	1.46 (0.79-2.69)	1.64 (0.83-3.26)	0.16	1.04 (0.60-1.80)	1.45 (0.77-2.71)	0.25
p53	*Negative*	1	1		1	1	
	Positive	1.90 (1.02-3.54)	1.54 (0.75-3.17)	0.24	1.21 (0.73-2.01)	1.07 (0.62-1.86)	0.81

Hazard ratios for Overall Survival from OPSCC stratified by HPV-status

		UHS Cohort				PFT/BLT Cohort			
		HPV-negative n=82	**HPV-positive TIL_{low} n=15**	**HPV-positive TIL_{mod} n=35**	**HPV-positive TIL_{high} n=51**	**HPV-negative n=51**	**HPV-positive TIL_{low} n=8**	**HPV-positive TIL_{mod} n=20**	**HPV-positive TIL_{high} n=22**
		Number (%)	*Number (%)*	*Number (%)*	*Number (%)*	*Number (%)*	*Number (%)*	*Number (%)*	
Gender	*Male*	56 (68.3)	13 (86.7)	26 (74.3)	38 (74.5)	37 (72.5)	6 (75.0)	15 (75.0)	15 (68.2)
	Female	26 (31.7)	2 (13.3)	9 (25.7)	13 (25.5)	14 (27.5)	2 (25.0)	5 (25.0)	7 (31.8)
Age at Diagnosis	*<50*	11 (13.4)	6 (40.0)	8 (22.9)	14 (27.5)	7 (13.7)	1 (12.5)	5 (25.0)	6 (27.3)
	50-69	49 (59.8)	8 (53.3)	18 (51.4)	33 (64.7)	32 (62.7)	6 (75.0)	11 (55.0)	14 (63.6)
	70+	22 (26.8)	1 (6.7)	9 (25.7)	4 (7.8)	12 (23.5)	1 (12.5)	4 (20.0)	2 (9.1)
	Mean (SD)	61.87 (11.74)	54.93 (12.14)	60.09 (13.68)	55.31 (9.25)	59.98 (12.05)	56.88 (10.29)	57.30 (12.38)	56.64 (10.22)
Smoking	*Non-Smoker*	7 (8.5)	4 (26.7)	9 (25.7)	13 (25.5)	3 (5.9)	1 (12.5)	5 (25.0)	12 (54.5)
	Current Smoker <10/day	1 (1.2)	-	2 (5.7)	5 (9.8)	7 (13.7)	-	1 (5.0)	2 (9.1)
	Current Smoker >10/day	50 (61.0)	5 (33.3)	9 (25.7)	13 (25.5)	27 (52.9)	4 (50.0)	7 (35.0)	2 (9.1)
	Ex-Smoker	15 (18.3)	6 (40.0)	8 (22.9)	14 (27.5)	5 (9.8)	3 (37.5)	3 (15.0)	5 (22.7)
	Not Known	9 (11.0)	-	7 (20.0)	6 (11.8)	9 (17.6)	-	4 (20.0)	1 (4.5)
Alcohol	*Non/Ex Drinker*	12 (14.6)	3 (20.0)	4 (11.4)	7 (13.7)	5 (9.8)	1 (12.5)	2 (10.0)	3 (13.6)
	Current Drinker	57 (69.5)	8 (53.3)	21 (60.0)	35 (68.6)	22 (43.1)	5 (62.5)	11 (55.0)	15 (68.2)
	Not Known	13 (15.9)	4 (26.7)	10 (28.6)	9 (17.6)	24 (47.1)	2 (25.0)	7 (35.0)	4 (18.2)
Tumour Site	*Tonsil*	35 (42.7)	12 (80.0)	20 (57.1)	35 (68.6)	24 (47.1)	4 (50.0)	11 (55.0)	17 (77.3)
	Tongue Base	24 (29.3)	2 (13.3)	12 (34.3)	13 (25.5)	11 (21.6)	3 (37.5)	6 (30.0)	5 (22.7)
	Other Oropharynx	23 (28.0	1 (6.7)	3 (8.6)	3 (5.9)	16 (31.4)	1 (12.5)	3 (15.0)	0 (0.0)
Disease Stage	*I*	9 (11.0)	0 (0.0)	0 (0.0)	1 (2.0)	9 (17.6)	0 (0.0)	1 (5.0)	1 (4.5)
	II	12 (14.6)	1 (6.7)	2 (5.7)	3 (5.9)	9 (17.6)	2 (25.0)	0 (0.0)	1 (4.5)
	III	7 (8.5)	2 (13.3)	5 (14.3)	3 (5.9)	10 (19.6)	0 (0.0)	4 (20.0)	6 (27.3)
	IV	54 (65.9)	11 (73.3)	28 (80.0)	44 (86.3)	22 (43.1)	6 (75.0)	15 (75.0)	14 (63.6)
	Not Known	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
Grade	*Well/Moderately Differentiated*	44 (53.7)	3 (20.0)	10 (28.6)	6 (11.8)	31 (60.8)	4 (50.0)	7 (35.0)	3 (13.6)
	Poorly Differentiated	38 (46.3)	12 (80.0)	25 (71.4)	45 (88.2)	20 (39.2)	4 (50.0)	13 (65.0)	19 (86.4)
Treatment	*Surgery+/- Radiotherapy*	31 (37.8)	7 (46.7)	13 (37.1)	23 (45.1)	25 (49.1)	4 (50.0)	5 (25.0)	7 (31.8)
	Radiotherapy	25 (30.5)	3 (20.0)	8 (22.9)	7 (13.7)	9 (17.6)	1 (12.5)	2 (10.0)	2 (9.1)
	Chemoradiotherapy	20 (24.4)	4 (26.7)	13 (37.1)	21 (41.2)	12 (23.5)	3 (37.5)	12 (60.0)	12 (54.5)
	None/Palliative	6 (7.3)	1 (6.7)	1 (2.9)	0 (0.0)	5 (9.8)	0 (0.0)	1 (5.0)	1 (4.5)
EGFR	*Negative*	26 (31.7)	6 (40.0)	14 (40.0)	36 (70`.6)	8 (15.7)	3 (37.5)	9 (45.0)	10 (45.5)
	Positive	49 (59.8)	6 (40.0)	20 (57.1)	10 (19.6)	42 (82.4)	5 (62.5)	11 (55.0)	12 (54.5)
	Not Known	7 (8.5)	3 (20.0)	1 (2.9)	5 (9.8)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
p53	*Negative*	27 (32.9)	6 (40.0)	27 (77.1)	31 (60.8)	16 (31.4)	2 (25.0)	6 (30.0)	4 (18.2)
	Positive	49 (59.8)	8 (53.3)	7 (20.0)	15 (29.5)	33 (64.7)	6 (75.0)	14 (70.0)	18 (81.8)
	Not Known	6 (7.3)	1 (6.7)	1 (2.9)	5 (9.8)	2 (3.9)	0 (0.0)	0 (0.0)	0 (0.0)

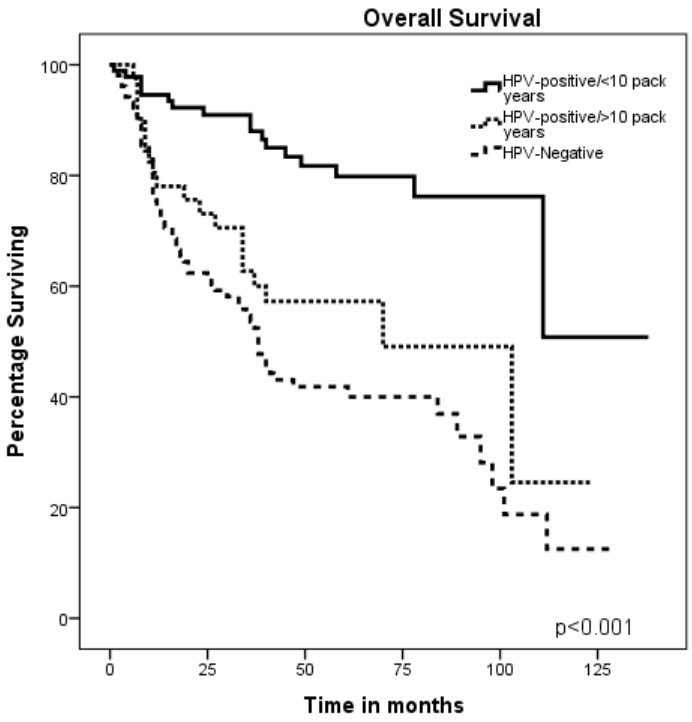
Demographics stratified by HPV/TIL-status



Kaplan Meier Curve for Overall Survival from OPSCC according to HPV/TIL-status

	Univariate HR (95% CI)	Multivariate HR (95% CI)	p-value
HPV-Negative	1	1	
HPV-Positive/TIL^{low}	0.93 (0.50-1.71)	1.36 (0.70-2.64)	0.37
HPV-Positive/TIL^{Mod}	0.49 (0.30-0.80)	0.62 (0.35-1.13)	0.12
HPV-Positive/TIL^{High}	0.15 (0.08-0.31)	0.19 (0.09-0.44)	<0.001

Hazard ratios for overall survival according to HPV/TIL-status



Kaplan Meier curve for overall survival from OPSCC according to HPV/Smoking status

	Univariate HR (95% CI)	Multivariate HR (95% CI)	p-value
HPV-Negative	1	1	
HPV-Positive/>10 pack years	0.67 (0.40-1.12)	1.08 (0.60-1.94)	0.81
HPV-Positive/<10 pack years	0.24 (0.14-0.41)	0.36 (0.20-0.65)	0.001

Hazard Ratios for Overall Survival from OPSCC according to HPV/Smoking status

	HPV-positive N=149	HPV-negative N=121	p-value
Total T-Cell (CD4+CD8)	111.7	53.7	<0.001
$CD8^+$	57.1	24.0	<0.001
$CD4^+$	54.0	29.9	<0.001
$Foxp3^+$	32.1	16.7	<0.001
Proportion $CD8^+$	51.1%	44.7%	<0.001
Proportion $CD4^+$	48.9%	54.0%	<0.001
Proportion $Foxp3^+$	31.2%	36.8%	0.08
CD4:CD8 Ratio	0.96:1	1.21:1	<0.001
Foxp3:CD8 Ratio	0.78:1	0.69:1	<0.001

T-cell subsets and ratios

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