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What is the role of the immune system, patient pathology and social deprivation on patients diagnosed with Head and Neck Cancer?

by

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ABSTRACT

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Adal Hussain Mirza

Table of Contents

List of Tables

List of Figures

DECLARATION OF AUTHORSHIP

I, ADAL HUSSAIN MIRZA

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

An Update in Head and Neck Cancer.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
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6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as: Mirza AH, King EV, Nouraei R, Repanos C. Impact of social deprivation on the outcome of major head and neck cancer surgery in England: A national analysis. *Head Neck* 2018 (doi: 10.1002/hed.25461) *and* Mirza AH, King EV. The Importance of the Immune System on Head and Neck Cancer. *Head Neck* 2019 (Accepted).

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And finally, a thank you to my Mother. I miss you.

Definitions and Abbreviations

5-FU	5-Flurouracil
AJCC	American Joint Committee on Cancer
APC	Antigen Presenting Cell
AUROC	Area under ROC curve
Bcl-2	B Cell Lymphoma 2
CD	Cluster of Differentiation
CT	Computed Tomography
CTL	Cytotoxic T Lymphocyte
CZI	Carl Zeiss Image
DAHNO	Data for Head and Neck Oncology
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DFS	Disease Free Survival
DSS	Disease Specific Survival
ECOG	Eastern European Oncology Group
ECS	Extra-capsular Spread
EGFR	Epidermal Growth Factor Receptor
FOM	Floor of Mouth
HNC	Head and Neck Cancer
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
HPV(+)	Human Papilloma Virus Positive Patients
HPV(-)	Human Papilloma Virus Negative Patients
ICD-10	International Classification of Statistical Diseases
IHC	Immunohistochemistry
ISH	In Situ Hybridisation
MDT	Multi-disciplinary Team
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NHS	National Health Service
OPCS	Office of Population and Censuses and Surveys
OPSCC	Oropharyngeal Squamous Cell Carcinoma
OR	Odds Ratio
OS	Overall Survival

p53	Tumour Suppressor Protein 53
PCR	Polymerase Chain Reaction
PEG	Percutaneous Endoscopic Gastrostomy
PDGF	Platelet Derived Growth Factor
PET-CT	Positron Emission Tomography-CT
PFS	Progression Free Survival
PY	Pack Years (Smoking)
ROC	Receiver Operator Characteristic curve
RT	Radiotherapy
QOL	Quality of Life
TIL	Tumour Infiltrating Lymphocytes
TNM	Tumour Node Metastasis
TOLS	Transoral Laser Resection
TOR	Transoral Resection
TORS	Transoral Robotic Resection
UHN	University Hospital Network (Princess Margaret Cancer Centre, Toronto)
UHS	University Hospital Southampton
UICC	Union for International Cancer Control
UK	United Kingdom
WHO	World Health Organisation
WISH	Wessex Investigational Sciences Hub Laboratory

Chapter 1: Introduction

1.1 Head and Neck Cancer

Head and neck squamous cell carcinoma (HNSCC) is a comprehensive term used to describe a collective of epithelial malignancies that occur in the oral and sino-nasal cavities, lips, nasopharynx, oropharynx, hypopharynx and larynx (see Figure 1-1) These subsites are recognised in the ICD-10 classification of diseases. Given their heterogeneous anatomical origins, the tumours often present and give rise to unique natural histories. Up to two-thirds of patients with HNSCC will present with disease of an advanced clinical stage, often based on the presence of regional metastasis (1). Distant metastasis is rare at presentation however and occurs in 10% of patients (1).

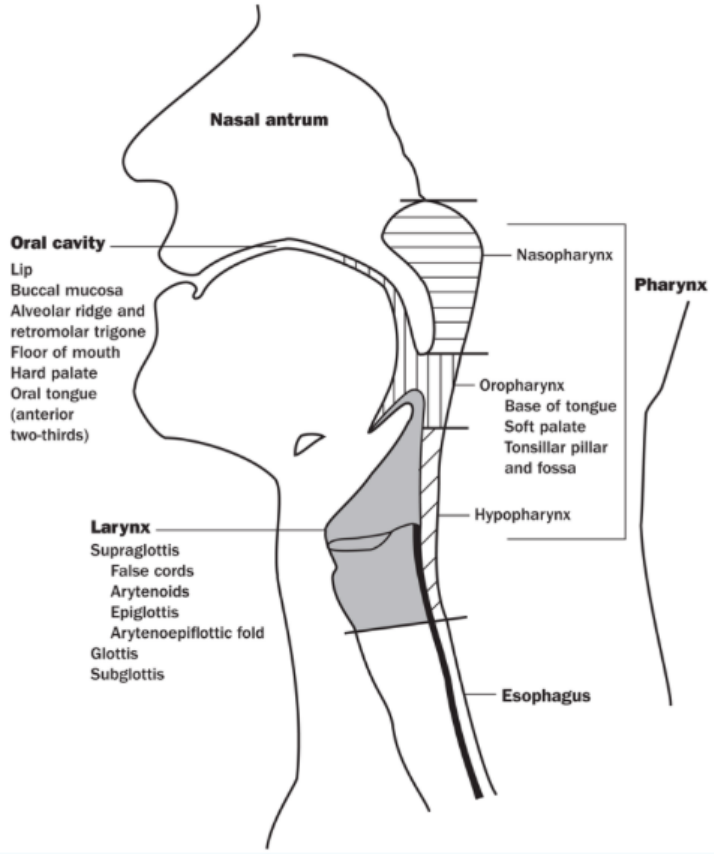


Figure 1-1: A schematic diagram demonstrating the regional anatomy of the upper aerodigestive tract and specifically the subsites located within each given anatomical site (2)

Whilst advances have been made in the diagnosis, investigation and treatment of HNSCC, outcomes for patients overall are largely unchanged. Five-year survival rate has improved somewhat from 52.7% in 1982-1986 to 65.9% in 2002-2006 (3), though some subsites fare considerably worse than others. Patients with a diagnosis of hypopharyngeal cancer for example

demonstrate survival of only 41.3% in the modern (post 1990) age. In patients where metastatic or recurrent disease is present, Overall Survival (OS) is less than a year (4).

1.2 Epidemiology of Head and Neck Cancer

Annually, there are over 500,000 incident cases of HNSCC worldwide and HNSCC is cited as the 6th most commonly diagnosed cancer (5, 6). In the United Kingdom (UK) alone there were 9,906 new diagnoses and 2,903 deaths attributed to HNSCC respectively (7). Although other histological subtypes exist, over 90% are squamous cell carcinoma (8). Non-squamous malignancy includes thyroid and salivary gland cancers but falls outside the remit of this text and is not discussed further.

The incidence of HNSCC appears to be related to geography and higher rates are reported in India, South America and Eastern Europe (9). In India, 45% of all malignancies are the result of HNSCC and occur most commonly in the oral cavity (9). Due to exposure to Epstein-Barr Virus (EBV) and inhaled carcinogens in cured fish, particularly high rates of nasopharyngeal carcinoma are seen in Southeast Asia (9, 10). In England, crude rates per 100,000 suggest that oral cavity (9.6) and larynx (7.9) are the most common sites of disease, followed by the pharynx (5.6) (11).

Men are two to three times more likely to be diagnosed with HNSCC than women and over 98% of cases occur in patients over the age of 40 (9, 12), with a median age of 60 years (1). Prior to this, the ratio was even higher (5:1) but contemporary reports suggest that incidence is increasing in females whilst decreasing in males (12).

Traditionally the most important carcinogens have been tobacco exposure, both active and passive and alcohol consumption. For men, the risk is multiplicative with tobacco and alcohol exposure combined, giving a higher proportion of HNSCC cases, than either alone (9). For women, the effect of alcohol alone presents a greater number of cases than tobacco and alcohol combined. The sum of the population-attributed risk is 72%; 33% and 4% from tobacco and alcohol alone and 35% synergistically (13).

1.2.1 Age and Head and Neck Cancer

Worldwide, incidence of HNSCC has increased, alongside increasing population age and 98% of all cancers are seen in patients aged 40 and above (14). For most patients, the median age at presentation is their early 60s (1, 15). In some subsets, e.g. oropharyngeal cancer, the age at presentation has decreased, and this is discussed further in Chapter 1.2.2. Outcomes are related to age, with increasing age at presentation related to worse outcomes.

1.2.2 Oropharyngeal Cancer

Oropharyngeal cancer occurs in the oropharynx, the space inferior to the nasopharynx and superior to the hypopharynx. Its anatomy is limited antero-superiorly by the oral cavity and antero-inferiorly by the tongue and valleculae. Posteriorly, it is limited by the 3rd cervical vertebra. Its lateral walls contain the palatine tonsils and the fauces and inferiorly it is in continuity with the hypopharynx.

The incidence of oropharyngeal cancer has increased significantly in developed countries (16). In the UK, the incidence of oropharyngeal cancer doubled between 1990 and 2006, doubling again in the time again between 2006 and 2010 (16). It is projected that by 2020, HPV-associated oropharyngeal squamous cell cancer will be more prevalent than cervical cancer (17).

In 1995, the International Agency for Research on Cancer (IARC) demonstrated a carcinogenic potential of HPV subtypes 16 and 18 in human patients (18). Subsequently, a causative link between HNSCC and HPV was first identified in 2007 and today HPV(+) related HNSCC is thought to comprise up to 90% of all oropharyngeal diagnoses (19, 20). Between 1970 and 2002 the incidence of tonsillar HNSCC was noted to have increased in Sweden by a factor of 2.8; associated closely with a 2.9 fold increase rate of HPV(+) HNSCC (21). Similar changes in the rate of oropharyngeal tumours were observed in the USA (5) and the UK, where age-standardised rate of oropharyngeal SCC per 100,000 increased 2-fold between 1990 and 2006 (10). The identification of a new clinico-pathogenic entity was confirmed, one that was distinct from its counterpart, caused by smoking and alcohol consumption.

Although many HPV(+) patients present with an advanced UICC TNM version 7 stage, in comparison to HPV(-) cases, their prognosis is in general, better (22). At 5 years, disease specific survival (DSS) is approximately 80% in HPV(+) disease, versus 40% in HPV(-) cancers, respectively (16, 23). Ang et al. demonstrated a 58% risk reduction of death compared with HPV(-) patients (HR 0.42, [95% CI 0.27-0.66]) (24). This has been subsequently and consistently reported since. McDonald et al. showed that in the years of 1991 and 2001, survival from oropharyngeal cancer increased (OR 1.46 [95% CI 1.28-1.66]), a phenomenon not demonstrated in other head and neck cancers (25), such as Hypopharyngeal Cancer, discussed further in section 1.2.3.

1.2.3 Hypopharyngeal Cancer

Hypopharyngeal cancer occurs in the aerodigestive tract, lateral and posterior to the larynx (see Figure 1-1). The hypopharynx is a continuous area, opening superiorly into the oropharynx and inferiorly into the cervical oesophagus, via the cricopharyngeus (superior oesophageal sphincter).

Surgically, the superior limit is marked by the hyoid bone (endoscopically the level of the pharyngo-epiglottic fold) and inferiorly, at the caudal aspect of the cricoid cartilage. The hypopharynx is subdivided into three areas; the post-cricoid region (extending from the arytenoid cartilages down to the inferior border of the cricoid), the pyriform sinus (extending from the pharyngo-epiglottic fold to the superior aspect of the oesophagus) and the posterior pharyngeal wall (to the level of the crico-arytenoid joints) (26).

Hypopharyngeal cancer is rare, representing 0.5% of all human malignancies. In the upper aerodigestive tract, 3-5% of all malignancies occur in the hypopharynx (9). Worldwide, the highest incidences of 2.5/100,000 are seen in Indian subcontinent, Brazil and Central and Western Europe. The lowest incidences of 0.5/100,000 are seen in East Asia, Africa and Northern Europe. Trends in its incidence over the last 20 years, certainly in the UK, suggest a static picture (10). In the UK an age-standardised incidence of ≈1/100,000 is seen per annum (9, 27); the highest occurring in Merseyside and Cheshire (1.14) and the lowest in Dorset (0.32) (10).

Typically, these cancers present most commonly in men, in individuals older than 50 years, with a peak incidence in the 6^{th} and 7^{th} decades. The site most commonly involved is the pyriform sinus (66-75%), followed by the posterior pharyngeal wall (20-25%) (27).

Of all the cancers within the head and neck, hypopharyngeal carcinoma carries the worst prognosis (28, 29) with a 5-year survival of approximately 30% (27, 30). 80% of these tumours present with TNM 7 AJCC UICC Stage III/IV (27, 29) and 20-30% of patients are inoperable on presentation (31). Posterior pharyngeal wall and cervical oesophageal cancers confer the worst survival (11% at 5 years, p=0.0152) with no significant difference between pyriform fossa and post-cricoid lesions (p=0.0410) (32). Increasing T stage in all hypopharyngeal cancers is associated with worse survival, with T1 tumours exhibiting a 46% survival at 5 years and T4 with 15% only (32)(see Figure 1-2). Multi-modal treatment is essential to provide the best chance of cure (33).

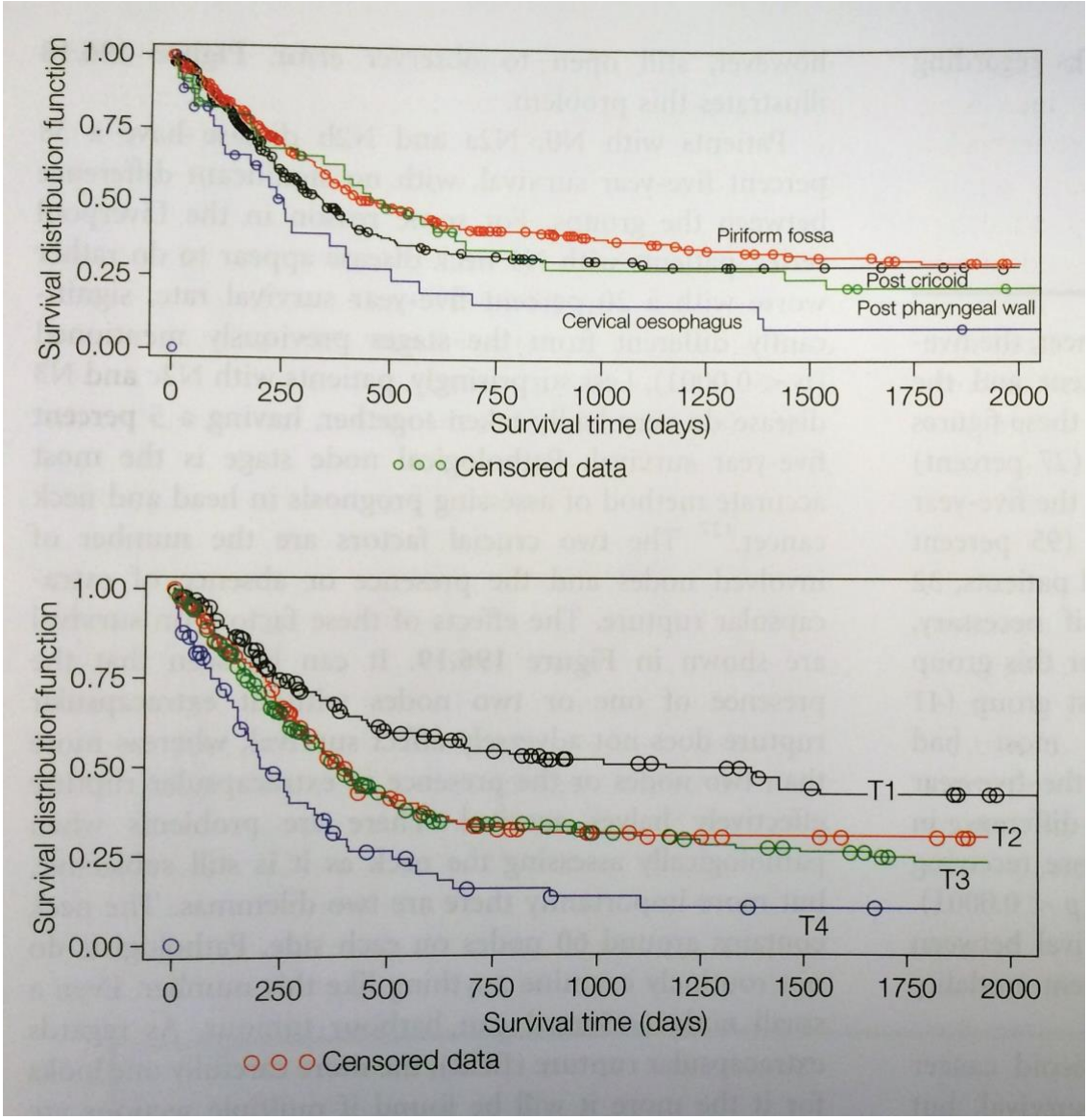


Figure 1-2. Survival outcomes post hypopharyngeal cancer diagnosis; Above - Subsite, demonstrating worst outcomes for cervical oesophageal and posterior pharyngeal wall cancer and Below - T stage, demonstrating worst outcomes for larger local tumours. Taken from Scott Brown's Otorhinolaryngology Head and Neck Surgery (32)

However, there is evidence to suggest, that outcomes may be subtly improving for hypopharyngeal cancer, perhaps in association with more refined diagnosis, management and care. In a three-decade population study by Newman et al., average 5-year survival was shown to improve from 37.5% before 1990, to 41.3% after 1990 ($p<0.0001$) (33). This study drew information from the SEER 17 (Surveillance, Epidemiology and End Results) database and included 6,647 patients with SCC of the hypopharynx (33). The best OS was seen in combination surgical and radiation therapy patients (48.5%) as opposed to those managed with either modality alone.

Interestingly, this dataset suggested that, while the posterior wall subsite was the least commonly affected, with the worst prognosis, there was also a decline in its mortality during the study

period, declining to 38.5% from 35.5% (p=0.1817) (33). Although the disease was usually seen in Caucasian patients (79% overall), African Americans diagnosed with the disease were associated with a worse outcome (41.2% vs. 28.9% p<0.001) (33).

1.2.3.1 Plummer Vinson Syndrome

Of note, in the post-cricoid region, the incidence of carcinoma is higher in women than for men (26, 31, 34). One explanation is Plummer-Vinson (Paterson-Kelly) Syndrome (PVS), a triad of dysphagia, hypochromic anaemia and oesophageal webs, first associated in a population of Swedish women, demonstrating a high incidence of both the syndrome and carcinoma (34). Subsequent longitudinal studies, have quoted the incidence of post-cricoid carcinoma in patients with chronic dysphagia in PVS as 10-16% (35, 36). 4-6% of patients with hypopharyngeal carcinoma have an accompanying diagnosis of PVS (26).

Overall however, PVS is a rare finding or precursor in patients with hypopharyngeal cancer and indeed head and neck cancers overall. Some of the more common aetiologies and factors related to HNSCC are discussed further below.

1.3 Patient based aetiologies in Head and Neck Cancer

1.3.1 Tobacco

The carcinogenic effect of tobacco and subsequent effect on prognosis and outcome is well established (37). In contemporary medicine, smoking is one of the most common and preventable causes of cancer and historically has been associated with up to 90% of patients with HNSCC (9, 14, 38). In combination with alcohol, it derives a multiplicative effect, the result of increased mucosal absorption of its constituent carcinogens (9).

Tobacco, in the western world, is most commonly smoked in the form of rolled cigarettes (14), the dried leaf of the plant *nicotiana tabacum*. It can additionally be consumed orally (as smokeless tobacco), via chewing tobacco or nasally as snuff, the former most common in the South Asian continent. Both within the UK (see Figure 1-3) and worldwide, cigarette consumption is declining, with 41.5 million current smokers worldwide in 2011, a decline of 4 million from 2005 (39).

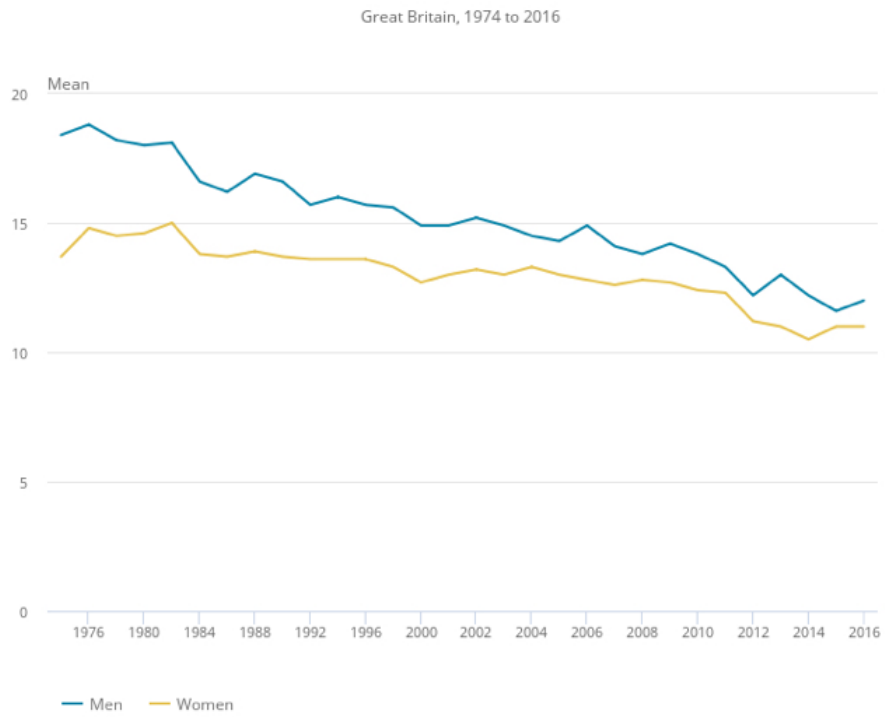


Figure 1-3. Chart to demonstrate the decreasing average daily consumption of cigarettes among current smokers for men and women aged 16 and over in the UK. From https://www.ons.gov.uk/ Accessed 20/07/17

A causal association between smoking and head and neck cancer was first introduced in a seminal series from Wynder et al, following introductory studies into lung and laryngeal cancer (37). This association has since been reinforced, with multiple studies underlining the strength of this relationship (13, 14, 40, 41). Wyss et al., using data from the INHANCE (International Head and Neck Cancer Epidemiology) Consortium, a collective of 13,935 cases and 18,691 controls, found an overall odds ratio (OR) of HNSCC, in *ever* compared with *never* cigarette smokers of 3.46 (95% CI 3.24-3.70) (40). This association has also been demonstrated for involuntary passive smoke exposure (38).

Pooled analysis from Lee et al. for example found a significant relationship for passive exposure for >15 years, both at home (OR =1.60, [95% CI 1.12-2.28]) and at work (OR 1.55, [95% CI 1.04-2.30]) (42). This association was strengthened for those who had never consumed both tobacco *and* alcohol in comparison to those with passive exposure who had, (OR =1.75, [95% CI 1.06-2.90]) and (OR 2.59, 95% CI 1.35-4.95) for home and work respectively (42).

Tobacco smoke is thought to contain over 4,000 different compounds, 200 of which are known to be poisonous and 60, to be carcinogenic (39, 43-45). Such carcinogens include polycyclic aromatic hydrocarbons (PAH) and *N*-nitrosamines (14) such as nitroso-nor-nicotine (NNN), nitroso-pyrrollodine (NPYR), nitroso-dimethylamine (NDMA) and 4-methyl-nitrosoamino-1-3-pyridyl-1-

butanone (NNK) (14, 43).The latter compounds are nicotine derivatives which although intrinsically lack carcinogenicity, ensure a longevity of exposure via addiction (see Figure 1-4). Importantly both PAH and *N*-nitrosamines require metabolic activation in order to exert their carcinogenic effect and the different rate at which this occurs (or indeed doesn't) in individuals, will dictate the subsequent risk of carcinogenesis (44).

These compounds interact in local keratinocyte stem cells to form DNA adducts and point mutations which subsequently result in epigenetic changes (mutations in RAS, TP53 and other genes) and abnormal cell physiology (46). Concurrent to this, receptor binding (e.g. PKA activation) takes place, leading to reduced apoptosis and increased angiogenesis. Finally the suppression of tumour suppressor gene creates a heightened carcinogenic environment, the cumulative outcome of all these effects being carcinoma (see Figure 1-4) (47).

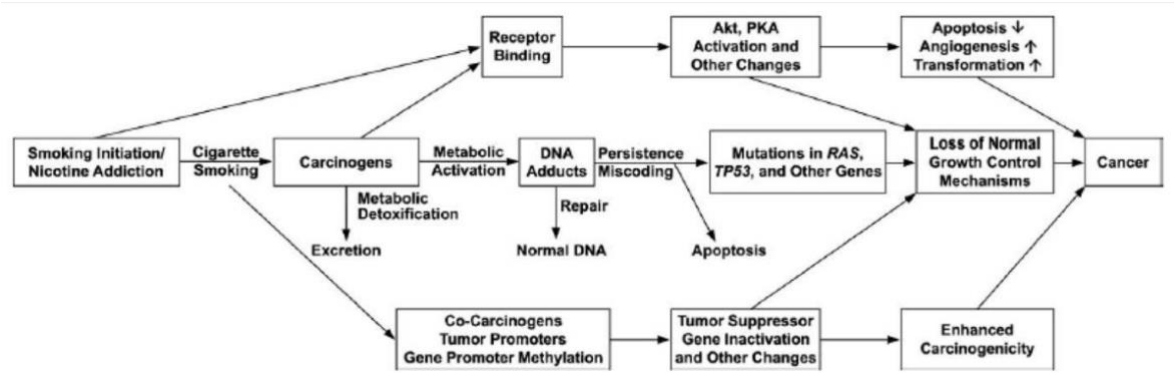


Figure 1-4. A schematic diagram representing the association of nicotine addiction and carcinogenesis in cancer. The central stem represents the importance of chronic addiction from nicotine leading to continued exposure to carcinogens and the formation of DNA adducts (carcinogens that have been metabolically activated and subsequently bound with DNA via covalent bonds). Repair is possible and in most circumstances, this returns the DNA to its normal undamaged state. Persistence of adducts during DNA replication can cause miscoding and hence a permanent mutation in the DNA sequence. Taken from Hecht 2003 (47)

A dose dependent relationship exists between smoking and the relative risk (RR) of developing cancer. In those smoking ≤7 cigarettes, a RR of 2.4 exists, compared to 16.4 in those smoking ≥25 (12). Of note, 5 years of abstinence is associated with a 50% risk reduction and the risk of developing oral cancer approaches that of lifetime *non*-smokers, following a decade of abstinence (9, 39).

1.3.2 Alcohol

Alcohol intake is associated with an increased risk of cancers of the upper aero-digestive tract (14, 48). Ethanol acts as a solvent and its consumption enhances contact of tobacco carcinogens with

the mucosa of the upper aero-digestive tract (48-50). Additionally, chronic alcoholism is associated with atrophy and lipomatous change in the salivary glands, leading to an impaired flow and increased viscosity of saliva (50). This may be associated with an inability to wash away local carcinogens, exacerbated by those using tobacco.

A study by Tuyns et al. in 1976 demonstrated a fall in the incidence of HNSCC during a period of decreased alcohol consumption in the Second World War; noteworthy, given that consumption of tobacco remained unchanged (51). This discrepancy between tobacco and alcohol was further reinforced by Hashibe et al. who demonstrated a population attributable risk of 72% in patients who drank *and* smoked, compared with risks of 4% and 33% for alcohol and tobacco alone (13).

There is a positive dose-response relationship suggesting that duration of exposure is more important that intensity (6, 52-55), but risk to discrete anatomical subsite, alcohol type, ethanol metabolising gene polymorphisms and the effect of alcohol cessation remain unclear.

In a large case-control series of 1,751 patients (HNSCC patients = 811, controls = 940), alcohol consumption was reviewed in an East Asian population with HNSCC (52). A positive dose-response relationship was demonstrated, with moderate (OR 1.47 [95% CI 1.02-.11]) and heavy (OR 2.21 [1.61-3.02]) drinking associated with a greater risk of HNSCC (52). The strongest positive association was seen for hypopharyngeal cancer, maintained at even low frequency and volume drinking: weekly (OR 13.47 [95% CI 3.98-45.63]) and light drinking (OR 6.63 [95% CI 2.03-21.63]) (52).

10 years of abstinence from alcohol consumption was associated with a reduced risk of HNSCC, comparable to occasional or never consumers (52). Furthermore, abstinence of up to 20 years is required to reduce level of risk to that of a non-consumer (38).

The significance of alcohol beverage type remains unclear. Some studies show an increased risk of HNSCC in distilled alcohol drinkers, compared to beer or wine drinkers(52, 56), but this has not been demonstrated by all groups (57). Purdue et al. pooled the collective results of 15 case-control series (HNSCC cases = 9,107, controls = 14,219) and found similar associations for beer and wine only drinkers and liquor consumers (58). For drinkers of ≤5, 6-15, 16-30 and >30 units, odds ratios were 1.6, 1.9. 2.2 and 5.4 in the beer only group and 1.6, 1.5, 2.3 and 3.6 in the liquor only drinkers respectively (58).

1.3.2.1 Pathogenetic mechanism of Alcohol

On consumption, ethanol is converted to acetaldehyde by mucosal alcohol dehydrogenase (ADH) and cytochrome P-450 (CYP2E1), see Figure 1-5 (15). Acetaldehyde is the molecule responsible for

the carcinogenic effect of alcohol. Acetaldehyde is ultimately metabolised to acetate by acetaldehyde dehydrogenase (ALDH). Mutation of the ALDH gene leads to ineffective ALDH enzyme (ALDH2), allowing for more elevated levels of acetaldehyde in some people.

Elevated levels of acetaldehyde are carcinogenic. Point mutations are caused in the *hypoxanthine-guanine-phosphoribosyl transferase* locus in leukocytes, alongside chromosomal aberrations. Furthermore, *06 methyl-guanyltransferase*, an enzyme important for the repair of adducts (protein and lipid by-products joined together by covalent bonds which can impair function of proteins and cause DNA mutations) created by alkylating agents, is inhibited. Acetaldehyde binds to cellular proteins and DNA that results in both morphological and functional impairments of the cell. This adduct is stable enough to allow longevity in replication errors and mutations in oncogenes or tumour suppressor genes.

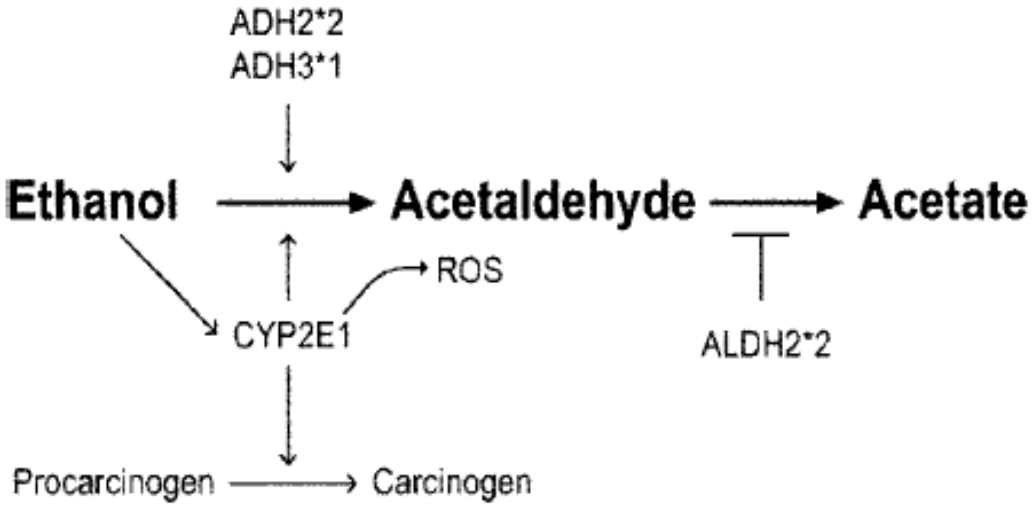


Figure 1-5. Mechanism of carcinogenesis of Alcohol, demonstrating the metabolism of ethanol to the carcinogenic acetaldehyde. ADH2*2 and ADH3*1 are polymorphisms of ADH. Taken from Seitz et al. (50)

As a consequence of the above events, inflammation and metaplasia of the aerodigestive epithelium is seen. DNA synthesis and repair is negatively affected and hyper-regeneration causes an increased risk of cellular injury.

Certain groups of people, predominantly far eastern populations, have been shown to be predisposed to alcoholism due to genetic alterations to the acetaldehyde dehydrogenase gene, located on chromosome 12 (52, 59). Low volume drinkers are more susceptible to alcohol side effects such as facial flushing, tachycardia and nausea and this may be related to inactivity of ADH2 (60). Additionally inactivity of ALDH2 could contribute to a higher risk of alcoholism (60).

1.3.3 Diet and the development of HNSCC

The role of diet in HNSCC has been explored and notable associations have been demonstrated (61-65). In a pooled analysis from Chuang et al. using data from the INHANCE Consortium, an inverse relationship of risk of HNSCC was demonstrated for fruit (OR 0.52 [95% CI 0.43-0.62]), non-starchy vegetables (OR 0.68 [95% CI 0.51-0.90]) and white meat (61). Contrarily, an increased association between consumption of red meat (OR 1.40 [1.13-1.74]), pork (OR 1.48 [1.19-1.83]) and eggs (OR 1.44 [1.12-1.86]) was also seen (61).

1.3.3.1 BMI

BMI has to date, been variably associated with risk of HNSCC (62, 66, 67). In a large prospective study by Maasland et al., 120,852 patients were followed up over 20 years. In this population, an inverse association between BMI and development of SCC was demonstrated on multivariate analyses; OR 3.31 [95% CI 1.40-7.82] comparing BMI of <18.5 to 18.5-<25. A BMI of ≥30 appeared protective (OR 0.48 [95% CI 0.22-1.03]) (68).

Outcomes of patients also seem dependent on BMI and this was illustrated in a study of 1279 patients by Gama et al. 2017 (69). Being overweight (HR 0.45 [95% 0.3-0.6]. p<0.01) was seen to be protective to OS in comparison to normal weight (69). For underweight patients, OS was seen to reduce significantly (HR 2.24 [95% CI 1.4-3.6])(69). Recurrence was also seen to be higher in underweight (HR 1.98 [95% CI 1.2-3.3]), than overweight (HR 0.69 [95% CI 0.5-1.0]) patients (69).

Such results should be interpreted in caution, in a patient group who invariably present underweight and in a state of malnutrition. Although both a high and low BMI can be associated with malnutrition, it may be that the above results suggest a relatively better nutritional state in patients with a raised BMI and this may be the underlying cause of the improved outcomes in this patient group.

1.3.4 Genetic susceptibility

Cells can harbour a genetic susceptibility to mutate and develop oncogenic potential. These events are random and infrequent enough to be normally policed by the patient's healthy immune system. However, with the addition of environmental carcinogens (tobacco, alcohol, betel nuts, and human papillomavirus) the risk of cancer transformation increases (15, 70).

The susceptibility of a particular individual to the carcinogens identified above is dependent in part on hereditary genetic factors. Not all patients exposed to carcinogens in the upper aerodigestive tract, go on to develop HNSCC and therefore other factors are at play (71). An

observational study by Foulkes et al. compared first-degree relatives of patients diagnosed with HNSCC and the first-degree relatives of their spouses (72). An increased relative risk of 3.79 (p=0.034) was identified in the HNSCC relatives compared with relatives of their spouses, having adjusted for ethnic group, sex, tobacco and alcohol consumption (72).

The glutathione S-transferases (GSTs) are a family of enzymes with many metabolic roles, and importantly, an ability to detoxify the highly mutagenic 7,8-diol-9,10-epoxide (BPDE) and other polycyclic hydrocarbons commonly found in tobacco smoke (46, 71). This family, made up of at least five different gene classes, have been seen to develop specific polymorphisms, in particular at the *GSTM1* and *GSTT1* genotypes which are important to the susceptibility of HNSCC.

Both the polymorphic *GSTM1* and *GSTT1* result in a null genotype, which subsequently offers no functional enzymatic activity. This genetic phenomenon is relatively common with 50% and 15% of US Caucasians demonstrating homozygosity for *GSTM1* and *GSTT1* (71). In some cases, null *GSTM1* and *GSTT1* genotypes have been linked with a higher risk of HNSCC (46, 73) and elsewhere, not (74).

In addition to sporadic genomic alterations, other genetic alterations occur frequently enough to be associated with HNSCC. Some of these include p53, retinoblastoma, epidermal growth factor receptor (EGFR) and the PI3 Kinase pathway.

1.3.4.1 Genetic Cancer Syndromes

Several cancer syndromes are known to exist in the head and neck, but sporadic neoplasia is the norm. Fanconi anaemia (FA) is an autosomal recessive instability disorder, known to cause germ line mutations in at least 15 DNA repair genes (*FANCA/B/C/D/D1/D2/E/F/G/I/J/L/M/N/O/P*) (75). A particular hyper-sensitivity is observed to the toxic effects of DNA cross-linking agents (71) and a mutation in p53 gene (76, 77). Clinically, patients present with bone marrow failure, congenital malformations and a higher risk of developing malignancies at a young age, particularly of acute myeloid leukaemia and HNSCC, which is the most frequently diagnosed solid tumour in FA (75). Patients present with neoplasia in the third decade, with a median age of 31 (75). Primary tumours most frequently present in the oral cavity with a disposition for malignancy in male populations. Overall, Fanconi anaemia is more common in women by a 2:1 ratio.

Bloom Syndrome (BS), another autosomal recessive condition, is associated with an increased incidence of autosomal breaks and re-arrangements (78, 79). Affected patients carry both copies of the *BLM* gene and suffer from abnormal BS protein function, the effect of which is not entirely clear. Patients are small in size, with a distinctive facial flush (79) and a notable proportion are seen to exhibit neoplasia at a particularly early age, with a mean age of 21 years (78). In their

seminal paper on Bloom syndrome and head and neck cancer, Berkower and Biller reported that all deaths in patients older than 7 years in the Bloom's syndrome registry were attributable to cancer (78).

Xeroderma Pigmentosum is a rare dermatosis presenting early in childhood, characterised by a deficit in nucleotide excision repair. Exposure to ultraviolet radiation results in a significantly increased risk of skin neoplasia. In patients under the age of 20, there is a 10,000 fold increased risk for non-melanoma skin cancer (80). The mean age for skin cancer in affected patients is 8 years and the most commonly affected site is the head, neck and scalp (80). Patients are also observed to develop oral squamous cell carcinoma of the anterior tongue with noteworthy frequency (81).

Elsewhere, there are several syndromes of note that present with head and neck cancer. Lynch II syndrome, an exceedingly rare disease, is known to present with multiple laryngeal cancers (71). Ataxia-telangiectasia, characterised by an increased number of spontaneously induced chromosomal aberrations, has a distinct entity type that presents with solid tumours of the oral cavity. Finally dyskeratosis congenita, a type of bone marrow failure, results in oral cavity and nasopharyngeal squamous cell cancers (76).

1.3.5 Human Papillomavirus (HPV)

HPV are a distinct family of viruses, ubiquitous in humans, of which there are over 150 fully sequenced sub-types (15, 76, 82). Infection with HPV is associated with squamous epithelial cells and often results in epithelial lesions, most of which are benign in nature. Mucosal sub-types are also identified and can be broadly differentiated into high- and low-risk forms. High-risk (HR) subtypes have been identified in HNSCC and HPV genotype 16 is now a well-established independent risk factor, occurring in up to 87% of all oropharyngeal HNSCC (15, 83, 84). Although other oncogenic genotypes have also been identified in HNSCC (e.g. genotype 13 and 18 in less than 3%) they are almost negligible in incidence (85).

Within the cervix and anogenital tract the HR HPV subtypes include HPV 16, 18, 31 and 45 (85). These latter subtypes are associated with over 99.7% of all cervical cancers (86). Much of what is understood regarding the oncogenicity of HPV relates to cervical cancer, where the virus has been extensively studied.

In adults, HPV infection follows a bi-modal distribution pattern, with peaks as a young adult and again in the sixth decade. Lifetime prevalence approaches 1% (85). Studies in women with cervical HPV infection suggest most clear the infection within weeks to several months (87). Up to 10-15%

fail to clear the infection via adequate immune response, establishing a chronic infection (87), the latter being the first step in a potential pathway to genomic instability. The viral genome enters the host with the potential for subsequent host mutations.

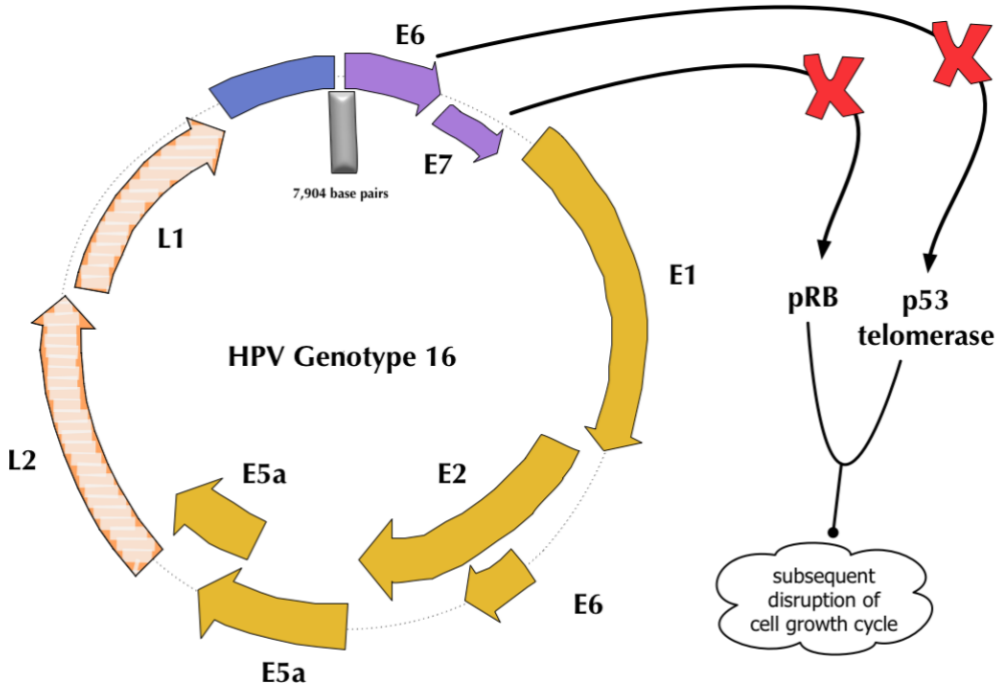


Figure 1-6: Schematic Diagram of HPV genome illustrating 6 'early'genes (E1-E6) and 2 'late' genes (L1-L2). The early genes are overwhelming involved in viral transcription, transcriptional regulation and the maintenance of the genome itself. E6 and E7, as demonstrated bind and inactivate p53 and Rb in their role as oncogenes.

Following acute infection, a long latency period is often observed prior to full transformation, suggesting that other events may be important in this process (88). In many benign lesions, the HPV genome manifests itself as an episome (an extra and non-essential portion of genetic material which can often exist independently), contrary to HR types where DNA is often, although not always, integrated (89, 90). With reference to cervical cancer models, 100% of HPV-18, 80% of HPV-16 and 81% of HPV-31 demonstrate integration (89, 90). The remaining minority can progress to invasive cancer forms in an episomal form, reinforcing the notion that while cervical cancers commonly follow viral integration, this is not absolute.

Subsequent to this, viral gene replication leads to the production of six early non-structural regulatory proteins (E1, E2, E4, E5, E6, E7) and two late structural proteins; L1 (major) and L2 (minor). These proteins are named to reflect their order of expression in the host, hence early and late. E6 and E7 are notable as they bind p53 and pRb respectively (see **Error! Reference source**

not found.). Both p53 and pRb are tumour suppressor genes (TSG) and when bound are unable to function thus tumourigenesis is more likely.

The site of viral integration is typically the E2 gene region. The E2 region codes for the E6 and E7 regulatory proteins and if deregulated at the time of integration, E6 and E7 oncoproteins are often over-expressed as a result (88). As is often quoted, genomic integration may result in a series of subsequent events that ultimately result in cellular immortalisation (88). Importantly and interestingly, integration is not a requisite part of the viral life cycle and in fact, once integrated, the virus is unable to formulate any viable progeny (90).

Although the main areas of concern relating to HPV in the head and neck are justifiably related to its HR strains (e.g. HPV-16), its LR strains are also of note and in the larynx (HPV-6 and -11) are associated with recurrent laryngeal papillomatosis, a relatively rare (1-4 cases per 100,000 depending on juvenile or adult population)(91) but debilitating disorder. HPV types 6 and 11 are known to reside in cells of the anogenital region and the infants suffering this condition are typically born to mothers infected with these types (88). Patients suffering this condition remain susceptible to airway obstruction and often undergo multiple surgical interventions. The adult presentation has an insidious clinical onset (92) and broncho-pulmonary extension has an associated mortality of up to 75% (93).

Specifically, HPV has been associated with the rise of squamous cell carcinoma of the oropharynx (1)and this is discussed in Chapter 1.2.2.

1.4 Molecular based aetiologies in Head and Neck Cancer

1.4.1 p53

The p53 protein, is known as the guardian of the genome (94). It is a transcription factor (encoded by the *TP53* tumour suppressor gene in the short arm of chromosome 17p13.1) and is activated during periods of cellular stress. Its usual function is as a monitor, screening for damage to DNA. If present, the cell cycle is interrupted so to allow DNA repair, glucose metabolism or apoptosis in non-recoverable events (95).

During periods of homeostasis and indeed any point at which the cell is not under stress, the activity of p53 protein is negatively buffered via the E3 ubiquitin-proteasome pathway, by its regulator MDM2. At periods of stress however the MDM2-p53 interaction is interrupted by stress-induced acetylation and on activation, p53 facilitates subsequent cell cycle arrest or apoptosis.

The significance of p53 in oncogenesis is integral in most cancers (95) including over 60% of HNSCC (76, 96). Mutations of the p53 gene are linked to its over-expression in tissue and associated poor outcome. Its absence in pre-cursor lesions, demonstrated in cases of colorectal polyposis or intraductal breast carcinoma, suggests that its resulting effect on cellular dysfunction may only be a late stage phenomenon, as opposed to the initial neoplastic step (96).

Finally, the therapeutic potential of p53 as an adjunct to traditional treatment may hold future prospect. In mouse models, the combination of p53 gene therapy with platinum-based chemotherapies was beneficial, compared to platinum based treatment alone (96). A retrospective study, where 62 hypopharyngeal cancer patients were treated with induction chemotherapy (cisplatin + 5-fluorouracil), found to the contrary however (97). Positive staining was demonstrated in 73% of cases. Overall response rate was 66% (complete = 19%, partial = 47%). An association for p53 negativity and better chemotherapy outcome was demonstrated, however this was short of significance (p=0.07) (97).

1.4.2 Retinoblastoma (Rb) and $p16^{INK4A}$

Transit of the cell cycle, from G1 phase (interphase, succeeding mitosis) into the S phase (DNA replication) is policed by the retinoblastoma protein (76). This is facilitated via the use of two accompanying proteins, RBL1 and RBL2, which together, bind and inactivate E2F, a transcription factor essential to S-Phase DNA replication (98).

A mitotic stimulus within the cell allows the phosphorylation of Rb via cyclin D1 or CDK4 or CDK6. Rb subsequently dissociates from E2F, permitting cellular progression into S Phase. Contrarily $p16^{INK4A}$, the protein product of the *CDKN2A* gene, works to activate CDK4 and CDK6 proteins that prevent the inhibitory phosphorylation of Rb, with a resulting cellular senescence (99). It is this inactivation of the Rb pathway by *CDKN2A*, via cell cycle regulators $p16^{INK4A}$ and p14/Arf/INK4B that is frequently seen in HNSCC. This genetic insult can be seen in somatic mutations (5-15%), homozygous deletions (30-60%) and epigenetic silencing (10-20%) (76, 98-100).

Immunohistochemical analysis suggests that a p16 absence is significant of underlying Rb phosphorylation in around 80% of HNSCC cases (76).

1.4.3 Epidermal Growth Factor Receptor (EGFR)

EGFR is a member of the membrane-bound receptor tyrosine kinase family, which is associated with the natural ligands of EGF and transforming growth factor (TGF-α) (70). Its genetic coding locus is found on chromosome 7p12 (101). On binding to its ligands, a dimer is formed which

subsequently permits a long series of intracellular signalling events, including activation of mitogen protein kinases, mammalian target of rapamycin, signal transducer and activator of transcription (STAT), phosphoinositide 3-kinase (PI3K) and protein kinase C pathways (70).

This signalling leads to cell migration, adhesion and proliferation. Over-expression of EGFR ligands is a phenomenon commonly observed in HNSCC and has been identified as a prognostic marker correlated to size of tumour, increased risk of recurrence (70, 101) and a reduction in survival after radiation therapy. In a study of 140 cases with laryngeal SCC, 5-year survival was 81% in EGFR non-expressing tumours, in comparison to 25% in those expressing EGFR ($p<0.0001$) (102).

Therapeutic strategies exist to counter the expression of EGFR in HNSCC. Competitive inhibition by monoclonal antibodies such as Cetuximab® can prevent the initiation of complex intracellular signalling pathways. The agents preferentially bind to the extracellular receptor, precluding binding of EGFR to its ligands and also subsequently downregulating EGFR expression downstream. Definitive clinical benefit however, has not been demonstrated (103).

In the phase III ECOG study of 117 patients with metastatic/recurrent HNSCC, cases were randomised to cisplatin with Cetuximab (Arm A) or Placebo (Arm B) every 4 weeks (103) in a first line setting. Hazard ratio of progression, between arm A and arm B was 0.78 (95% CI 0.54-1.1). OS ($p=0.21$) and PFS ($p=0.09$) were not shown to be different between the two arms. Median PFS was 2.7 months (95% CI 1.9-3.8) for Arm B and 4.2 months (95% CI 3.71-5.55) for Arm A (103), see Figure 1-7. Of note, tumours over-expressing EGFR in comparison to low-moderate staining those with low-moderate staining were found to show a reduced response to treatment; 9% vs. 27% ($p=0.05$) (103).

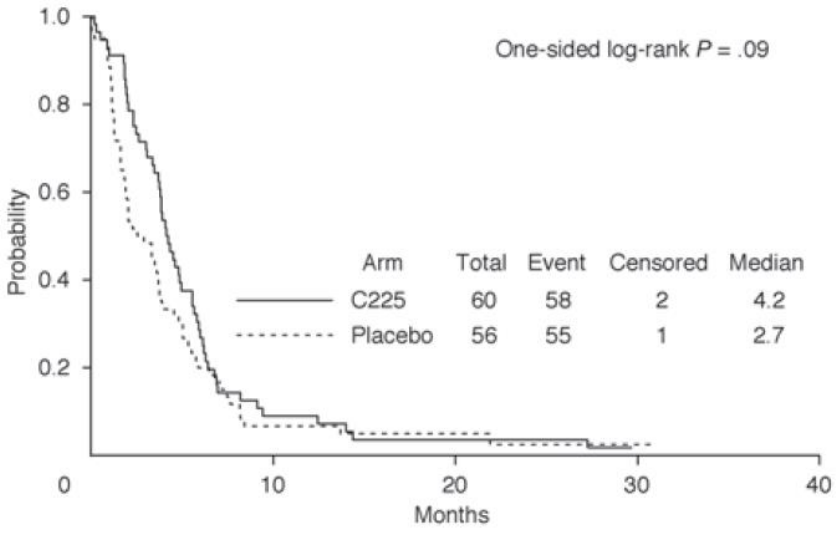


Figure 1-7. Kaplan Meier Chart demonstrating differences in Progression Free Survival (PFS) between Cisplatin + Placebo and Cisplatin + Cetuximab in a first line recurrent/metastatic setting of

HNSCC. There was no significant difference between either of the two groups. Taken from Burtness et al. 2006 (103).

Contrarily, the most recent update of the open-label randomised phase III trial of Radiotherapy +/- Cisplatin vs Cetuximab (De-ESCALATE) found that although overall and severe toxicities were not considerably different between the two treatment arms, there was a considerable difference between Cisplatin and Cetuximab at both 2-year OS (97.5% vs. 89.4% and HR 5.0 [95% CI 1.7-14.7, p=0.001]) and 2-year recurrence (6.0% vs. 16.1% and HR 3.4 [95% CI 1.6-7.2]) (104).

1.5 Treatment of Head and Neck Cancer

Treatment of head and neck cancer is well established in the form of surgery (ablative and reconstructive), radiotherapy and chemotherapy either alone or in combination. Certain immunotherapies (e.g. Nivolumab) have also been NICE approved for recurrent HNSCC lesions but fall out of the remit of this work. A short discussion on this topic can be found in Appendix A.

Choice of therapy is subject to the decisions of a local specialist head and neck cancer MDT, which largely follows national guidelines (105). TNM classification (see Appendix B) is used to determine tumour stage, which subsequently determines severity of disease (16). Recent changes in the AJCC v8.0 have significantly down-staged HPV driven oropharyngeal cancer (106). This is discussed further in Section 3.4.

In AJCC v7.0, Stage I and II disease without metastasis is seen in approximately 40% of patients (17) and is usually treated with a monotherapy, whilst the more advanced Stage III and IVa/b disease is usually treated with multi-modal therapy in combination. Metastatic disease is usually treated on the basis of patient performance status, with a good performance status treated with combination chemotherapy and poor performance status treated with single agent chemotherapy or best supportive care (17). Median survival for patients with recurrent or metastatic disease remains poor and is often less than 1 year (4).

1.5.1 Surgery

1.5.1.1 Surgery to primary site

Early stage disease (Stage I and II) can be treated successfully with both surgery and chemoradiotherapy and outcomes are largely comparable (27). Advantages of surgical management include immediate treatment and shorter recovery time (in general), an ability to accurately stage the patient and confirm disease clearance on a histo-pathological basis (27).

Although traditionally, late stage disease (Stage III and IV) was difficult to treat with surgical means, the relatively modern approaches of pedicled myocutaneous and free micro-vascular flaps, endovascular and robotic surgeries ensure that even the most poorly accessible and large lesions can be resected and reconstructed successfully (8). Due to the poor prognosis of these tumours however, treatment is inevitably multi-modal and unless indicated for palliative lesions, most late stage lesions are accompanied by chemo-radiotherapy. Despite the advances in the aforementioned approaches, mortality of HNSCC overall is largely unchanged (10).

1.5.1.2 Surgery for regional disease

Surgery is the mainstay of treatment for cervical metastasis (8), most commonly being a dissection of the lymphatics of the neck. Optimal management of the neck is important. The presence of occult disease, is seen in up to 40% of patients (16, 27) and long term survival in the presence of known nodal metastasis can be reduced by up to 50% (8). This said, modern ablative surgeries of the neck have moved away from traditional radical approaches, instead opting for so called selective neck dissections; organ preserving and respective of post-operative function.

1.5.2 Radiotherapy

Radiotherapy is based on the linear acceleration of high-energy photons of electromagnetic radiation. On interaction with tissue, orbiting electrons are emitted as high-energy photoelectrons, which serve to create secondary ionising events within the tissue, a photoelectric effect. This energy is absorbed in part by the host tissue causing molecular damage within the cell. Normal DNA replication and cell division is inhibited as a result.

For lethal damage to occur, a double strand DNA break is required (107). Additionally a *Compton Effect* is witnessed, whereby a photon collides with a “free” electron and both photon and electron and subsequently scattered (107). The photon continues to undergo additional interactions, albeit at a lower energy and the electron begins to ionise with the energy transferred from its original collision.

The most common means of application is via dose fractionation. By dividing the radiation dose into independent fractions, the re-population of normal cells is enabled, whilst also allowing tumour cells to reassert themselves into a more radiosensitive cell cycle phase, with resulting greater therapeutic effect (107). Continuous treatment without interruption is required, to preserve this effect (1). Furthermore, fractionation enables oxygenation of tissues, critical for preserving the life of free radicals (e.g. hydroxyl radicals) and thereby increasing the likelihood of successful DNA damage (108).

Intensity Modulated Radiotherapy (IMRT) is now the gold standard of radio-therapeutic treatment in HNSCC (105), allowing minimal morbidity to adjacent tissue. Radiation toxicity is known to be less in IMRT patients, particularly when considering symptoms such as xerostomia. In the PARSPORT Trial, 94 patients with HNSCC (T1-T4, N0-N3) were randomly assigned either IMRT or conventional radiotherapy in a prospective 1:1 controlled trial (109). At 12 months, although 73 of 82 living patients reported xerostomia, it was significantly lower in the IMRT group (38%) than the conventional therapy group (74%), p=0.0027. This effect persisted at 24 months (83% vs. 29%, p<0.0001) (109).

Although IMRT has a significantly improved toxicity profile, it is still far from ideal. Positional alterations of both the tumour and patient during treatment mean irradiation of adjacent normal tissue is still possible. Adaptive radiotherapy, with its real time adjustments to patients' weight, tumour size and position may prove beneficial in this respect in the future (110). Other regimens such as accelerated (normal dose over a shorter time period) or hyper-fractionated (2-3 low dose fractions/day) therapies have been beneficial in demonstrating some locoregional control, but have not demonstrated reliable improvements in OS (111). The phase III RTOG 0129 trial randomised 721 patients to 3 groups; conventional fractionation, accelerated fractionation (72 Gy in 42 fractions) and accelerated fractionation with a cisplatin boost (112). No differences were for OS were demonstrated (112). No evidence of significant late toxicity was seen in the accelerated groups (112).

Conventional therapy dictates single daily fractions of 1.8-2 Gy in a 5-day week, totalling up to 70 Gy in a 6-7 week period (113).

Currently there is no Level 1 evidence suggesting the superiority of conservative surgery against radiotherapy in early stage HNSCC, or indeed vice versa (111, 113). Chemotherapy given in conjunction with radiotherapy has been shown to improve loco-regional control in comparison to radiotherapy alone, resulting in a 6.5% increase in OS (114).

1.5.3 Chemotherapy

Chemotherapy is used in the treatment of patients presenting with advanced stage or recurrent/metastatic disease. Its role was established following two seminal trials that demonstrated an equivocal effect on locoregional control in comparison to surgery, but with the benefit of organ preservation (20, 115).

The Veterans Administration (VA) larynx preservation study was a prospective randomised trial of advanced (stage III and IV) laryngeal cancer, comparing induction chemotherapy and radiation

therapy and laryngectomy with adjunctive radiotherapy. Survival was comparable in both groups (two year survival for both groups being 68%, p=0.9846) but with the added benefit of laryngeal preservation (seen in 64% of those randomly assigned to induction chemotherapy) (115). Since this seminal work, the outcome has been replicated in trials of different subsites in the head and neck (17, 111).

Chemotherapy can be given as a neo-adjuvant agent, where its actions are thought to both shrink tumour and reduce micro-metastases (15, 116). Alternatively, it is given concurrently in three divided doses at days 1, 22 and 43 of RT (116). Cisplatin 100mg/m^2 is the regimen of choice and can be accompanied by 5-FU (116). In elderly populations, cisplatin has an increased toxicity profile and is often replaced with carboplatin or the monoclonal antibody, cetuximab(17).

HNSCC that is recurrent or metastatic in nature is difficult to treat, reflected by a median survival of approximately 12 months. Recurrent loco-regional disease can be treated with salvage treatment options, if available, with a view to treating distant disease independently, often with adjuvant chemotherapies. Ultimately patient performance status and disease burden dictate the place of chemotherapy in the patient with distant metastasis and there is no chemotherapeutic package that has been shown to significantly extend survival in this scenario (116). A response rate of 30% can be expected and the most commonly used agent is Carboplatin; utilised for its relatively forgiving toxicity profile.

To date, HPV does not necessitate any alteration in chemotherapeutic regimen although de-escalation studies are underway to examine the role of reduced intensity therapies in low risk HPV positive patients (117).

1.5.4 Immunotherapy and Novel Therapies

Immunotherapy is an evolving therapeutic practice in HNSCC and is currently approved for practice in selected indications whilst also being studied in various phase II and III trials worldwide. Its background, practice and current role are discussed further in section 1.7 and Appendix A.

1.5.5 Treatment of Oropharyngeal Carcinoma

Our understanding of HPV driven disease has divided OPSCC into two distinct diseases, exhibiting notable biological and phenotypical differences. Given the significant side effects of aggressive curative treatment, a movement has begun towards targeting discrete treatments for people with high risk (HPV[-]) and low risk (HPV[+]) disease. Smoking (118), T-stage (22), nodal status (16, 22)

and poor tumour infiltration of immune cells (22) have all been demonstrated as reducing survival outcomes of HPV(+) OPSCC patients and are all considered as high risk.

1.5.5.1 Early stage disease (Stage I and II)

Patients with early Stage disease (T1/T2, N0) disease can be successfully treated with either surgery or radiotherapy and currently no evidence exists to suggest superiority of one method over another. Monotherapy is the rule, though adjunctive treatments can be used for patients demonstrating signs of aggressive disease (1).

The development of transoral methods (TOLS/TORS) has re-introduced surgery as an option in early stage disease. Where supported, TOLS and TORS are now offered as part of routine management plans. Ipsilateral neck dissection of anatomical Levels II-IV should be offered as routine, given the high incidence of occult regional metastasis of approximately 30% (16). The presence of nodal disease (N1-N2b TNM v7) necessitates a *modified* neck dissection encompassing potential resection of level IIb, the accessory nerve, sternocleidomastoid muscle and the internal jugular vein.

Radical radiotherapy can be used where organ preservation is key. A dose equivalent of 70 Gy in 35 fractions is used for curative treatment and is usually administered over six weeks (1). For post-operative treatment, doses are smaller and usually dependent on histological outcome (e.g. positive tumour margins and extracapsular spread). Most centres advocate a regime of 60 Gy in 30 fractions (16). To date, there is limited data supporting the role of post-operative radiotherapy in patients with HNSCC. What exists is mostly in the form of retrospective observational studies.

1.5.5.2 Late stage disease (Stage III and IV)

Advanced disease (Stage III and IV) is common and can be seen in up to two thirds of patients (1). Treatment can be in the form of primary surgery with adjuvant radiotherapy (with the possible addition of platinum based chemotherapy) or primary concurrent chemo-radiotherapy with or without neoadjuvant chemotherapy (1). Choice of treatment is often guided by organ function, likely functional result and patient preference. For now, organ preservation is championed where possible and advanced HNSCC is commonly treated with primary chemo-radiotherapy, resorting to salvage surgery where required (16, 49).

Palliative cases are routinely treated with palliative doses of chemo-radiotherapy alone (1).

1.5.6 Treatment of Hypopharyngeal Carcinoma

Treatment of hypopharyngeal cancer requires accurate staging of the disease and an honest appraisal of a patient's physiological state. Patients are known to present late (1, 105, 119) and as such, often present with significant dysphagia (120) and malnutrition. Assessment by a speech and language therapist and a dietician is required for optimisation prior to management. The patient's performance status is sought, in order to guide the most appropriate management options. Both surgery and radiotherapy mandate a minimal physiological reserve in order to ensure success (8, 27, 119).

Surgery or radiation can be used to treat early stage (I and II) with comparable outcomes (15, 27). In the UK, an emphasis is placed on organ-sparing techniques where possible (15). Given the natural history of its presentation, there is little high-quality evidence available to compare the modalities.

1.5.6.1 Surgery

Prior to offering definitive surgery, pre-operative surgical planning is paramount to ensure both tumour resectability and adequate ablative margins. Assessment whilst under anaesthetic allows for optimal inspection of the post-cricoid region which can be difficult to examine in the awake patient (120). This is complimented by 3-dimensional radiography in the form of high-resolution cross-sectional CT, MRI and PET-CT (see Figure 1-8).

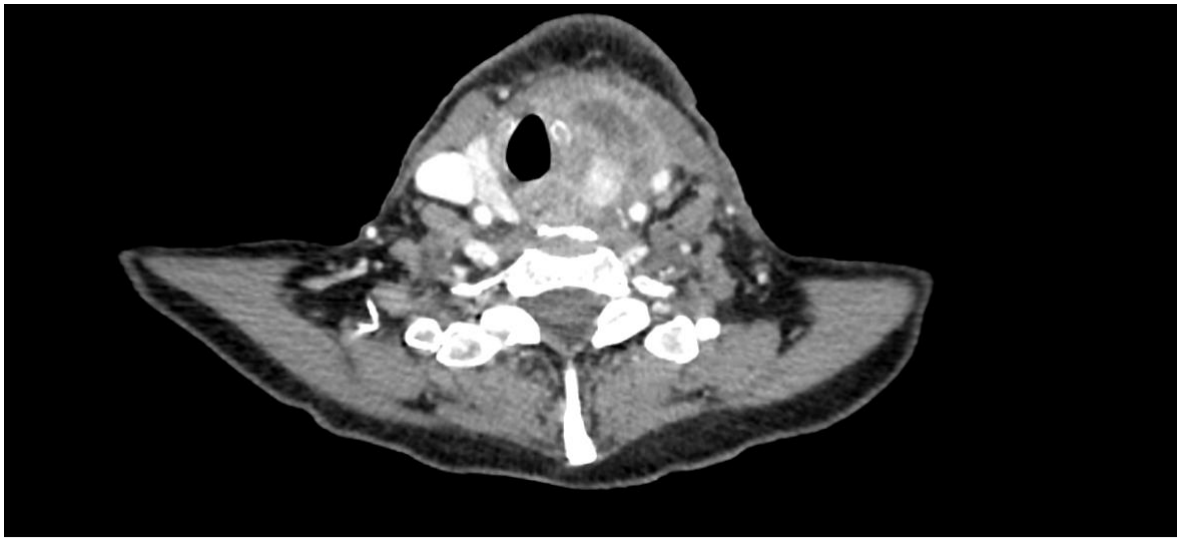


Figure 1-8. Axial CT scan demonstrating large hypopharyngeal cancer in left pyriform fossa. Image source: Poole Hospital Foundation Trust.

Surgical management is guided by the lesion's location, its extension and potential invasion into nearby structures and need for reconstruction. Very little data exists to guide surgical approaches to the hypopharynx (32).

Disa et al. devised a system for assessment of the hypopharyngeal deficit, which can broadly indicate the method of reconstruction required (121). Five types are described as follows;

- Type 0: Minimal defects of the pharyngo-oesophageal segment that *can* be closed primarily.
- Type I: Defects affecting <50% of the pharyngo-oesophageal segment that *cannot* be closed primarily.
- Type II: Defects affecting >50% of the pharyngo-oesophageal segment.
- Type III: Longitudinal lesions that extend across adjacent anatomical subsites (oropharynx, floor of mouth or jaw).
- Type IV: Defect extending into cervical oesophagus.

In order to minimise the risk of a fistula or stricture following reconstruction, an updated classification scheme was proposed by Urken et al, specifying a requirement for a strip of native pharyngeal mucosa at the site of resection (121, 122). Superscript letters of *i* and *s* are used additionally to indicate a patient with likely poor or problematic wound healing and the need for reconstruction requiring a tissue flap respectively (120, 122).

- Type 0: Minimal defects of the pharyngo-oesophageal segment that *can* be closed primarily.
- Type I: A non-circumferential defect that on resection will leave behind a strip of native pharyngeal mucosa, 2cm in width.
- Type II: A circumferential defect that is limited superiorly by the vallecula.
- Type III: Any defect (non-circumferential/circumferential) is limited by vallecula superiorly.
- Type IV: Any defect that extends beyond the vallecula superiorly

Neck dissection and irradiation are inevitably part of routine treatment, with or without clinically apparently locoregional nodal disease, given the considerable incidence of occult metastasis (123). Lesions crossing the midline, involving the posterior pharyngeal wall or post-cricoid region require bilateral neck dissections and irradiation due to a high incidence of contralateral neck failure (27).

Transoral approaches can be used, but these are limited to lesions that are accessible via an endoscope (119). Hypopharyngeal cancer is known to present with sub-mucosal disease in 60% of cases, with extension up to 20mm from the lesion (27). Resection margins must be accounted for via this approach, with 15mm superiorly, 30mm inferiorly and 20mm laterally (27).

In a systematic review by Takes et al. the oncological results of TOLS operations appeared comparable with open approaches, with a 5-year OS rate of 50 to 70% in Stage I-II disease and 40-50% in Stage III-IV (123). Today, TORS offers an alternative means of access and but is largely limited to partial pharyngectomy (17, 124). Lorincz et al. reported on 5 cases of T1-T2 hypopharyngeal cancer treated with TORS (125). At time of publication, 1 patient had died from another cause, with the remaining 4 alive and free of tumour (125). 3 of these 4 did not require adjunctive treatment (125). Elsewhere, Park et al. followed up 23 cases over 3 years with hypopharyngeal cancer (pyriform sinus [n=18], posterior pharynx [n=4] and post-cricoid [n=1]} treated with TORS (126). Positive margins were seen in 2 (8.7%) patients (126). OS at 3 years was 89% and DFS was 84%. Swallowing outcomes were favourable with a functional outcome swallowing scale of 0-2 in 96% (126).

For T3-T4 cases that involve more than two-thirds of the circumference, the traditional ablative procedure is total pharyngo-laryngectomy, an operation removing the entire larynx, the pharynx and varying amounts of the oesophagus (123). An interpositional conduit, in the form of a free microvascular tissue flap is usually used for reconstruction and can include a jejunal flap or a tubed antero-lateral thigh flap. Some centres will advocate a gastric “pull up”, the literal caudal mobilisation of the gastro-intestinal system. As in all complex, multi-disciplinary surgery, the number of surgical cases performed reflect patient outcome (127). An ipsilateral hemi-thyroidectomy, and neck dissection of levels IIa-IV are also performed (27).

1.5.6.2 Chemoradiotherapy (CRT)

Although historically, this was a disease treated with surgery, several large retrospective studies demonstrated that primary radiotherapy with salvage surgery delivered a comparable survival outcome, with the benefit of being laryngeal sparing, in those patients who do not require salvage surgery.

However, patients with intractable dysphagia, requiring supplemental or total enteral feeding, recurrent aspiration events and subsequent lower respiratory tract infections and those with tumour volume causing airway obstruction, may require induction chemotherapy before chemoradiotherapy. Treatment differs widely but usually involves the use of Cisplatin (30mg/m^2) to allow for radio-sensitisation followed by 3-5 cycles of Cisplatin (20mg/m^2) +/- 5-fluorouracil (1000mg/m^2) (27). However, in keeping with NICE guidelines, patients who have significant laryngeal involvement at time of diagnosis, resulting in aspiration and/or a fixed hemi-larynx are likely to benefit from primary surgery as there is a significant chance that they will be left with a non-functional larynx after CRT.

In cases where both CRT and primary surgery are contra-indicated, EGFR blockade (i.e. Cetuximab) can be used (27).

1.6 The Immune System

A tumour is now known to be a symbiosis of various elements, not merely a collection of autonomous cancer cells. Tumour growth is not simply related to the activity of the tumour cells themselves, but also the tumour *stroma*; a collection of cells recruited by the tumour from the adjacent environment. Tumour stroma can act in a regulatory manner, by ensuring stable tissue structures facilitated by tight junction proteins and cell adhesion molecules. Local fibroblasts can be used to modulate progression of transformed cells (128).

The microenvironment is also powerful enough to convert cells with tumorigenic potential to a less aggressive phenotype (128). On occasion however, the tumour evades these inhibitions and instead manipulates local cells and signals for its own benefit, allowing tumour progression and invasion (128, 129). In keeping with this, chronic infection and inflammation has long been associated with tumourigenesis and in one study, chronic infection was related to 18% of all cancers worldwide (130). In age matched populations, patients on immunosuppressive treatment following transplantation, harbour a higher rate of *de novo* neoplasia, with up to a 10-fold increase (131, 132).

1.6.1 Innate Immunity

Overall, the immune system is divided into two communicating systems; innate and adaptive. The innate system delivers a non-specific, rapid-response to a pathogen within the first minutes to hours of an encounter (133, 134). In evolutionary terms it pre-dates any adaptive immune response; developing long before the separation of vertebrates from invertebrates, with similar responses seen in plants.

Innate immunity encompasses a group of proteins and phagocytic cells that recognise discrete motifs on pathogens (133). Cells included within this response are phagocytic leukocytes (macrophages and neutrophils), dendritic cells (DC) and natural killer cells (NK) cells. Recognition of non-self in phagocytes occurs through presentation of *pathogen-associated immunostimulants*, surface molecules unique to pathogens and not found on the host (135). These are recognised and responded to via toll-like receptors on cells of the innate system (134).

Further actions include harnessing and delivery of products such oxygen metabolites, chemokines and cytokines, mediators that recruit, mobilise and direct additional innate and adaptive

modulators. Other non-immunological sponsors of the inflammatory process, such as fibroblasts and endothelial cells, are also subsequently activated (129).

The complement system, another integral part of innate immunity, is a circulating set of protein molecules. It is typically activated via the classical pathway, by binding factor C1 to an antigen-antibody complex (IgM or IgG), or via the alternative pathway via exposure of C3/C3b to bacterial wall lipopolysaccharides. This said, other pathways e.g. *The Lectin Pathway* are also known to exist but are often similar to the two traditional mechanisms (136). The role of complement in tumourigenesis, although curious, should also be noted. Mice deficient in C5 or C5a have demonstrated decreased tumour growth when compared to wild type counterparts (137). It is postulated that while complement derived, membrane attack complexes are detrimental to cells in 'lytic" doses, in 'sub-lytic' doses, they are proliferative and inhibit apoptosis (136, 137).

Further to basic surveillance and phagocytic activities, the innate system produces soluble products such oxygen metabolites, chemokines (138) and cytokines (e.g. TNF-α, IL-1,IL-6)(139); mediators that recruit, mobilise and direct further innate and adaptive modulators (139, 140) (see Table 1). Other non-immunological sponsors of the inflammatory process, such as fibroblasts and endothelial cells, are also subsequently activated.

Major contributory cytokines in Innate Response	
Interleukin 1	Macrophage and T cell activation. Raised local temperature.
Interleukin 6	B and T cell activation
Interleukin 12	NK cell activation.
Interleukin 16	Chemotaxis
Tumour Necrosis Factor α	Increased vascular permeability, local inflammation & raised local temperature
Interferon a & g	Inhibition of viral replication.

Table 1: Actions of major contributors of the Immune System

1.6.2 Adaptive Immunity

The adaptive immune system responds to discrete antigenic targets, activating and expanding specific clones of B and T cells. It can take up to a week before the responses are effective,

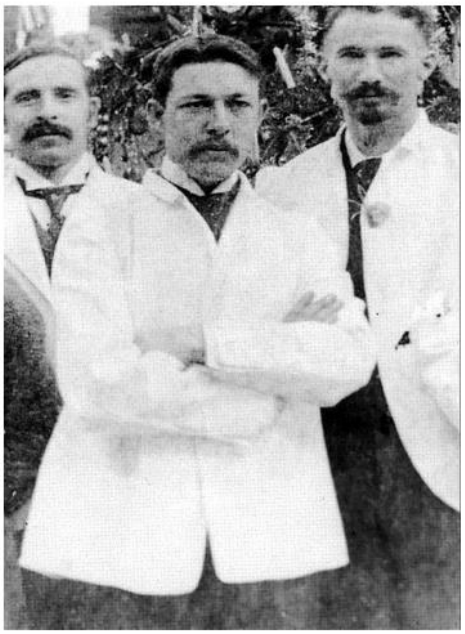
depending on whether a primary or secondary response is prompted, this contingent on whether the pathogen has been encountered before.

The humoral response is manifested by myeloid B cells, which subsequently differentiate into plasma cells, producing specific antibodies. Cellular immunity is conferred via T cells, which can be broadly divided into cytotoxic- ($CD8^+$) or helper-T cells ($CD4^+$). The antigen is processed by, and presented on, antigen presenting cells, (APC). Endogenous antigens are presented in combination with Major Histocompatibility Complex (MHC) Class I molecules for presentation to CD8+ T cells, and MHC class II molecules present exogenous antigens to CD4+ T cells (141, 142). The polygenic, polymorphic nature of MHC makes a formidable asset to the adaptive immune system, offering a large selection of potential binding sites for foreign antigens (142). Until they have been presented their specific antigen, most commonly by DCs, $CD8^+$ and $CD4^+$ are present only in a naïve state. DCs, almost comparable to macrophages in terms of their phagocytic capacity are vitally important in the specialised activation of naïve T cells.

1.7 Immunology in HNSCC

With the evolution of immunotherapies in contemporary medicine, there has been a notable move in oncology to identify the role of the immune system in cancer. Immunotherapies however are not entirely new and as early as 1898, a surgeon named William Coley published his seminal work on the treatment of sarcoma patients with a vaccine composed of dead bacteria, *Streptococcus Pyogenes* and *Bacillus Prodigosus* (See Figure 1-9) (143). In his lifetime, Coley treated many hundreds of patients; some who seemingly achieved complete cure, having been previously identified as palliative cases only (143). Largely overshadowed by the dawn of radiotherapy, his son propagated Coley's vaccines following his death, but the idea of immunotherapy had not won favour.

Once an abandoned idea, the notion of immuno-surveillance has once again come to the fore (136). It is now understood that an increased number of tumour infiltrating lymphocytes (TILs) relates to an improved prognosis in both HPV(+) and HPV(-) head and neck tumours (22, 144). Transcriptomic data of immune infiltrate suggests a similar T cell profile, but a distinct B cell population when comparing HPV driven HNSCC with HPV independent tumours (144). More widely, the numbers, ratios and location of T cells are also of importance in other solid tumours (145).



New York Times - July 29, 1908

ERYSIPELAS GERMS AS CURE FOR CANCER

Dr. Coley's Remedy of Mixed Toxins Makes One Disease Cast Out the Other.

MANY CASES CURED HERE

Physician Has Used the Cure for 15 Years and Treated 430 Cases— Probably 150 Sure Cures.

Following news from St. Louis that two men have been cured of cancer in the City Hospital there by the use of a fluid discovered by Dr. William B. Coley of New York. It came out yester-

Figure 1-9. Historical newspaper article of Dr Coley's original trials using streptococcus to treat sarcoma (Picture from https://www.slideshare.net/manojsci/the-immunotherapy-of-cancer-past-present-the-next-frontier, Accessed 10/10/17)

Inherent checkpoints within the immune system are used to curb physiological immune responses and these are exploited by tumours to enable immune escape. Two of the best-described pathways include Programmed cell death protein-1 (PD-1) and CTLA-4.

PD-1, a surface T cell co-inhibitory receptor protein normally bound to the protein ligands PD-L1/B7-H1 and/or PD-L2/B7-H1 is used to downregulate the activity of T cells via apoptosis (see Figure 1-10) (146). PD-L1 (B7-H1) is widely present on both immune (e.g. T and B cells) and non-immune tissue (e.g. muscle and tumour cells) (147). Binding of PD-1 to any of its ligands (PD-L1 or PD-L2) results in inhibition of T cell activation and function. PD-L2 has been difficult to quantify in tissues (in immuno-histochemical terms) and is currently thought to play a less important role in immune modulation (147).

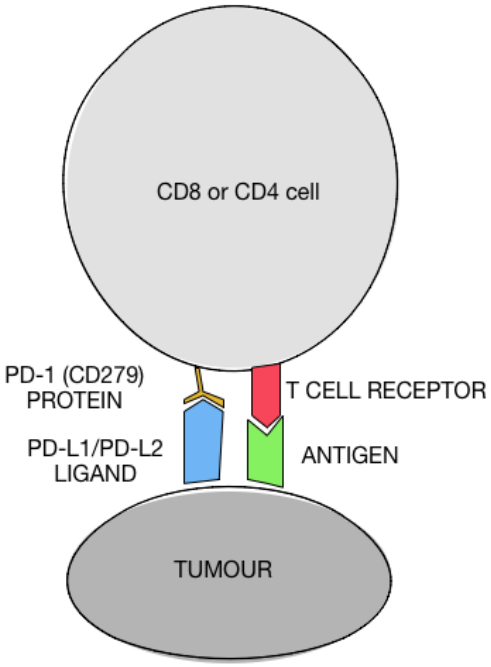


Figure 1-10. Schematic diagram showing the relationship of PD-1 protein to PD-L1 or L2 ligands, down-regulating the immune response. Original Diagram.

In solid tumours, when PD-L1 is up-regulated it confers tumour protection by increasing apoptosis of CD8 T cells and CD4 T-reg activity (146) and its presence has been negatively correlated to survival (148), particularly in patients with recurrent and metastatic disease (149-151). Importantly however, this is not absolute (notably in the case of melanoma and colon cancer, where it was shown to be protective) (148). The detection and presence of PD-L1 still lacks a consistency of outcomes in the head and neck (152, 153), but as outlined in the Checkmate 141 study, increased expression was linked with better survival (154).

Additionally, CTLA-4, a surface glycoprotein expressed on cytotoxic T cells can be used to physiologically downregulate the host immune response, and thus prevent auto-immunity. Following activation, CTLA-4, which has a greater affinity for the B7 (CD80/CD86) receptor (on APCs) than the co-stimulatory CD28, overrides the T cell activation, inhibiting further action of the T cell.

1.7.1 Window of Opportunity Studies

Traditionally, novel therapeutic agents (NTAs) have been trialled on patients with end-stage disease, often having exhausted conventional management options. This approach mitigates the risk of undetermined side effects and limits recruitment. Importantly, our understanding of how

these agents work is limited to patients with inherently poor immune systems (155). This raises the question as to whether alternate clinical trial designs, such as window trials, are better suited to evaluating NTAs.

In window studies, patients receive a NTA prior to beginning conventional therapy. This approach allows for their appraisal without risk of other confounding treatments. Studies can be designed so to incorporate tissue sampling both prior to and following NTA delivery, simultaneously analysing ongoing molecular changes in tumours and potentially identify predictors of biological response.

Most centres are wary of delays to instituting definitive therapy and as a consequence, all efforts are made to begin therapy before the 31 days required for definitive cancer treatment in the UK (156). Our group performed a short study looking at the effect on outcome based on time to treatment and this is discussed further in Appendix C. These data demonstrated no impact on patient survival when considering time to delivery of definitive treatment (see Appendix C).

Another important consideration with window studies is the potential delay to definitive treatment due to NTA toxicity. This toxicity can be hard to predict and has the potential to significantly delay the potentially curative treatment offered to a patient. Honest discussions with any patient pre-trial entry are required to define potential benefits or harms for any trial.

1.8 Social Deprivation in HNSCC

In general, health outcomes have improved over the course of the 20th century in England, particularly following the Second World War and institution of the NHS. Life expectancy in the UK has increased for both women and men and is currently 77 years for men and 81 years for women (157).

This trend however, has not been reflected between different socioeconomic groups and the divergence appears to be increasing. In the period between 1972-76 the difference in survival between professional and unskilled manual cohorts was reported as 5.5 years in men and 5.3 years for women (158). Most recently, in 1997-99, the difference had risen to 7.4 years and 5.7 years respectively (158). This relative survival can also be demonstrated regionally and indeed, in the period between 1999 and 2001, there was a 10 year difference in survival between the lowest socio-economic local authority (Glasgow, 69 years) and the highest (North Dorset, 79 years) (158).

One of the more difficult parameters to model, most modern definitions of deprivation rely on a composite of varying factors, each designated a comparable weighting and proven as indicators of poor socioeconomic status. In the UK, these are defined from the English Index of Multiple

Deprivation (IMD) 2015 (159). 7 domains are listed including; income, employment, education, health (comprised of indices including years of potential life lost, comparative illness and disability ratio, acute morbidity and mood and anxiety disorders), crime, barriers to housing and services and finally living environment. The IMD then ranks 32,844 individual areas (defined as Lower-layer Super Output Areas[LSOA]), which house an average of 1500 residents each (159). Overall this can build up a global picture of social deprivation and can allow quick and easy comparisons between LSOAs.

Socioeconomic status also plays an important role in the incidence (160, 161), presentation(162), management and prognosis of cancer (25, 163); from entire global regions to local communities and neighbourhoods (160, 161, 164, 165). In a recent publication from the National Cancer Intelligence Network (NCIN), incidence and mortality of all cancers combined were notably higher in the most deprived populations of society, compared to the least (166).

If cancer incidence rates were adjusted for deprivation group, 15,300 fewer cancers would have been diagnosed in the period between 2006-2010 (166). In a similar period (2007-2011) there was an excess of 19,200 deaths (166). In short, social deprivation is an important prognosticator for outcome in cancer and by extrapolation, of HNSCC too.

In their 2010 case control study, Conway et al. identified 103 patients with HNSCC (n=103) alongside age and sexed matched controls (n=91) from local general practitioners (167). Socio-economic measures were based on educational level, occupational social class and local area postcode measures of deprivation. People living in the most deprived areas (OR 4.66 [95% CI 1.79-12.18]) and the unemployed (OR 2.27 [95% CI 1.21-4.26]) were at a significantly higher risk of developing cancer than those with higher educational attainment (OR 0.17 [95% CI 0.05-0.58]) (167).

Bryere et al. demonstrated a similar outcome in a population of over 68,000 people (164). Using quintiles from the European Deprivation Index, quintile 5 (most deprived) had a relative risk of 2.05 (95% CI [1.77-2.05]) for cancers of the lips-mouth-pharynx, when comparing to quintile 1 (least deprived) (164).

Similar to this, Auluck et al. demonstrated an increased incidence of both oral and oropharyngeal cancers over time, with a highest rate of increase seen in patients of the lowest social quintiles (q5), see Figure 1-11 (160). For oropharyngeal cancer, annual percentage changes were highest in the lowest quintiles (q4 = 0.87 [p=0.006] and (q5 = 0.98 [p<0.001]) (160).

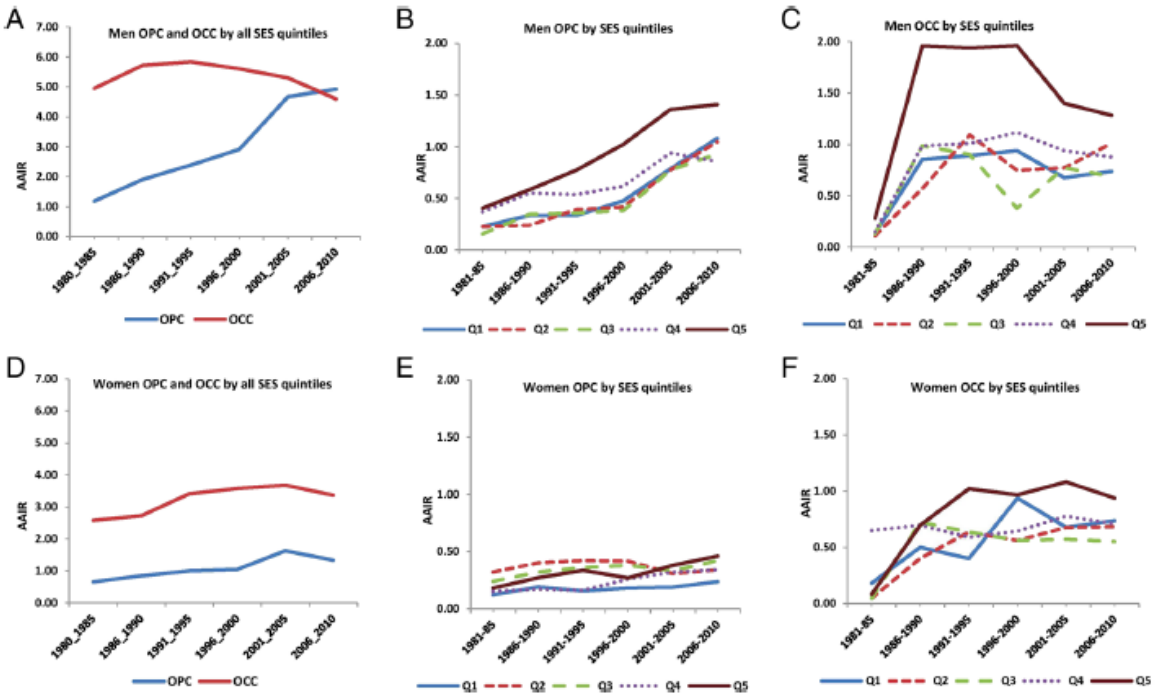


Figure 1-11. Temporal trends for age adjusted incidence rates 1981-2010 for oropharyngeal carcinoma (OPC) and oral cavity cancers (OCC) by socioeconomic status (SES) and sex. A – Change in incidence for OPC and OCC for men, B – temporal trends for changes in OPC based on SES quintiles for men, C – temporal trends for changes in OCC based on SES quintiles for men, D – Change in incidence for OPC and OCC for women, E – temporal trends for changes in OPC based on SES quintiles for women, F – temporal trends for changes in OCC based on SES quintiles for women. Taken from (160)

HPV driven penile, vaginal, female oral cavity and oropharyngeal cancers appear to demonstrate a significant association between poor education and increasing incidence (161). For male oropharyngeal and oral cavity cancers, there is a significant association with increasing incidence and poverty (161). In their study, McDonald et al. noticed a reducing gap of survival between the most and least deprived quintiles in census data from 1991 and 2001 (25). In 2001 this was only 72% of the equivalent ratio in 1991 (p=0.05) (25), a phenomenon that could potentially be explained by the emergence of HPV(+) disease and its purported predisposition for higher socio-economic groups.

As clinicians, our role within secondary care is more often with the management of disease and less so in its prevention. As surgeons, it is important to anticipate the effect of confounding variables upon the outcomes of common operations. Head and Neck cancer procedures are often invasive and debilitating and demand a considerable physiological reserve, a requirement not always delivered in the patient population. Our study, discussed in Chapter 4: discusses the impact of socio-economic status on outcomes of patients undergoing major Head and Neck procedures in the UK.

1.9 Summary of Introduction

Head and neck cancer is a highly dynamic disease process that is influenced and effected by a host of different factors . Patient outcomes in oncological medicine and surgery are myriad and are not uncommonly, related to a variety of factors which are related intimately to the patient.

This thesis aims to explore how these occur at differing levels, beginning with the immune system and its effect on a particularly aggressive subtype of cancer, hypopharyngeal cancer. The author hope to investigate the relationship between immune infiltrate and survival outcomes, similar to the method in which its importance has been confirmed in OPSCC. Additionally, the role of the tumour stroma and its effect in nourishing and supporting the tumour will also be reviewed.

By looking at patient pathology at the time of diagnosis, we want to confirm the importance in staging a patient appropriately and demonstrate, as has been noticed, that the contemporaneous staging system (version 7) was established at a time preceding the knowledge of HPV (+) disease as a separate clinical entity. We hope to confirm the utility of the newer staging system (version 8, introduced in 2018) and highlight areas of importance for stratifying patients more appropriately in the future.

Finally the author hopes to address the role of socio-economic circumstances at the time of treatment and how these continue to exert an effect on patient outcomes, both immediately and in the longer term. It has long been clear that deprivation plays an un-deniable role in health inequalities, from incidence of tobacco consumption to access to public health resources. This has a subsequent and important effect on patient morbidity and its subsequent outcomes. Whilst this has been made clear on a general basis, this investigation hopes to identify the specific effect on patients diagnosed with HNSCC, undergoing major surgery.

1.10 Aims and Hypotheses of this Thesis

Patient characteristics are an important factor of consideration in managing the patient with head and neck cancer. These include parameters such as immune infiltrate, HPV status and socio-economic factors. The aims and hypotheses of this thesis are to explore these, as follows and are discussed in the succeeding chapters.

1.10.1 Chapter 2

Hypopharyngeal cancer is known to be an aggressive cancer with poor treatment and survival outcomes. We predict hypopharyngeal cancer, unlike oropharyngeal cancer, is a malignancy that is not affected in the same way by tumour infiltrating lymphocytes. Therefore, it is not appropriate for targeting by the way of novel immunotherapies in the future. We intend to define the role of other important biomarkers in hypopharyngeal cancer and subsequently identify whether these may be associated with survival.

1.10.2 Chapter 3

We predict that the AJCC/UICC TNM version 7 staging system is an inappropriate staging system for the staging and treatment of patients with HPV (+) head and neck cancer. We intend to review the nodal stage of the TNM staging system in HPV(+) surgical patients undergoing neck dissection in order to demonstrate that the TNM version 7 staging system does not appropriately capture the true natural history of HPV(+) disease.

1.10.3 Chapter 4

We predict that social deprivation may negatively influence outcomes on patients undergoing head and neck surgical operations. To look at the importance of socio-economic status in head and neck cancer and define the hypothetical effect upon patient outcomes following major surgery.

Chapter 2: The role of the immune system on outcomes of hypopharyngeal cancer

2.1 Introduction

Immune infiltrate within solid tumours is linked with improved survival in the head and neck. This has been demonstrated in oropharyngeal SCC (22, 24), where high immune infiltrate is synonymous with improved response to patient treatment and OS. These so called immune-rich tumours are often associated with virally driven disease; human papillomavirus and current clinical trials are collecting data to ascertain if patients with HPV-driven tumours can be successfully treated with transoral surgical options, and tailored adjunctive treatment (117) in reduced treatment intensity format. Interestingly, at this time, immune infiltrate is not considered as a pathological stratifier within these trials (PATHOS and BEST-OF) (168).

Late stage presentation of HNSCC is not unusual, and up to two thirds of patients can present in this way (1). This is particularly the case for cancers of the hypopharynx (33). Hypopharyngeal cancers commonly present insidiously, obfuscated due to their location of origin and include tumours on the posterior pharyngeal wall, the pyriform fossae and the post-cricoid region.

According to data, survival outcomes in hypopharyngeal cancer appear largely unchanged over a period of 30 years (see Figure 2-1) (33). A subtle improvement has been made and this is most likely due to refinements of diagnosis and treatment. What is clearly a highly aggressive disease process, requires further work addressing why it is associated with such a poor outcome and if by understanding the reason(s) behind this, helping to tailor treatments accordingly.

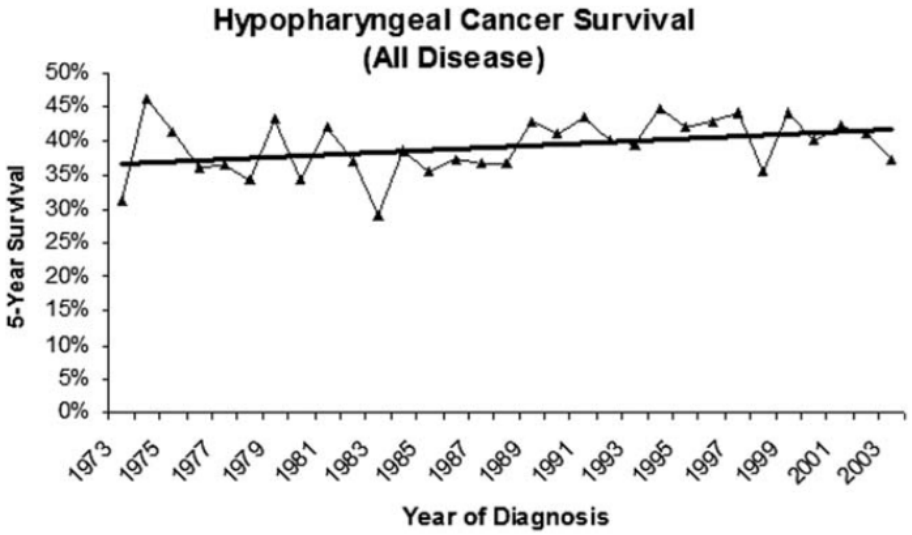


Figure 2-1. 5-year survival chart demonstrating how little change there has been in outcome for hypopharyngeal SCC (p <0.0001). Taken from Newman 2015 (33)

Following our observations that tumour infiltrating lymphocytes (TILs) in oropharyngeal tumours were linked with improved survival (see Figure 2-2) (143), we hypothesised a similar but reversed effect in hypopharyngeal carcinoma also, in that patients with hypopharyngeal cancer were more likely to have tumours that were either immune-cold or were not likely to demonstrate any divergent outcome based on immune infiltrate.

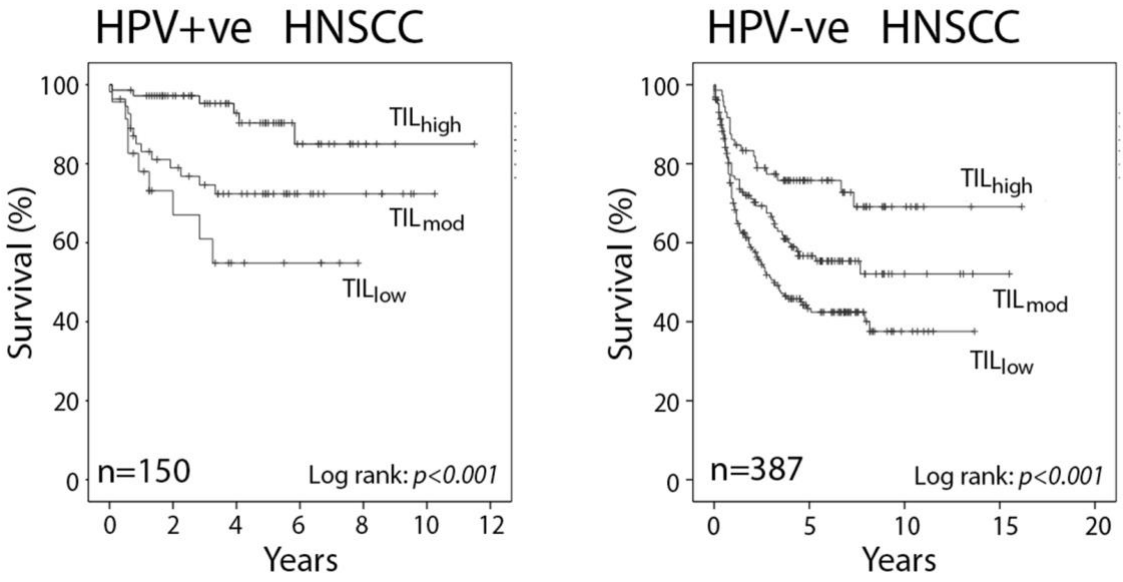


Figure 2-2. Survival chart for Oropharyngeal Carcinoma patients, divided by level of immune infiltrate demonstrating clear survival advantage to those tumours with high immune infiltrate than low immune infiltrate. Taken from Wood et al. (143)

2.1.1 Immunology in Hypopharyngeal Cancer (Literature Review)

Evading the host immune system promotes tumour development and 60% of HNSCC harbour an ability to evade the immune system, suppressing it both directly and indirectly and mediating the actions of suppressor cell populations (169).

Loss of HLA class 1 molecules leads to reduced surveillance by cytotoxic T cells (141). Down-regulation of co-stimulatory B7 molecules dampen the immune response and HLA-G expression inhibits natural killer cells, antigen presenting cells and T cells (170). Directly, the tumour may express cytotoxic ligands (e.g. CTLA-4, PD-L1, PD-L2, Fas/FasL) and releases immunosuppressive factors into the tumour milieu, e.g. IL-10, IL-6, prostaglandin E2, TGF-β and vascular endothelial growth factor (VEGF) (170). Functional maturation of antigen presenting cells e.g. dendritic cells can be inhibited (171) and finally, the recruitment of regulatory T cell populations promotes immune tolerance and unfettered growth (170).

The presence of tumour-infiltrating immune cells in the tumour milieu, have been well established as a marker of oncological outcomes (22, 145). $CD8^+$ cells in particular have been demonstrated as a relatively reliable means of indicating a good outcome, with high $CD8^+$ infiltrate associated with improved survival (22). Contrarily, high populations of intra-tumoural regulatory cells ($CD4^+$ $CD25^+$ T Reg) have been shown to exhibit a negative prognostic effect.

The effects of the immune system in hypopharyngeal cancer have yet to be explored (172). HPV positivity, the hallmark of the contemporary immune tumour does not seem to feature in the diagnosis of hypopharyngeal disease (173, 174) though this has been contested (172).

A retrospective assessment of 75 patients with Stage IV hypopharyngeal cancer was performed to assess the clinical utility of p16, p21 and p53 (172). The patient sample was relatively homogeneous, all undergoing treatment with primary surgery and subsequent radiotherapy only. 7 (9%) of cases were positive for p16. This was a dichotomous expression, i.e. highly expressed, or no expression (see Figure 2-3).

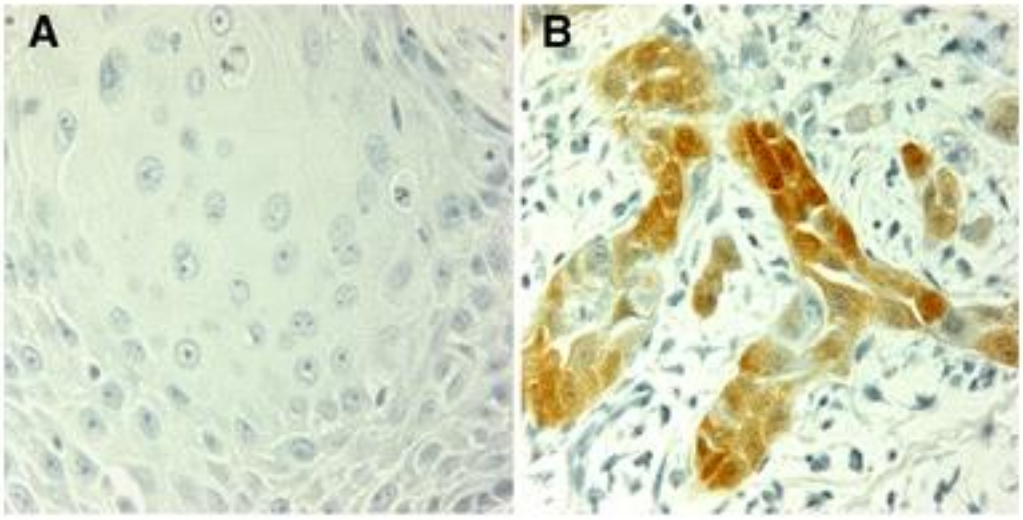


Figure 2-3. An example of immunohistochemical p16 expression. A - low/no expression, B - high expression. Taken from (172)

p16(+) cases had a greater 5-year DFS (100% vs. 58%) than p16(-) cases, but this was not statistically significant (p value not specified) (172). p16 expression is traditionally linked with cell senescence and it may well be upregulated in this dataset, for that reason, rather than representing HPV-driven tumours.

2.1.1.1 The Immunogenicity of HPV(-) and HPV(+) cancers of the Head and Neck

HPV(-) and HPV(+ cancers are diseases with distinct genetic and molecular profiles. To date, HPV(+) disease is recognised as harbouring a greater immune infiltrate than HPV(-) cancer. In keeping with higher immune activation, expression of perforin, granzyme A and granzyme B was greater in HPV(+) disease(175). Cumulatively, the net effect of this is a greater cytolytic score, indicating higher level of $CD8^+$ cell activation.

Additionally, whilst PD-1 and PD-L1 expression was comparable within both disease populations, a significantly high expression of the immuno-inhibitory receptor CTLA-4 was demonstrated in HPV(+) cancers. Finally a higher proportion of infiltrating regulatory T cells and regulatory T cell: $CD8^+$ ratios were seen in HPV(+) disease. Altogether, this supports the notion that therapies specifically targeting the immune environment in HPV(+) may be instrumental in targeting and addressing tumour immune checkpoint blockade(175).

63 (84%) of cases were contrarily found to be positive for p21 but this did not have any significant effect on patient outcomes with a 5-year DFS (p21[+] 60% vs. p21[-] 70%) (172). 27 of 73 cases (37%) were seen to over-stain for p53 in their nuclei. Intriguingly, p53(+) patients were shown to

demonstrate a significantly reduced 5-year DFS in comparison to their p53(-) counterparts, (48% vs. 73%, Log-rank p=0.008), see Figure 2-4 (172).

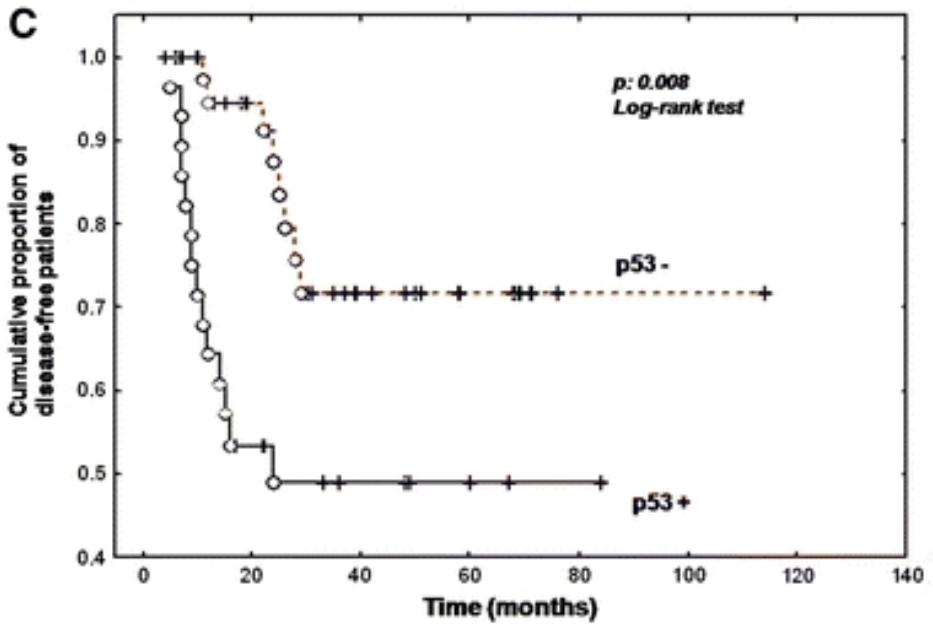


Figure 2-4. Kaplan Meier chart demonstrating improved Disease Free Survival in p53(-) patients with HNSCC. Taken from (172)

A cohort study by Dalianis et al. also addressed the role of HPV16 DNA and p16 expression in the tissue of patients with hypopharyngeal carcinoma (176). In this study 3/82 patient were HPV-positive which was associated with improved OS (Log-rank p=0.0185) and DSS (Log-rank p=0.0581), (see Figure 2-5) (176). It may be that these patients' tumours originated in the oropharynx and progressed distally into the hypopharynx.

Intranuclear p53 staining and its effect on survival was assessed by Chien et al (94). High expression of p53 (> 50% of tumour cells) correlated significantly with advanced stage of tumour (Stage IV vs. Stage II-III, p=0.002) and was associated with a reduced 5-year OS (High 51.6% vs. Low 80%, p=0.028) (94). Cox's regression analysis did not show a significant impact on survival (HR 1.971 95%CI [0.656-5.925], p=0.227) (94).

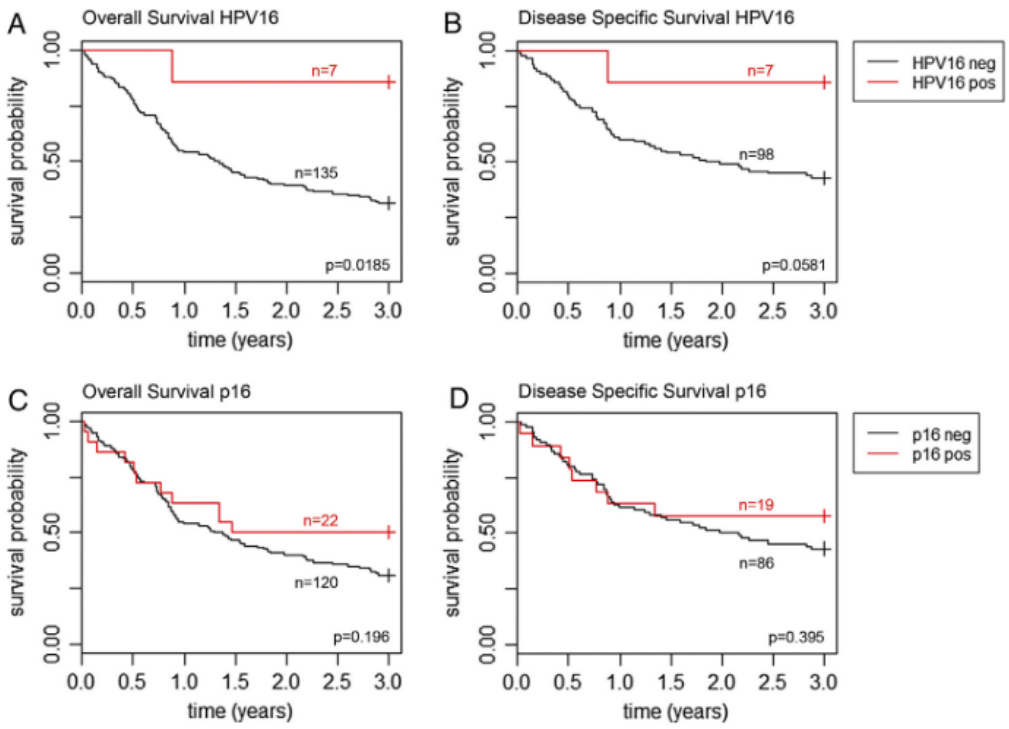


Figure 2-5. Survival curves for HPV16 and p16 in patients with Hypopharyngeal cancer. Top left, HPV 16 OS (p=0.0185), Top right, HPV 16 DS (p=0.0581), Bottom left p16 OS (p=0.196) and Bottom right p16 DSS (p=0.395). From (176)

2.1.1.2 T cells in Hypopharyngeal cancer

Many solid tumour types with high numbers of TILs are associated with improved survival (22, 144, 145). In this context, the TILs are generally $CD4^+$ and $CD8^+$ T cells. A specific subset of $CD4^+$ T cells are notable, as they regulate the T cell activity, regulatory T cells ($CD4^+CD25^+FoxP3^+$) which act to maintain immune homeostasis and tolerance (177).

The nuclear expression of FoxP3 is thought to be the most specific marker for T regulatory cells and indicates their immunosuppressive function. Physiologically, transcription factor forkhead box P3 (FoxP3) or *scurfin* is an important intracellular molecule for the function and development of T-regulatory cells. In normal conditions, $FoxP3^+CD4^+$ cells are used to suppress the host immune system and therefore, maintain immunological tolerance. In the case of many solid tumours, an increased infiltrate of these cells has been observed; in both the general tumour mass but also the draining lymph nodes and blood. The presence of this increased infiltration is associated with an unfavourable outcome and may be useful as a biomarker of prognosis (178).

In murine studies, $FOXP3^+$ expression has been associated with tumour suppression, in contrast to clinical studies, where $FOXP3^+$ expression has largely been associated with reduced survival (178, 179). A large meta-analysis of 59 studies with 12,563 cases, identified a significant association of lower OS with higher $FOXP3^+$ expression (pooled OR 1.46 [95% CI 1.19-1.78, p=0.0002]) (178). This

included breast, gastric and oesophageal cancer, melanoma and bladder carcinoma (178). The exception to this is HER2-positive breast carcinoma; where increased expression was associated with tumour suppression (180). In hypopharyngeal cancer, the role of FoxP3 is undefined, besides a few small descriptive studies (179).

One such study by Weller et al. assessed the effect of FOXP3$^+$ cells in oropharyngeal and hypopharyngeal cancers (n=89) collectively, in comparison to laryngeal lesions alone (n=83) (181). Tumour cell expression was allocated as weak, medium and strong. 5-year survival outcomes based on log-rank tests using Kaplan Meier curves suggested that FoxP3$^+_{strong}$ expression was associated with a significantly worse survival than FoxP3$^+_{weak}$, for both laryngeal (log-rank p=0.035) and oro-hypopharyngeal cohorts (log-rank p=0.001), see Figure 2-6 (181). On multivariate analysis, only oro-hypopharyngeal cancer gave a significant survival effect (HR 2.59 [95% CI 1.30-5.19], p=0.007) (181).

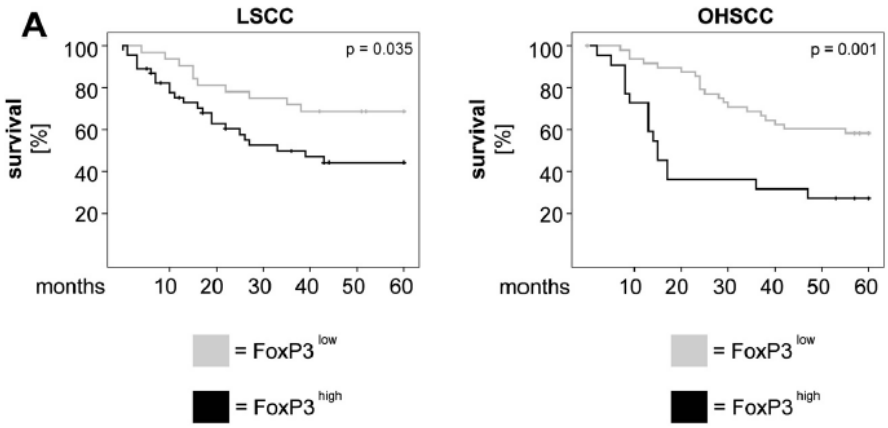


Figure 2-6. FOXP3$^+$ expression in laryngeal cancer (left, log-rank p=0.035) and oro-hypopharyngeal cancer (right, log-rank p=0.001), demonstrating an improved survival association in patients who were FOXP3$^+$ low.. Taken from (181)

Shang et al. performed a meta-analysis of 76 studies (n=12,563) assessing the prognostic effect of FoxP3$^+$CD4$^+$ cells on survival, including 2 head and neck studies in their cohort (178). A significant association was noted between FoxP3$^+$CD4$^+$ cells and survival (p=0.0002), with a high density associated with a lower pooled OS (OR 1.46, [95% CI 1.19-1.78]). Of note, there was considerable heterogeneity within the trials (I^2=81%, p<0.001) (178). A similar outcome was confirmed for the 37 studies (n=8,460) that assessed DFS outcomes (I^2=77%, p=0.0003).

Once again a high FoxP3$^+$CD4$^+$ infiltrate, was associated with a decreased survival (OR 1.23 [95% CI 1.01-1.50], p=0.003) (178). Interestingly within the subgroup of head and neck patients alone, FoxP3$^+$CD4$^+$ was found to be associated with *improved* survival (OR 0.69 [95% CI 0.50-0.95], p=0.024). This was also the case for colorectal and oesophageal cancer (178).

This positive effect on survival was corroborated by Russell et al (n=39) who showed a statistically significant decrease in HNSCC-specific mortality, in patients demonstrating high intra-tumoural and invasive margin infiltration by $FoxP3^{+}CD4^{+}$; (HR 0.66 [95% CI 0.24-1.84]) and (HR 0.44 [95% CI 0.15-1.31]) respectively (169). This was independent of HPV status (See Figure 2-7).

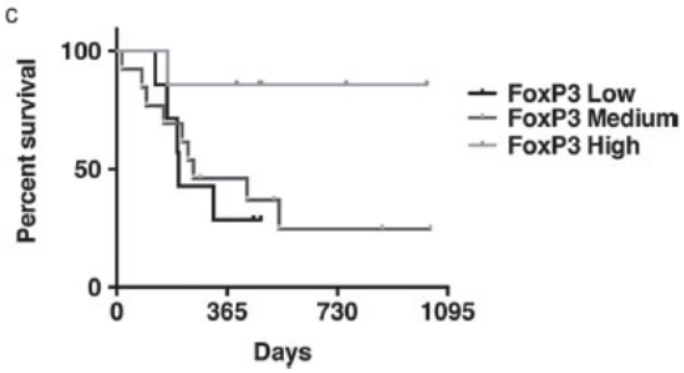


Figure 2-7. Kaplan Meier survival chart comparing patients' outcomes on the basis of $FoxP3^{+}CD4^{+}$ infiltrate expression. Contrarily this suggests improved survival in FOXP3+ high patients. Taken from (169)

Whether the presence of high $FoxP3^{+}CD4^{+}$ infiltrate is a surrogate for outcomes in HNSCC has yet to be confirmed, though there does seem to be a considerable discrepancy between outcomes for HNSCC and other solid body tumours.

2.1.2 $CD1a^{+}$

Gene methylation is a uniquely important contributor to the molecular heterogeneity of HNSCC (182). This is true for the cyclin-dependent kinase inhibitor p16, which demonstrates hypermethylation in cases of HPV-associated HNSCC and today fundamentally dictates its management across the world.

The *CD1A* gene encodes a transmembrane protein responsible for presenting non-peptide antigens by dendritic Langerhans cells to T cell lymphocytes (182, 183). CD1a density is used as a marker of dendritic cells and subsequently over-expression is associated with positive outcome with a series of cancer subsites, including the tongue (184), nasopharynx (185) and lung (186).

346 patients with HNSCC (hypopharynx =3%) were retrospectively reviewed by Virani et al., comparing the expression of gene methylation markers and tumour recurrence to survival (182). Differences in methylation were significantly associated with OS in all HNSCC patients (Log-rank p=0.005). The lowest quartile of methylation of CD1a was associated with the lowest probability of OS and the highest, with the highest probability (at 24 months, 0.68 and 0.80 respectively). On

multivariate analysis, HPV(+) patients demonstrated a 3.34 times higher hazard of death when comparing lowest quartile to those in 3rd and 4th quartiles (95% CI 1.88-5.93) (182).

2.1.3 CD34⁺ Cells

CD34 is a transmembrane glycoprotein that facilitates both cellular adhesion and transduction (187). C*ancer associated fibroblasts* (CAFs), the stromal cells linked with poor outcome (188) may originate from CD34 encoded antigen presenting cells. Although this has yet to be confirmed, $CD34^+$ cells represent stem cells capable of a variety of subsequent cellular differentiations.

One hypothesis outlines a transformation of $CD34^+$ cells of normal mucosa, to SMA^+ cells of CAFs in invasive carcinoma (189). In this way, the cellular composition of tumour-free and tumour-laden stroma is thought to be entirely distinct (188). This has been described in high-grade intraductal breast carcinoma (187, 190), invasive SCC of the skin (191), cervical SCC (191) and colorectal carcinoma (192). Only two similar articles exist for hypopharyngeal lesions.

A study of 42 larynx and hypopharynx HNSCC cases was reviewed (189). In normal mucosa, scattered $CD34^+$cells were demonstrated across the stroma, which were absent entirely from the mucosa of invasive carcinoma. Contrarily both the peri-tumoural zone and within tumour itself SMA^+ cells were seen, supporting the idea of an evolving desmoplasia (189), see Figure 2-8.

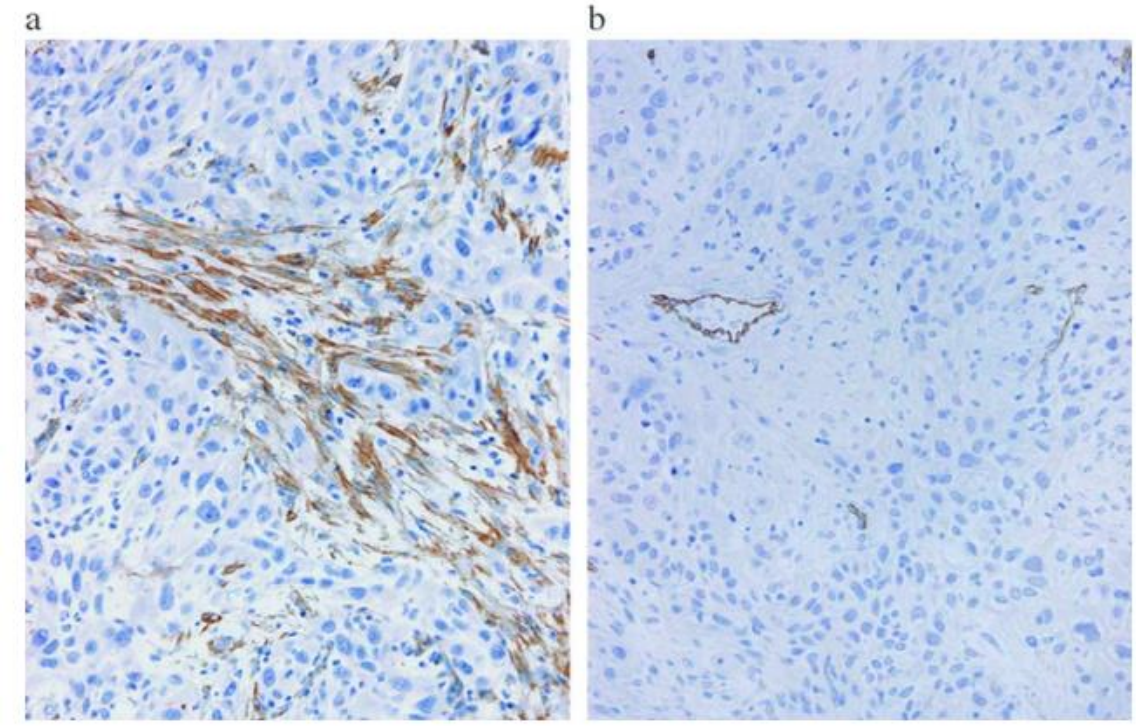


Figure 2-8. Immunohistochemical demonstration of SMA positive staining in SCC laryngeal tissue. Of note, blood vessel endothelia stain positive. Image A - scattered positive SMA staining in stroma. Image B - No CD34 staining in a case of invasive SCC. Taken from (189)

In addition, Barth et al. studied 39 HNSCCs, 6 of which were hypopharyngeal (188). The majority were oral cavity (n=16) and oropharyngeal lesions (n=13). The peri-tumoural border of invasive SCC was characterised by an abrupt reduction of $CD34^+$ cells and in 33 cases, it was completely devoid (188). The tumour stroma itself was replaced by an infiltration of α-SMA positive cells, in all but 8 cases (188).

2.1.4 α-Smooth Muscle Actin (SMA)

As early as 1889, Paget *et al.* had already drawn attention to the importance of the tumour microenvironment, to the support of the tumour; a theory "of seed and soil"(193). Since then, focus of research has largely been concerned with the cancer cell, addressing loci of genetic and epigenetic alterations, polymorphisms, environmental traumas and the subsequent carcinogenesis, invasion and metastasis.

Desmoplasia is associated with epithelial neoplasia: It is an orchestrated fibrosis in the stroma of the tumour. Within this stroma, there is evidence of neovascularisation and deposition of extracellular matrix proteins and myofibroblasts. The latter myofibroblasts (CAFs), demonstrate both secretory and contractile abilities and most widely express SMA (194).

Increasingly it is felt that these normal stromal cells are differentiated, subsequently recruited by tumour, and act to preserve its viability. CAFs facilitate a number of tumour supporting functions and have been associated with poor prognosis in a number of carcinoma types, including breast, colorectal (195) and ovary (196).

In the head and neck, there is evidence that α-SMA over-expression is associated with a poor outcome in the oral cavity (197). In addition, Kojc et al. reviewed the expression of $CD34^+$ and SMA in squamous epithelial lesions of the hypopharynx and larynx; comparing normal tissue to tumour stroma (189). The conversion of $CD34^+$ cells of normal mucosa to SMA^+ cells in SCC was used as a hypothesis. Normal mucosa was found not to stain for SMA, in comparison to SCC that universally stained for SMA (189). Contrarily, and as hypothesised, $CD34^+$ was found diffusely in the normal stroma but not in the adjacent SCC (189). No association with clinical outcome was shown. Finally over-expression of α-SMA was seen to demonstrate poor prognosis in patients with Nasopharyngeal carcinoma(198).

α-SMA was therefore investigated as a marker of over-expression, which where present might indicate that hypopharyngeal carcinoma was a cancer reliant on the tumoural stroma for continued growth and development.

2.1.4.1 $p16^{INK4A}$ (p16)

p16 has been shown as a marker of prognostic significance in OPSCC (22). Cases of p16 over-expression in OPSCC (supporting a HPV-driven tumour) have been associated with an improved prognosis on both OS and DSS (22, 24, 174). Tumours in other head and neck subsites can express p16, but this is not associated with an improved survival (199).

Wilson et al. studied the association between hypopharynx cancer and p16 in 27 patients, primarily treated with chemotherapy (n=22) (200). p16 was considered positive for cases of nuclear and cytoplasmic staining of >60% of tumour cells (200). 67% of all cases were p16 negative. All 13 p16 negative cases were confirmed by immunohistochemistry (200). More women were in the p16 positive than negative group (33% vs. 0%, p=0.03) (200). No significant difference was demonstrated for any succeeding outcome, either OS (56.7% vs. 58.7%, p=0.88), LRC (65.3% vs. 76.8%, p=0.52) and DFS (45.8% vs. 54.2%, p=0.60) (200).

A survival analysis was performed on 142 patients diagnosed with hypopharyngeal cancer by Dalianis et al (176). p16 positivity was considered for cases of greater than 75% of tumour cell staining (176). p16 over-expression was seen in 15.5% of cases. 3.7% of cases were both p16 positive *and* HPV DNA positive. For cases of p16 *and* confirmed HPV-16 DNA positivity, both overall and disease specific survival were significantly improved, (p=0.0185 and p=0.0581 respectively) (176). This was not the case for p16 alone (p=0.196 and p=0395) (176).

51 cases of hypopharyngeal carcinomas were assessed for HPV DNA positivity and p16 overexpression by Yang et al (201). HPV positivity was seen in 26.1% of cases and p16 over-expression in 39.1% (201). There was positive correlation between HPV infection and p16 expression (coefficient = 0.437, p=0.002) (201). Median OS was 43.5%. Differences between HPV infection and p16 overexpression were both significant for OS (51 and 75 months, p=0.001 and 53 and 69 months, p=0.001) (201).

As demonstrated, the literature on p16 in the hypopharynx is contentious. Hypopharyngeal cancer appears to comprise a cohort of patients with p16 positivity and this may be associated with improved outcomes. Varying definitions of positive staining may reflect the discrepancies of results. In addition, there may be some uncertainty regarding the tumour origin in these HPV-driven cases. They may have originated from either the lower pole of the tonsil or supraglottis, explaining their viral origin.

2.1.4.2 p53

Overall p53 over-expression appears equivocal for cases of HNSCC, with studies demonstrating both positive and negative associations with survival (202-205). Specific studies addressing hypopharynx carcinoma alone are limited.

Chien et al. followed 58 hypopharyngeal cancer patients, treated primarily with surgical treatment (94). The greatest majority of cases were of tumour stage T3 and above (93.1%). High p53 expression was identified in 69% of the tumours, predominately localised to cancer cell nuclei (94). No p53 staining was demonstrated in the adjacent normal tissue. High expression of p53 (>50% of tumour cells) was significantly correlated to advanced T stage ($p<0.001$) and advanced overall stage ($p=0.002$) (94). OS at 5 years was 64.3%.

Based on rate of survival, patients with a high expression of p53 were found to have a significantly reduced chance of survival compared to those with low expression, (51.6% vs. 80%, Log rank $p=0.028$) (94). This effect was not seen to persist on multivariate Cox's regression analysis (HR 1.97 [95% CI 0.66-5.93], $p=0.227$) (94).

A study by Frank et al. assessed 43 hypopharyngeal cancer patients for expression of p53 (96). Expression of p53 was identified in 37% of patients. Normal squamous epithelium, similar to the findings of Chien et al., did not stain for p53 (94, 96). Patients positive for p53 were largely found to be younger than those who were negative (median age 54.9 vs. 64.1 years, $p=0.025$) (96). All p53 positive patients were of an advanced overall clinical stage (Stage III and IV, $p=0.035$). Follow up was a median of 43 months. 5-year survival of p53 positive vs. negative groups was 21% vs. 26%, though this was not significant on univariate or multivariate cox-regression analysis (96).

A study by Lassaletta (n=62) found similar results (97). 5-year relapse-free survival rate was 20% with a median survival rate of 10.6 months. Survival was consistently higher for p53-negative than p53 positive at 2, 3 and 5 years respectively (Log rank $p=0.03$, $p=0.01$, $p=0.007$) (97). Median relapse free survival was 16 months in those lacking p53 expression, in comparison to 9 months in those with high expression (97).

For HNSCC tumours, p53 over-expression appears to be associated with tumour aggressiveness and poor survival.

2.1.5 Aim of Study

We therefore sought to investigate hypopharyngeal tumours in a similar way in order to attempt to stratify cases according to TIL infiltrate. We intended to demonstrate that hypopharyngeal

cancer was likely an immune-cold cancer or one where immune infiltrate was unlikely to demonstrate any degree of differentiation based on survival outcomes. We looked at specific immune subsets within the hypopharyngeal tumours using a panel of markers for antigenic analysis via immunohistochemistry (IHC). In brief,

1. $CD3^+$: A 'pan' T cell marker
2. $CD4^+$: A T cell marker, used to identify *helper* T cells
3. $FoxP3^+$: A T cell marker, used to identify *regulatory* T cells
4. $CD8^+$: A T cell marker used to identify all *cytotoxic* T cells
5. $CD34^+$: A T cell marker of haematopoietic cell lines

We also chose to look at the following markers of importance

1. α-SMA: A myofibroblast marker, a subgroup of fibroblasts
    a. This was specifically in reference to work by Kojc et al. who had demonstrated the importance of the tumour *stroma* in patients with hypopharyngeal cancer(189). Specifically, the stromal environment had changed from one of normal $CD34^+$ expression, to a marker associated with so called cancer associated fibroblasts.
2. p16: A surrogate marker for HPV E7 expression
    a. This was investigated to exclude the fact that hypopharyngeal cancer was a virally driven tumour
3. p53: A cell cycle regulator protein
    a. p53 was specifically investigated for its established importance within HNSCC as a mutated gene of relevance. Specific studies had not been performed to ascertain its role in hypopharyngeal cancer and therefore this study hoped to clarify whether patients with p53 mutations and overexpression were a factor of note.

2.2 Materials and Methods

Patients were retrospectively identified from seven institutions internationally, including Poole Hospital NHS Foundation Trust (England, UK), University Hospital Southampton (England, UK), Portsmouth Hospital Foundation Trust (England, UK), Aintree University Hospitals Trust, Liverpool (England, UK), Princess Margaret Cancer Centre (Toronto, Canada) and Sunnybrook Health Sciences Centre (Toronto, Canada). An eighth site which had been enlisted to recruit was removed from further review given limitations in identification of patient tissue (Sardinia).

Retrospective electronic searches were performed within six institutions by the author to identify patients diagnosed and treated with hypopharyngeal carcinoma. These patients were sourced using assistance from locally based MDT co-ordinators and head and neck nurse specialists. The seventh site (Aintree) possessed an established hypopharyngeal database that was accessed. A total of 363 patients were originally identified from these seven contributing hospitals (see Figure 2-9).

Portsmouth Hospital Foundation Trust, as a site, was removed subsequently due to a failure to establish capacity and capability approval from the trust-based Research and Innovation department. Additionally, patients in whom there were inadequate tissue stores or tissue was inaccessible, were excluded. Patients with incomplete outcome information were excluded. 95 patients were considered for final analysis (see **Error! Reference source not found.**).

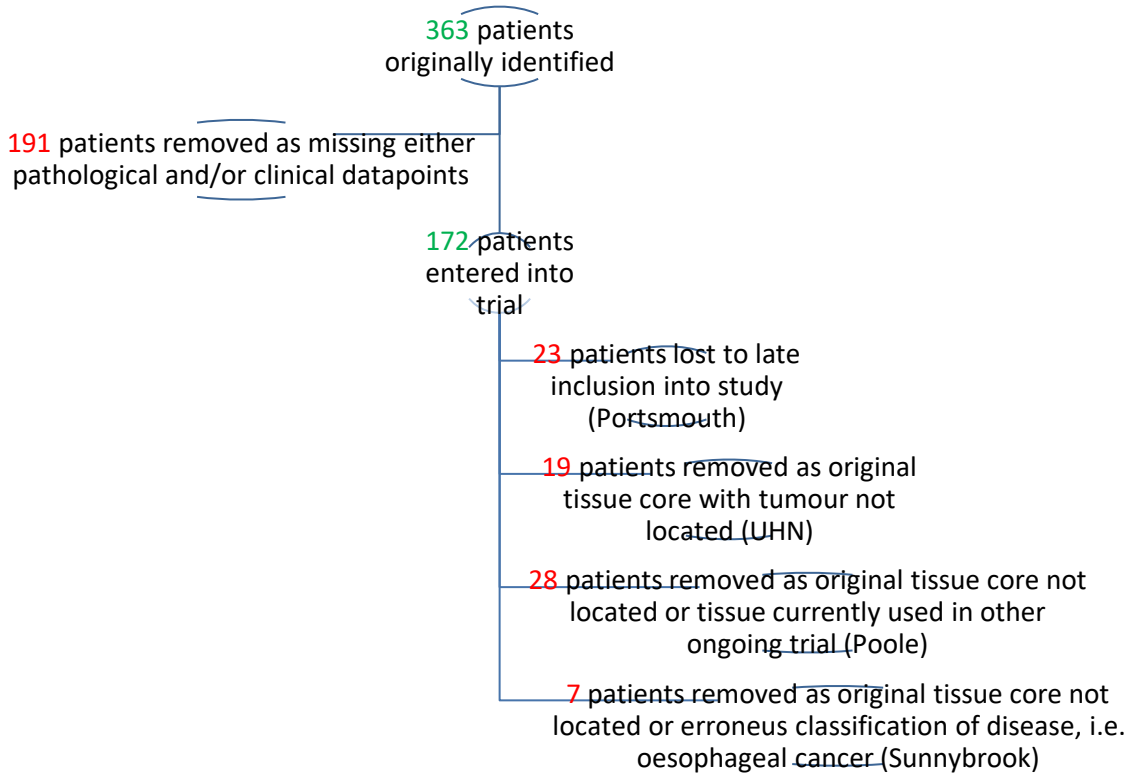


Figure 2-9. Flowchart demonstrating patient inclusions and exclusions into trial. 363 patients were demonstrated from the outset and 95 patients were considered for final inclusion.

Given the significant attrition of patients from the original cohort identified, the author acknowledges the significant risks of both recruitment bias based on the select nature of the final cohort of patients included and the subsequent odds of a type 2 error, possibly causing confounding results due to the former.

2.3 Construction of Database

A comprehensive database was created detailing patient, demographic, management and outcome data. Patient data was collected from the period inclusive of 1st January 2000 and 31st December 2016. The database was created and then subsequently built in Microsoft® Excel® for Macintosh 2011 Version 14.4.2, secured by an access password. Patient information was recorded using categorical and continuous data. Regarding the latter, for items pertaining to demographic and management data, this was recorded to single units; elsewhere, outcome data was recorded to the nearest decimal place. Categorical data was assigned a numerical value where appropriate. All parameters of interest are displayed in detail in Appendix D.

2.4 Ethical Approval

For the purposes of this study, patients were pseudo-anonymised within the medical database, each being assigned a successive study number from 1 to infinity, indicating their timing of inclusion within the study. This was corresponded to pathological data related to the TMA and its subsequent analysis. From this point, no unique identifiers were used within the context of the study and all statistical analysis was grouped and entirely anonymous.

This database was stored on a University of Southampton, Apple Inc. MacBook Air (1.6Ghz Intel Core i5, 4 Gb 1600 MHz DDR3) laptop with an encrypted hard drive and backed up to secure servers at both the University of Southampton and Southampton University Hospitals Trust.

2.4.1 Consent

Usually, recruitment into clinical trials involves a process of informed consent, whereby an individual subscribes to a clinical study, having been presented with sufficient information to make an educated choice about enrolment. For the purposes of this study however, consent was not formally obtained from individual patients. Given the natural history of hypopharyngeal carcinoma, it was presumed that most patients would be deceased and the process of contacting next of kin would not be appropriate, particularly since the study was making use of clinically available information to local trusts.

As per the Human Tissue Act (HTA) 2004, this was supported by clause of Statutory Exemption. Section 1 (9) of the HTA indicates that existing holdings (i.e. the body or material of a deceased person) are exempt from consent provisions in the situation that the tissue was held or collected

prior to the commencement of the Act, on 1st September 2006. Or, as per Section 3, if tissue and cells are sought within a research project with appropriate ethical approval and anonymity (206).

Ethical approval was sought and approved in the UK (REC: 16/EM/0497, Protocol no: P160921). In Canada, research approval was granted within the auspices of local research board evaluations at each included hospital (Princess Margaret: 16-6298 and Sunnybrook: 025-2017).

2.5 Tissue micro-array construction

Source specimen blocks were from a multitude of biopsies including original diagnostic biopsies and surgical resective specimens, depending on what material was available from the host hospital.

A TMA designer was used to design the TMA. A punch size of 1000µm was used to create the cores from the centre of each respective paraffin-embedded block, to enable a central tumour sample as opposed to an invasive front. Each core was spaced between 800µm-1000µm apart. From this a total number of core "spots" was totalled, a product of the rows and columns.

Subsequently a "TMA design", a Microsoft® Excel® document was downloaded, including the associated histology number and its relationship to the TMA "map". 3 cores were taken, where possible, from each block. The machine then removed a wax core from the designated recipient block, which then allowed subsequent insertion of the donor tumour core. This marked slide was placed on top of the donor block so to allow the inbuilt camera to match the computer-generated markers from the donor area, indicating cores for harvest to the underlying recipient core.

Finally, the new compilation TMA block was then heated in a 40°C oven overnight allowing wax adherence to the tissue core. Subsequent microtomy sections of 4µm were cut from this TMA and mounted electro-statically upon SuperFrost™ Plus slides (Thermo Fisher Scientific™). Slides were dried at 60°C at 20 minutes. Each slide was labelled to correspond to its contributory TMA and subsequent intended primary antibody.

2.5.1 Immuno-histochemistry

Mrs Monette Lopez, a biomedical scientist working in the academic histopathology department at UHS, performed the immuno-histochemistry for this study. The details of this are outlined below.

Sections from the appropriate TMA were cut and subsequently stained using the Leica BOND-MAX™ automated stainer (Leica Microsystems, UK) so to allow repeatable consistency in staining of the TMA. The reagants were pre-mixed and sourced from Leica Microsystems.

Sections were dewaxed using Bond™ Dewax solution (Novocastra Reagants, Leica Microsystems, UK) and hydrogen peroxide 3% was used for 10 minutes so to inhibit the activity of endogenous peroxidase. Heat or protein-induced epitope retrieval (HIER/PIER) methods were both used to retrieve specific antigens. HIER was performed using Bond™ Epitope Retrieval Solution 1 or 2 for 20 minutes at 98°C (Novocastra Reagants, Leica Microsystems, UK). PIER was performed using Bond™ Enzyme Pre-treatment kit (Novocastra Reagants, Leica Microsystems, UK). This 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 subsequently incubated for 10 minutes at 37°C.

Following this, specific sections were incubated with their relevant primary antibody for 20 minutes at 37°C. All primary antibodies were diluted in Bond™ Primary Antibody Diluent (Novocastra Reagants, Leica Microsystems, UK). Optimal antibody concentrations and retrieval methods had been previously determined for antibodies that were in routine clinical practice. The antibodies used and their concentrations and techniques for retrieval are shown in Table 2 below.

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	1AS	Novocastra	1:50	Bond Epitope Retrieval Solution 2
FOXP3	236A/E7	Ebioscience	1:20	Bond Epitope Retrieval Solution 2
CD1a	MTB1	Novocastra	1:60	Bond Epitope Retrieval Solution 2
α-SMA	αsm-1	Novocastra	1:50	Bond Epitope Retrieval Solution 1
P16	Ink4a	CINtec	1:3	Bond Epitope Retrieval Solution 2
P53	DO-7	DAKO Cytomation	1:50	Bond Epitope Retrieval Solution 1
CD34	My10	Becton Dickinson	1:80	IHC-Tek Epitope Retrieval Solution

Table 2. Table to illustrate the immunohistochemistry antibodies used within the context of this study

2.5.2 TMA scoring and automated cell counting using ZEISS Axio Scan.Z1 slide scanner and QuPath

Completed TMAs were digitised and scanned using a ZEISS Axio Scan.Z1 slide scanner at the WISH lab in Southampton University Hospitals Trust (see Figure 2-10). Each TMA was loaded manually into a modular tray located within the virtual microscope and scanner (see Figure 2-10). Slides were illuminated using a transmitted LED light (400-700nm) and photographed at a magnification resolution of x20 using a Hitachi HV F202 camera. Digitised images were rendered as high quality .CZI files, a ZEISS specific file type, used to incorporate Meta information.

Figure 2-10. Compilation image of ZEISS Axio slide scan.Z.1. Left image – slide trays, demonstrating capacity to run 25 individual trays of 4 concurrently. Right, top – open tray demonstrating capacity for loading 4 individual slides. Right, middle – detail of tray. Right, bottom – the scanner in situ in the WISH lab, University Hospital Southampton.

An open-source bio-image analysis software, *QuPath* (207) was used to subsequently count cells for T cell populations, having defined scripts unique to each individual population, as described below. Using this software, images were batch processed to deliver individual cell counts, which were then married to survival data accordingly.

1. Images were uploaded, as part of a complete pictorial TMA and de-arrayed (split up) to give individual core images as illustrated in Figure 2-11. The image exemplified demonstrates positive brown staining for one singular core belonging to a parent TMA slide. The resulting TMA slide was therefore analysed individually at each core level.

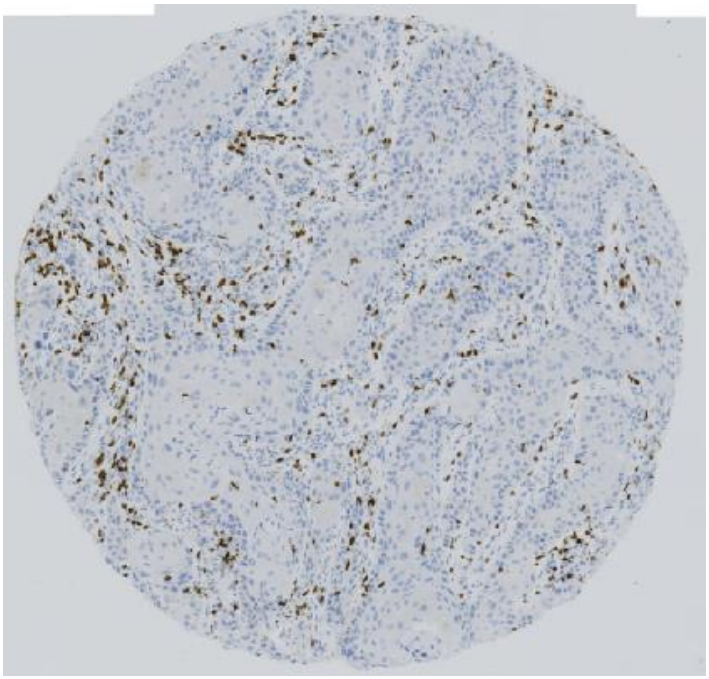


Figure 2-11. Individual tumour core taken from host $CD3^+$ TMA demonstrating final appearance post, staining for $CD3^+$ cells

2. Stain vectors were subsequently estimated, so to allow the most accurate assessment of staining for each particular TMA. As a computerised “white balance” this allowed the software to identify all required colours within that particular TMA. This process ensured that homogeneity was preserved within each grouped analysis. Individual array stain vector discrepancies were excluded to preserve consistency across the test group, see Figure 2-12.

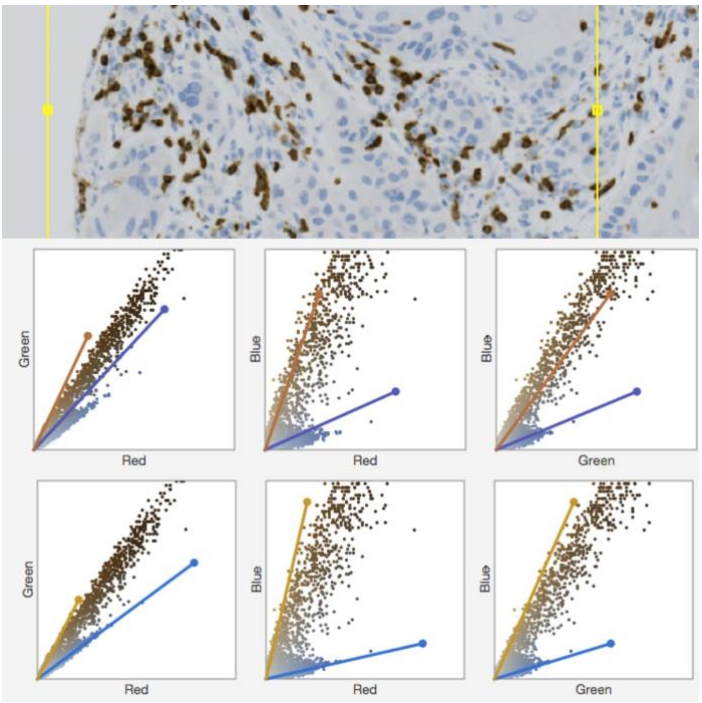


Figure 2-12. Example of stain vector analysis. Top - image is selected to incorporate area both outside and within core, giving a stain vector shown in the middle image. This is then re-calibrated to ensure all staining is adequately identified (image below).

3. Having established appropriate staining vectors, tissue detection was enabled; a process identifying areas suitable (i.e. pixels of the image representing actual tissue core), and not suitable (i.e. pixels of the image representing areas in between adjacent tissue cores) for analysis. Amendments to parameters of threshold tissue detection (i.e. the red, green and blue values of the image), pixel size (to adequately reflect resolution of image) and minimum tissue area (i.e. the smallest piece of tissue that QuPath would recognise as requisite of analysis, all smaller pieces being discarded), were made. The intended final area would hopefully encircle only the core in question and not any surrounding area and if successful, a final area for analysis would be shown as demonstrated, e.g. Figure 2-13.

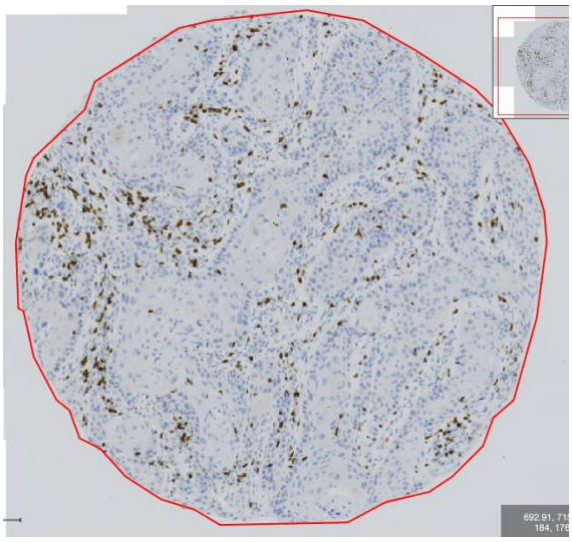


Figure 2-13. Red line demarcates the edge of the core indicating that tissue detection has been successful; that the area *within* the red line is to be used for analysis whilst the area outside of the red line is not to suitable for analysis.

4. Finally a cell count analysis was performed to distinguish positive cells, i.e. cells that had stained positively for that particular core. This was largely based on adjustment of a *nucleus:DAB OD mean* value, a ratio which corresponded to the amount of brown (DAB) staining within cell nuclei. A single binary threshold was used to distinguish positive and negative cells. From this a final image was created as shown in Figure 2-14 with further detail demonstrated in Figure 2-15.

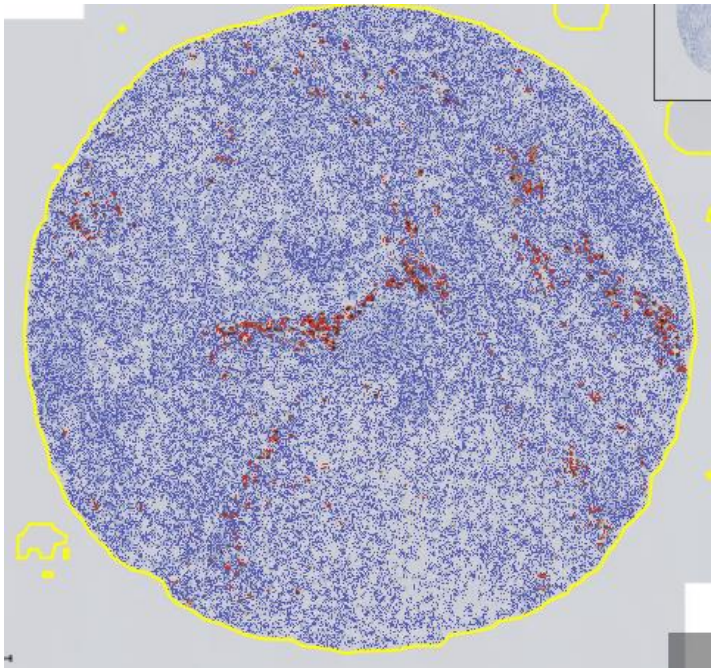


Figure 2-14. Final cell count analysis demonstrating the core selected for analysis and cells that had subsequently been identified as staining negatively (blue) and positively (red)

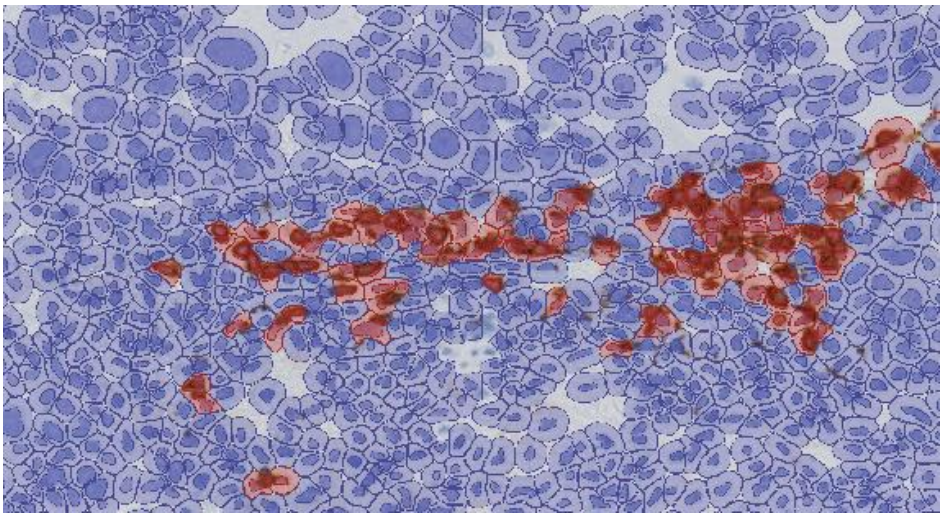


Figure 2-15. Zoom (x20) of image above, demonstrating negative (blue) and positive (red) cells

4. From this image a series of values were calculated as follows; total number of negative cells, total number of positive cells, percentage of positive cells and number of positive cells per mm^2. Final cell counts were based on number of positive cells per mm^2. This was produced as a singular .txt file which was amalgamated using MS-DOS command window, to give a spreadsheet of final results in Microsoft Excel™.

2.5.3 Cell Scripts

To expedite the process in 2.5.2, a series of computer scripts were designed to automatically analyse subsequent data sets. These were created using the programming language *Groovy*, within the QuPath Script Editor and were unique to each particular stain. A sample script for CD8 cells is given in Appendix E.

2.5.4 Assessment of Method (Script counting vs. Manual counting)

To ascertain whether script defined results were consistent with those from manual processing, comparisons of means were made for script harvested and manual counted results. An *annotations selector* in *QuPath* was used for the purposes of manual cell counting and this was performed by the author.

A single TMA was used (Poole) and comparisons were made for CD3, CD8 and CD4 stains. All cores within each stained slide were analysed. In order to measure the strength and direction of each cell counts, a bivariate Pearson correlation test was used to compare the results of the means. For each this demonstrated, moderate to strong correlation and this was significant (see Table 3).

TMA	Correlation	Significance
CD3	r=0.883	p<0.0001
CD4	r=0.901	p<0.0001
CD8	r=0.847	p<0.0001

Table 3. Correlation between hand counted cores and those measured with scripts for Poole TMAs of CD3, CD4 and CD8 stains

The author was not blinded to the sample being analysed (e.g. could identify which core or TMA arose from which stain and centre) but did not address each core in the context of its clinical outcome. To do this would have required access to the TMA screen maps and comparing the immunohistochemical and clinical screen maps together.

Once it had been confirmed that there was strong correlation between the hand counted and script counted cores, the decision was made to progress with the script counting method. Each script was run once for each TMA stain, given the automated nature of the process.

There was no indication to run multiple tests for the same stain in the context of this testing. Specifically, there was no sample regression (change to the TMA core being tested), no intermittence (external variables that might unduly affect the running of the script on the computer), no trial failure (suggestion that a script might not work as well on any given run) or study mutations (no change to the core in between potential tests).

2.6 Results

Tissue and outcome data were obtained for 95 patients (see **Error! Reference source not found.**). 77 (81.1%) patients were male and 18 patients were female (18.9%). Mean average of age at diagnosis was 65.4 years (± 10.9 years), see Table 4.

2.6.1 Demographics of Population

2.6.1.1 Gender

As expected for a cohort of patients with hypopharyngeal cancer, there was a greater male population than female, see Table 4. When dividing patients into age groups about the median

(see 2.6.1.2), the greatest proportion of patients were male, both at ages less than 67 years (84%) and greater than 67 years (80%).

2.6.1.2 Age

Age at diagnosis was defined as the years between patient's date of birth and date of diagnosis. 92 (96.8%) patients had information to calculate this and the following histogram demonstrates their distribution of age (see Figure 2-16). Mean age was 65.35 (± 11.68 years), with a range of 31 to 91 years. Median age was 63.9 years (see Table 4).

11 (11.6%) patients were diagnosed at ages 55 years and younger (mean age 50.8 years) and there was no association with HPV positivity, with only 3 (27.3%) patients testing weakly positive for p16 staining (p = 0.403, Fishers exact testing).

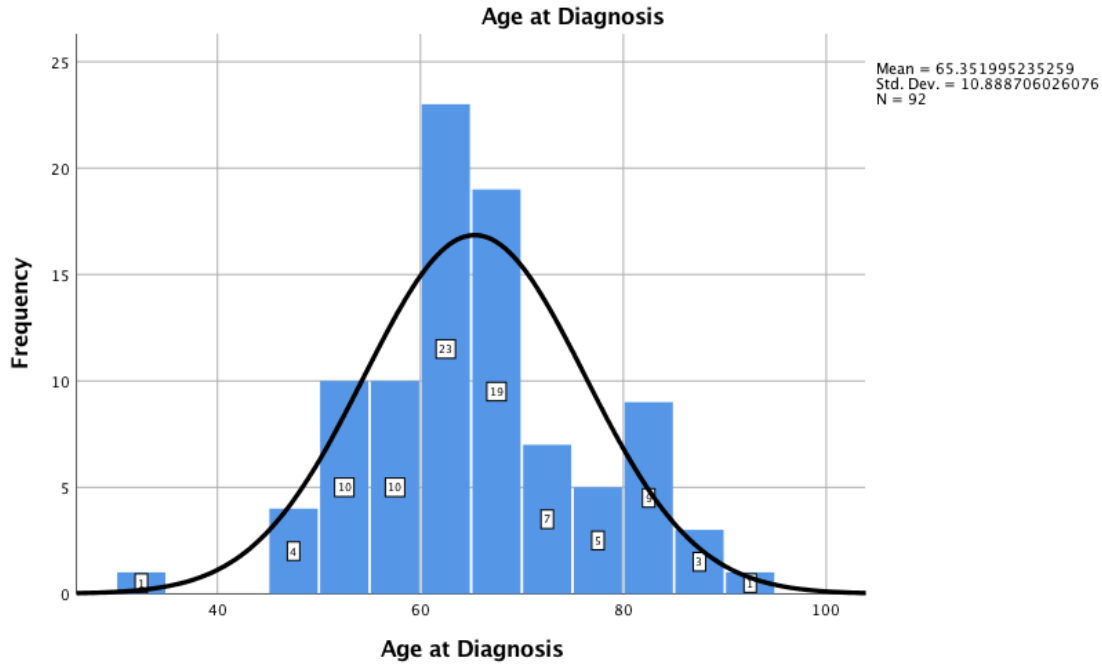


Figure 2-16. Histogram demonstrating a relatively normal distribution of ages of total population of hypopharyngeal carcinoma

2.6.1.3 Weight and BMI

Weight data was only available for 20 (21.1%) patients. The median weight was 60.05kg. The mean was 63.18kg (± 14.03), see Figure 2-17. The corresponding BMI of this group was 20.9kg/m^2 (± 6.48).

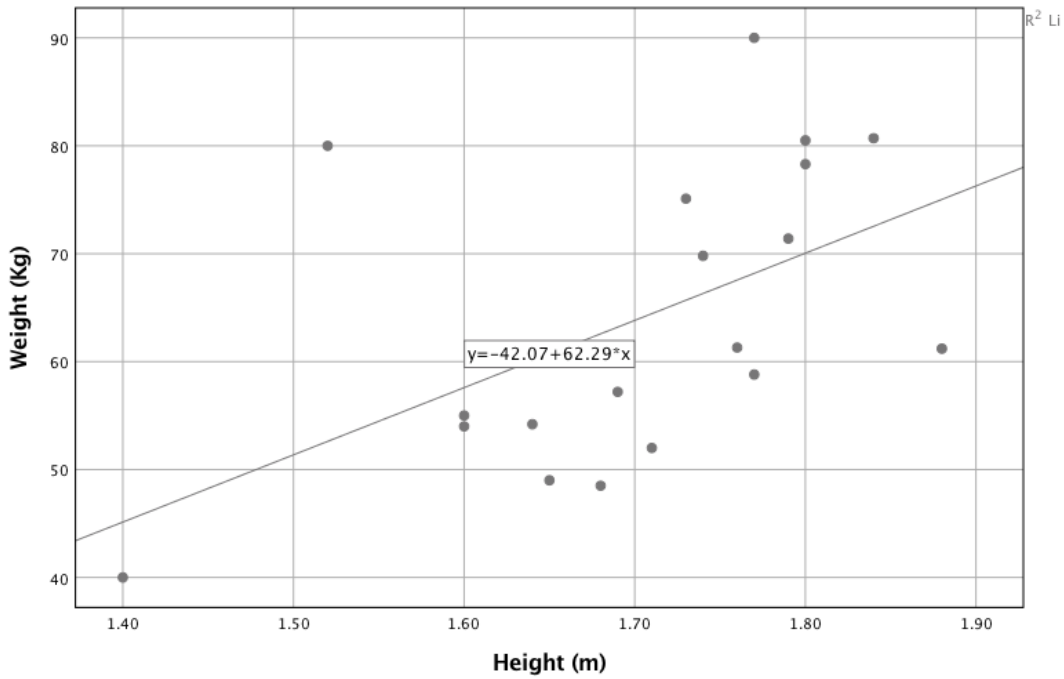


Figure 2-17. Relationship between height and weight for cases of hypopharyngeal cancer (n=20)

2.6.1.4 Tobacco Consumption

Smoking was categorised based on trial outcomes from Granata et al. (118) addressing risk stratification in a population with oropharyngeal squamous cell cancer.

From this classification, 27 (28.4%) patients were classified as low-risk and 33 (34.7%) as high risk. Smoking status was attributed a binary stratum, with low-risk being (non-smoker, ex-smoker, smoker <10 pack years) and high-risk (smoker of >10 pack years), given categories to be related significantly to OS (118). Information was not available on smoking status for 33 (34.7%) patients (see Table 4).

		Cohort Total (n=95)
		Number (%)
Hospital	Southampton Poole UHN Sunnybrook Aintree	26 (27.4%) 25 (26.3%) 17 (17.9%) 8 (8.4%) 19 (20.0%)
Age	<50 50-69 >70 *Median (± SD)*	5 (5.3%) 60 (63.5%) 28 (29.5%) *65.35 (± 11.68)*

Gender	Male Female	77 (81.1%) 18 (18.9%)
Smoking Status	Never Current Light Current Heavy Ex-Smoker Unknown	7 (7.4%) 16 (16.8%) 20 (21.1%) 17 (17.9%) 33 (34.7%)
Smoker Status (According to <10py/>10py)	<10 py, never/ex-smoker >10 py Unknown	27 (28.4%) 33 (34.7%) 35 (36.8%)
Alcohol	Non-drinker <10 units/week 10-20 units/week >20units/week Ex-drinker Unknown	5 (5.3%) 10 (10.5%) 5 (5.3%) 25 (26.3%) 7 (7.4%) 43 (45.3%)

Table 4. Demographics of Patient Population on entry into study. (*py, pack years)

2.6.2 Tumour Characteristics

The characteristics of the tumours within the database are summarised in Table 5 and expanded upon below.

2.6.2.1 Tumour Site

The majority of tumours arose from the pyriform fossae, followed by the post-cricoid region. Only 7 (7.4%) of tumours were found outside of these areas.

2.6.2.2 Synchronous Primaries and Incidence of Distant Metastatic Disease

Synchronous primaries were defined as discrete additional malignancies identified at the time of diagnosis. 5 patients (5.7%) were identified with synchronous tumours within this data set. Distant metastases were seen in 5 patients (5.7%) at diagnosis.

The pharyngeal tumours were grouped as a single subsite (lateral and posterior wall), no significance was demonstrated for predilection of synchronous primaries between the hypopharyngeal subsites (Pharyngeal walls vs. post-cricoid vs. pyriform fossae [p= 0.097, Kruskall Wallis Test]). This was similarly the case for distant metastatic disease (p=0.667, Kruskall Wallis Test).

2.6.2.3 TNM Staging

TNM staging was based on the AJCC/UICC v7 classification. For grouping purposes, 4 categories were considered for both tumour and nodal status (see Table 5). Tumour groups were populated in two predominant peaks at T2 (30.5%) and T4 (44.2%). The predominance of the patients with lower tumour stages may have reflected the fact that the database was primarily composed of surgical cases.

Most patients presented within a v7 nodal stage of N2 (61.1%). This was composed of 10 (10.5%) N2a cases, 34 (35.8%) N2b and 14 (14.7%) N2c cases. As expected, the greatest proportion of cases presented with AJCC/UICC Stage IV tumours (76.8%).

		Cohort Total (n=95)
		Number (%)
Tumour Site	Lateral Pharyngeal Wall Posterior Pharyngeal Wall Pyriform Fossa Post-cricoid Supraglottis Missing	4 (4.2%) 3 (3.2%) 55 (57.9%) 10 (10.5%) 1 (1.1%) 22 (23.2%)
Tumour Side	Right Left Bilateral Unknown	45 (47.4%) 34 (35.8%) 11 (11.6%) 5 (5.3%)
Synchronous Primary Tumour	No Yes Unknown	69 (72.6%) 5 (5.3%) 21 (22.1%)
AJCC/UICC Tumour Stage	Stage I Stage II Stage III Stage IV Unknown	4 (4.2%) 8 (8.4%) 8 (8.4%) 73 (76.8%) 2 (2.1%)
T Stage	T1 T2 T3 T4 Unknown	8 (8.4%) 29 (30.5%) 14 (14.7%) 42 (44.2%) 2 (2.1%)
N Stage	N0 N1 N2	28 (29.5%) 7 (7.4%) 58 (61.1%)

	N3 Unknown	1 (1.1%) 1 (1.1%)
Distant Metastases at Presentation	No Yes Unknown	89 (93.7%) 5 (5.3%) 1 (1.1%)

Table 5. Tumour characteristics of patients with hypopharynx cancer cohort

2.6.2.4 Tumour Histology

Histological information was sought from electronic pathological reports where possible. 3 (3.4%) cases were well differentiated. 29 (33.3%) of cases were of moderate differentiation and the majority of 43 (49.4%) were poorly differentiated. 12 (13.8%) had insufficient tumour grade information. Regarding cohesive margin status, 4 (4.6%) were seen to have a cohesive margin, 19 (21.8%) were discohesive. 2 (2.3%) were mixed with 62 (71.3%) with missing information.

Histological information regarding primary peri-neural, peri-vascular and lymphatic spread was only available for 12 (13.8%), 18 (20.7%) and 17 (19.5%) cases respectively and was not considered for further analysis.

53 (60.9%) patients underwent a neck dissection. 5 (5.7%) had missing information. Of these 53 cases, the mean total number of nodes was 22.98 (±25.18) with a median of 10.5. The range of total nodes harvested extended from 0-77.

The mean of the total number of positive nodes harvested was 1.75 (±1.68) with a median of 1. The full range of positive nodes extended from 0-6. The mean maximum lymph node diameter of these positive cases was 26.05mm (±11.95), with a median of 22m. The range of positive node diameters was 7mm-50mm.

19 (35.8%) of cases had extranodal extension (ENE) and 22 (41.5%) did not. 12 (22.6%) of pathology reports did not comment on this parameter.

There was no association between the binary presence of positive nodes and ECS (p=0.368, Chi-Square Testing). In addition, the lymph node ratio (defined by number of positive nodes divided by total number of nodes), was not linked with ENE when considering lymph node ratio groups of 1 or less than 1 (p=0.322, Chi-Squared).

Simple logistic regression was similarly used to predict for the dependence of ENE to lymph node size, total positive nodes and lymph node ratio. Of these, only lymph node diameter was seen to approach significance, with increasing size subsequently increasing odds of ENE (OR 1.07 [95% CI 1.009-1.14], p=0.025), see Appendix F.

2.6.2.5 Patient Treatment, Follow Up and Outcomes

All patient outcomes are summarised in Table 6 below. Overall, there was insufficient information present to build an understanding of treatment demographics. 32 (33.7%) patients were treated primarily with radiotherapy and 32 (33.7%) with surgery. 10 (10.5%) patients were not treated and the treatment status of 21 (22.1%) was unknown. Of the radiotherapy patients, 16 (50.0%) received concurrent chemotherapy, 3 (9.4%) neo-adjuvant and 1 (3.1%) both concurrent *and* neo-adjuvant chemotherapy. 10 (10.5%) of all patients refused treatment.

Treatment failure being residual and active disease following treatment (either in the form of surgery or chemo/radiotherapy) and late recurrence were relatively uncommon; seen in 13.7% and 15.8% of patients. Second primary tumours were similarly uncommon, seen in 5.3% of cases. Late distant metastasis was seen in 11.6% of the patient population. In summary, 34 (35.8%) of the entire patient cohort were seen to suffer treatment failure, late recurrence or late distant metastasis.

Survival outcomes were recorded for all 95 patients, confirmed either through electronic patient note search or via contacting the National Cancer Data Repository via Public Health England, where information was missing.

61.1% of the entire patient population died in the period of follow up. The rate of survival was 37.9% at 3 years and 17.9% at 5 years. The mean length of survival was 36.4 months (± 37.22). Median survival was 22 months with a range of 0-171 months.

		Cohort Total (n=95)
		Number (%)
Treatment Failure	No Yes Unknown	60 (71.3%) 13 (13.7%) 22 (23.2%)
***Late Recurrence**	No Yes Unknown	76 (80.0%) 15 (15.8%) 4 (4.2%)
Second Primary Tumour	No Yes Unknown	69 (72.6%) 5 (5.3%) 21 (22.1%)
Late Distant Metastasis	No Yes Unknown	58 (61.1%) 11 (11.6%) 26 (27.4%)

Treatment Failure, Recurrence or Metastasis	No Yes Unknown	36 (37.9%) 34 (35.8%) 25 (26.3%)
Life Status (At time of last follow up)	Dead Alive Unknown	58 (61.1%) 37 (38.9%) 0
Cause of Death	Died from Primary Tumour Still alive Died of other cause Unknown	43 (45.2%) 23 (24.2%) 7 (7.3%) 22 (23.2%)
3-year survival	Dead Alive	59 (62.1%) 36 (37.9%)
5-year survival	Dead Alive	78 (82.1%) 17 (17.9%)

Table 6. Patient outcomes for entire cohort. *late recurrence is defined as the presence of disease after a period of 5 years from initial treatment.

When assessing for relationship between radiotherapy and survival outcomes, patients had a worse outcome if they received post-operative radiotherapy as opposed to primary therapy or not at all (see Appendix G); as discussed later, this likely reflects the disease stage and pathological markers identified in the surgical specimen.

2.6.3 Relationship of Patient demographics, tumour characteristics, histology and cancer treatment to Survival Outcomes

Data pertaining to these aspects was assessed and discussed further in Appendix H as it was not directly pertinent to ths study.

2.6.4 Prognostic pathological Factors in Hypopharyngeal Carcinoma

2.6.4.1 $CD3^+$ cell count

100% of cases were available for $CD3^+$ analysis and cell counts were scored as mean per mm^2. A positive skewness was seen on the stem and leaf plot. (see Figure 5-20).

2.6.4.1.1 Identification of levels of significance in immune cell numbers

To date there have been no suitable levels of immune cell numbers of significance in the literature. We therefore sought to identify suitable levels of comparison, by performing multiple assessments, dividing the mean $CD3^+$ cell counts in various ways. OS was used a means of assessing differences in outcome and an example of these assessments in shown in Figure 2-18 below.

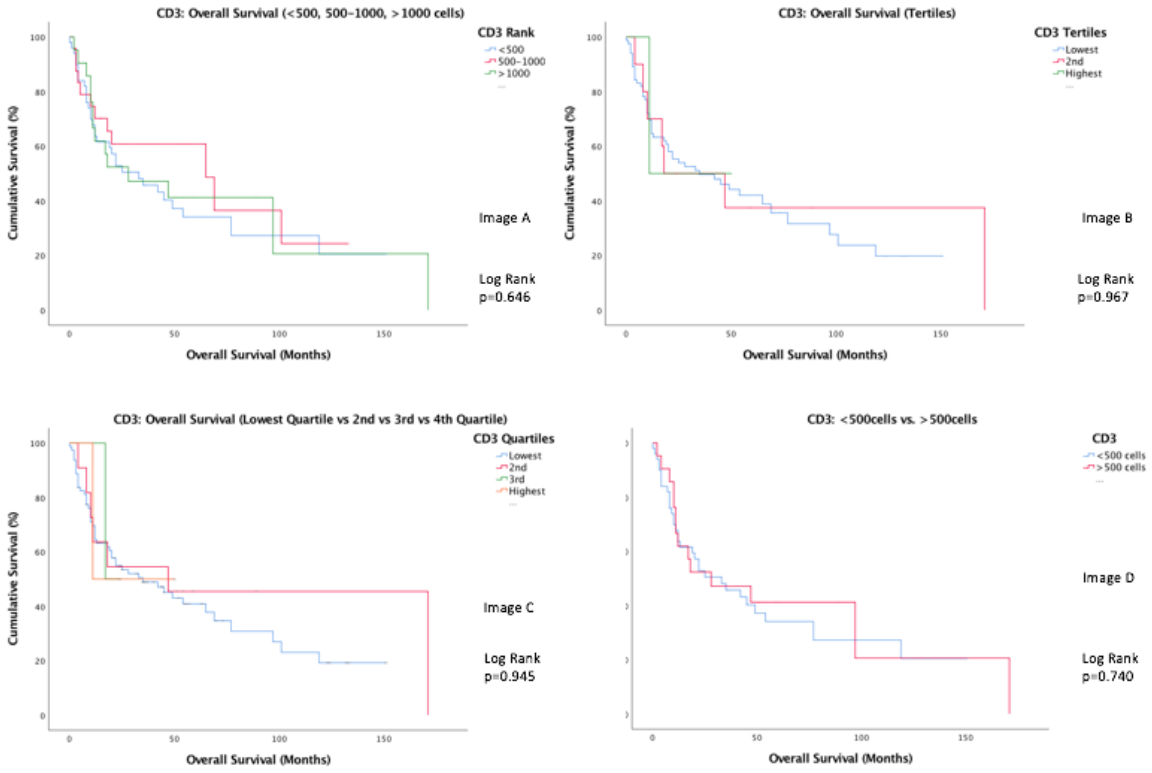


Figure 2-18. Examples of the different ways CD3 cells were divided prior to definitive analysis. Clockwise from top left; Image A (<500 cells, 501-1000 cells, >1001 cells), Image B (CD3 Tertiles), Image C (CD3 Quartiles) and Image D (<500 cells vs ≥500 cells).

Similar assessments were made for succeeding cell cohorts to identify the most statistically significant comparison groups. These were not always comparable, quantitively between Only these are discussed from this point.

For comparison, patients with high and low $CD3^+$ counts were divided numerically above and below 500/mm^2. All 95 cases were available for analysis. For patients with counts of greater than 500 cells, there was a trend towards better outcome, but this was not significant (HRs: OS 0.82, p=0.445, DSS 0.69, p=0.242), see Figure 5-17 and Table 8. Hazard ratios for OS and DSS for potential prognosticators of hypopharyngeal cancer

.

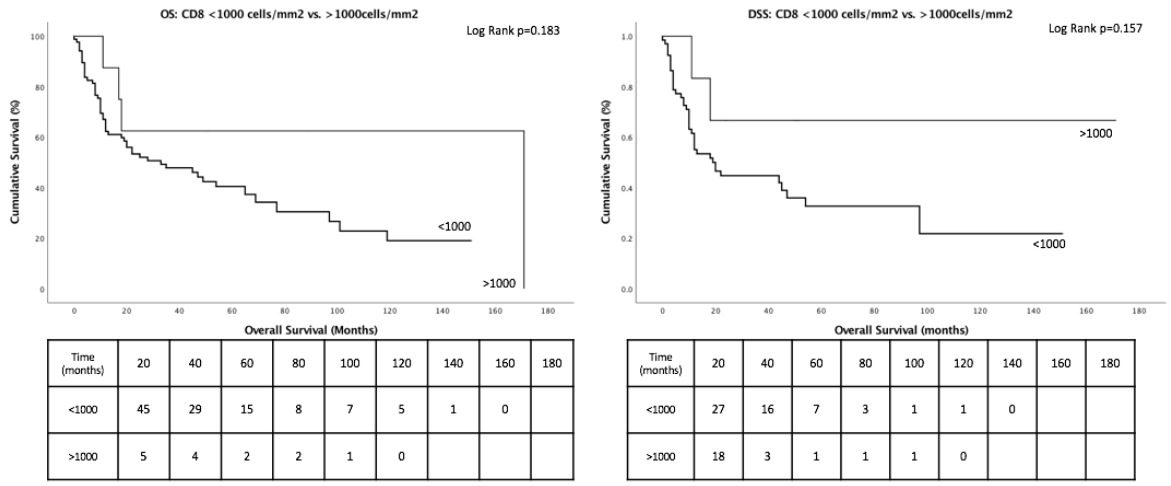
There was also a non-significant trend between high $CD3^+$ cells (OR 2.41 [95% CI 0.67-8.67], p=0.180). This is difficult to explain with current biological principles. Contrarily, fewer $CD3^+$ cells were associated with late recurrence (OR 0.50 [95% CI 0.16-1.60, p=0.243) and second primaries (OR 0.31 [95% CI 0.019-5.15], p=0.416), but these were similarly not significant.

2.6.4.2 $CD8^+$ cell count

92 (96.8%) of 95 cases were available for analysis. $CD8^+$ cell counts were scored for mean counts per mm^2. A positive skew was seen (see Figure 5-21).

$CD8^+$ counts were divided by counts below and above 1000/mm^2. There appeared to be a differentiation between the two groups based on both OS and DSS, favourable for a higher immune infiltrate, but this was not significant (HRs: OS 0.47, p=0.199, DSS 0.38, P=0.181), see Table 8 and Figure 2-19.

As described in 2.6.4.1.1, multiple assessments were made for other comparison groups but none were proven to demonstrate any greater significance than the comparison in Figure 2-19 below (e.g. $CD8^+$ OS Upper vs. Lower tertiles Log rank p=0.198, HR 0.47, p=0.208 [Data not shown]).



Time (months)	20	40	60	80	100	120	140	160	180
<1000	45	29	15	8	7	5	1	0	
>1000	5	4	2	2	1	0			

Time (months)	20	40	60	80	100	120	140	160	180
<1000	27	16	7	3	1	1	0		
>1000	18	3	1	1	1	0			

Figure 2-19. Kaplan Meier Survival Analysis for any $CD8^+$ cell count and survival in patients with hypopharyngeal SCC, left OS (p=0.183) and right DSS (p=0.157).

No association was made for increasing proportions of $CD8^+$ cells and treatment failure (0.99 [95% 0.99-1.00], p=0.485). This was similar for late recurrence (0.64 [95% CI 0.99-1.00], p=0.641) and second primary tumours (1.00 [95% CI 0.99-1.00], p=0.726).

2.6.4.3 $CD4^+$ cell count

84 (96.5%) cases were available for analysis (see Table 7). $CD4^+$ cell counts were scored for mean counts per mm^2. A stem and leaf distribution plot is shown in Figure 5-22. For the purposes of comparison, cell groups were divided into groups of $\leq$750 cells and >750 cells.

There appeared to be a survival benefit in cases of higher immune infiltrate but this was not seen to be significant on hazards testing (HRs: OS 0.81, p=0.527, DSS 0.60, p=0.197), see Figure 5-18 and Table 8.

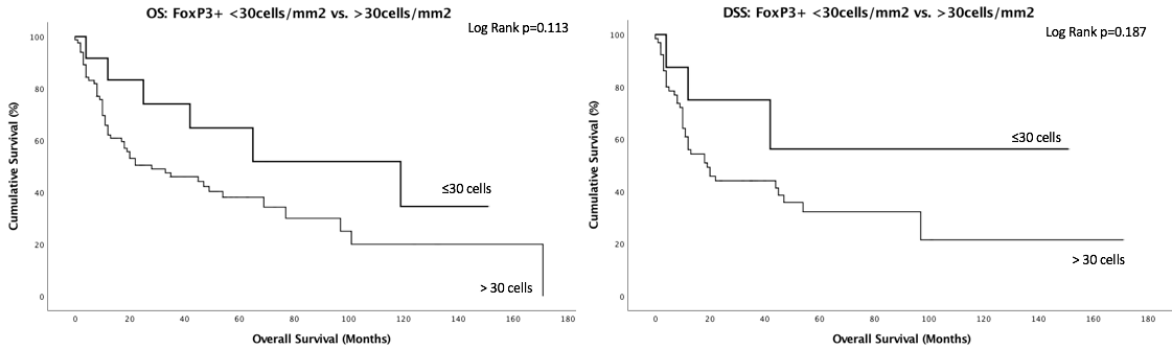
No association was demonstrated for $CD4^+$ cells and treatment failure (OR 1.00 [95% CI 0.99-1.00], p=0.706. This was similar for late recurrence (OR 1.00 [95% CI 0.99-1.00], p=0.427). Data could not be commuted for presence of a second primary.

2.6.4.4 $FOXP3^+$

All 95 cores were available for analysis of $FOXP3^+$ cell populations. $FOXP3^+$ cell counts were scored for mean and median counts per mm^2.

A positive skew was demonstrated on the steam and leaf plot, see Figure 5-23. Cells were divided into cohorts, for counts less than or equal to 30/mm^2 and greater than 30/mm^2. 12 (12.6%) cases were in groups of ≤30/mm^2 and 83 (87.4%) were greater, see Table 7.

Rate of survival was improved in the former cohort though this did not meet significance (HRs: OS 1.89, p=0.143, DSS 2.09, p=0.222), see **Error! Reference source not found.** and Table 8.



Time (months)	20	40	60	80	100	120	140	160
≤30 cells	9	8	5	3	3	2	1	0
> 30 cells	41	26	12	7	5	4	1	0

Time (months)	20	40	60	80	100	120	140	160
≤30 cells	5	4	2	1	0			
> 30 cells	28	16	6	3	2	1	0	

Figure 2-20. Kaplan Meier Survival Analysis for any FOXP3+ cell count and survival, left OS (p=0.113) and right DSS (p=0.187).

There was no association demonstrated for treatment failure (OR 0.73 [95% CI 0.13-3.98], p=0.713). No statistics could be computed for presence of second primary. There was however a non-significant association for FOXP3^{+} cell counts and late recurrence with a smaller infiltration associated with a higher rate of late recurrence (HR 0.39 [95% CI 0.13-1.15], p=0.088), see Figure 2-21 below). This was not particularly unusual, given that low FOXP3^{+} expression has been associated with both positive and negative oncological outcomes to date, for example over-expression demonstrating better outcome in both colorectal(209) and breast cancer(210).

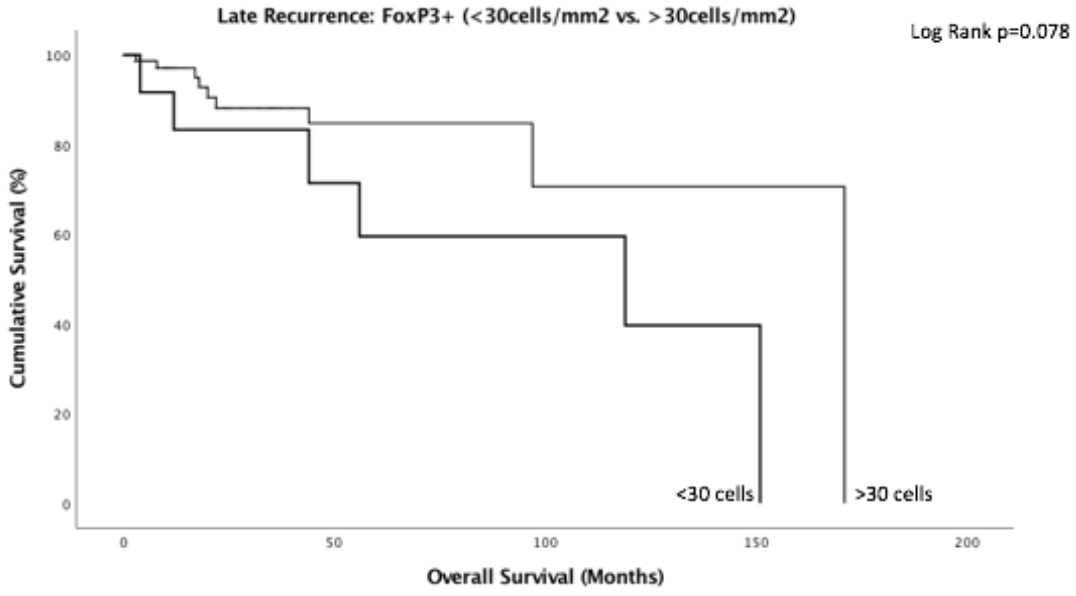


Figure 2-21. Kaplan Meier Survival analysis demonstrating that patients with lower FoxP3+ expression were more likely to develop a late recurrence (Log-rank p=0.078).

2.6.4.5 p16^{INK4a}

p16 ^{INK4a} immunostaining was scored manually and individually from each tumour core by the author. Multiple cores representing the same patient were tallied and averaged for a final count. p16 ^{INK4a} positivity was deemed for all cores staining greater than 70% as shown, see **Error! Reference source not found.**. This is in keeping with best practice internationally (208). If a core demonstrated at least 70% of positive (brown) staining, as exemplified in the image below it was deemed positive.

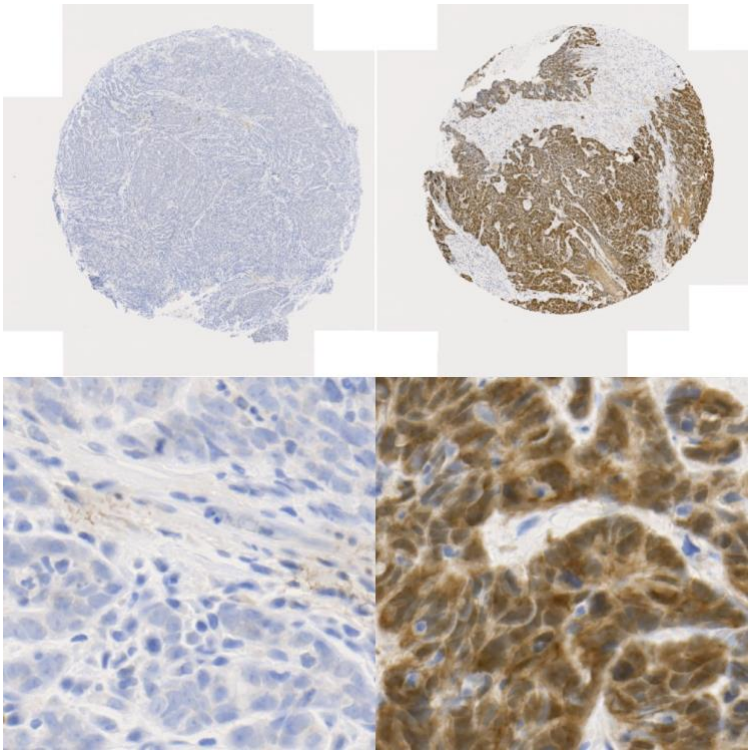


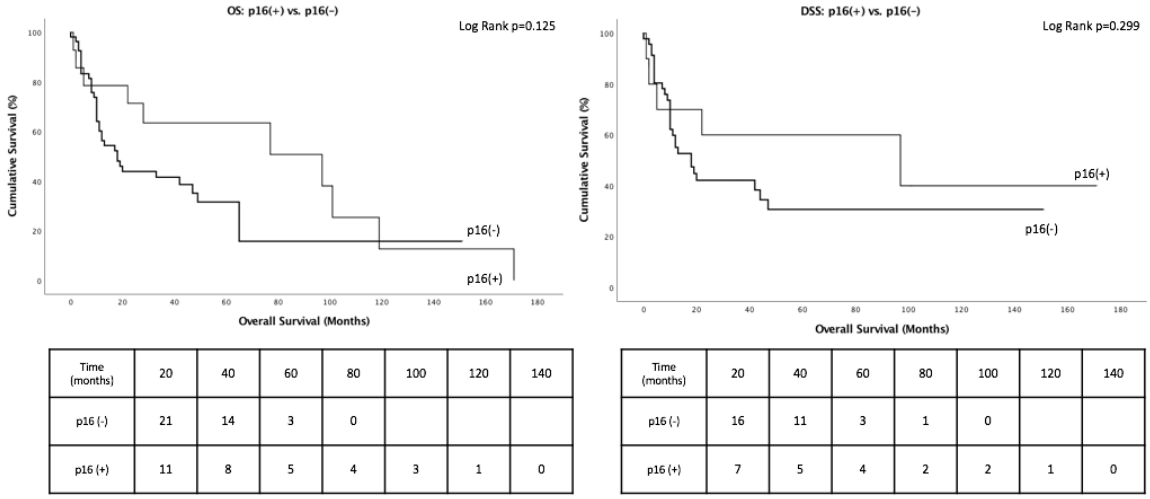
Figure 2-22. Examples of p16 ^{INK4a} /CDKN2A staining negativity and positivity on H&E staining. Top left; tumour core, negative for p16. Top right, tumour core, positive for p16. Bottom left; negative core magnified x20. Bottom right, positive core magnified x20.

p16 ^{INK4a} was positive in 14 (14.7%) of cases and negative in 54 (56.8%). There was no association between p16 and gender (p=0.470, Fisher's Exact Testing) smoking status (p=0.470, Fisher's Exact Testing) or patient age (p=0.422, Chi Square Testing).

Similarly, there was no association of p16 status to treatment failure (p=0.246, Fisher's Exact Testing) or indeed *any* treatment failure, second primary or disease recurrence (p=0.398, Chi Square Testing). Patients with a positive p16 status were less likely to incur a late recurrence (p=0.005, Chi Square Testing).

On Kaplan Meier survival analysis, a positive p16 status was associated with better survival, but this was not proved to be significant (HRs: OS 0.54, p=0.133, DSS 0.60, p=0.311), see Table 8. Hazard ratios for OS and DSS for potential prognosticators of hypopharyngeal cancer

and Figure 2-23.



Time (months)	20	40	60	80	100	120	140
p16 (-)	21	14	3	0			
p16 (+)	11	8	5	4	3	1	0

Time (months)	20	40	60	80	100	120	140
p16 (-)	16	11	3	1	0		
p16 (+)	7	5	4	2	2	1	0

Figure 2-23. Kaplan Meier Survival Analysis for any p16 status and survival in patients with hypopharyngeal SCC, left OS (p=0.125) and right DSS (p=0.299).

There was no association between p16 status and treatment failure (OR 0.31 [95% CI 0.04-2.66], p=0.286). There was a strong association between p16 positivity and late recurrence (OR 6.43 [95% CI 1.61-25.65], p=0.008). There was no association for p16 status and second primary tumours (OR 4.17 [95% CI 0.53 -32.65], p=0.174).

2.6.4.6 α-SMA

All 95 cases were available for SMA analysis. Cases were differentiated by percentage of sample stained with 70% staining equating to a positive stain, see Figure 2-24. 57 (60%) cases tested positive, see Table 7. Table demonstrating cut-offs for analysis for subsequent testing on included hypopharyngeal tumour cores

. Although there was evidence of improved survival in the negative cohort, there were insufficient numbers to draw statistical significance (HRs: OS 1.39, p=0.231, DSS 2.14, p=0.205), see Table 8. Hazard ratios for OS and DSS for potential prognosticators of hypopharyngeal cancer

and Figure 2-25.

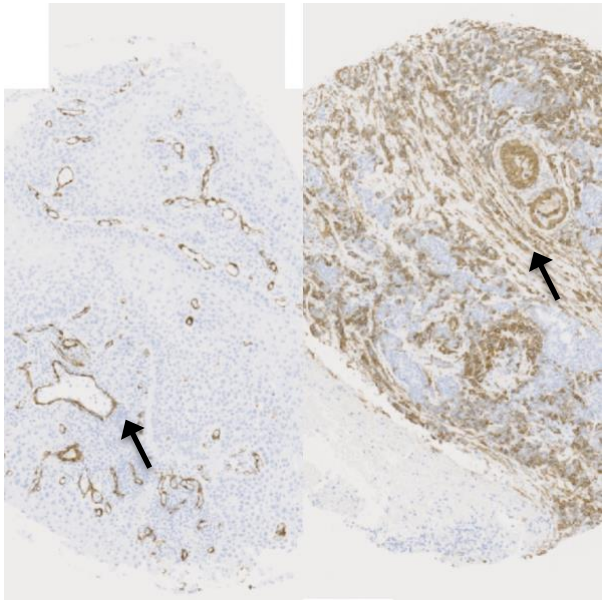
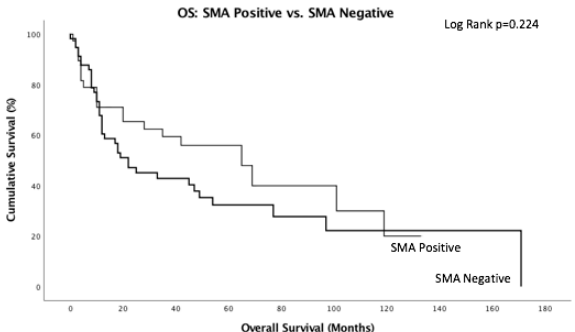
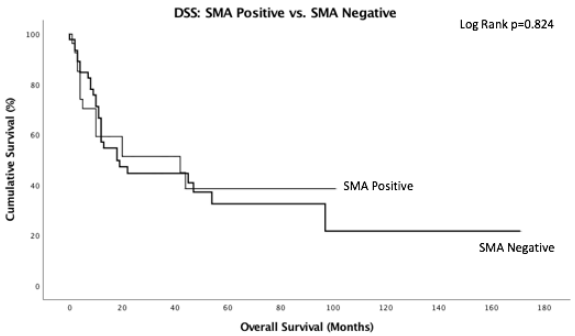


Figure 2-24. Images of SMA cytoplasmic staining in patients with Hypopharyngeal SCC. Left; core, negative for SMA. Staining uptake is centred around smooth muscle vascular cells and does not indicate positivity. Right; core, positive for SMA. Staining uptake in stromal cytoplasm.

There appeared to be an association with SMA negativity and treatment failure, but this did not reach significance (OR 0.31 [95% CI 0.09-1.08], p=0.07. This relationship was reversed for late recurrence (OR 1.72 [95% CI 0.48-4.92], p=0.471) and presence of a second primary (OR 0.91 [95% CI 0.14-5.79], p=0.918).



Time (months)	20	40	60	80	100	120	140	160	180
SMA Positive	23	17	9	4	4	2	0		
SMA Negative	27	17	8	6	4	4	2	1	0



Time (months)	20	40	60	80	100	120	140	160	180
SMA Positive	13	8	3	1	1	0			
SMA Negative	19	12	5	3	2	1	1	1	0

Figure 2-25. Kaplan Meier Survival Analysis for any SMA positivity and survival, left OS (p=0.224) and right DSS (p=0.824).

2.6.4.7 p53 positivity

There were 46 (48.4%) p53 counts available for analysis. p53 positivity was based on a positive stain of 70% per core. On this basis, only 1 (1.1%) core was found to be positive for p53 suggesting a high risk of false negatives. Even allowing for adjusting to a positivity score of 50%, only added

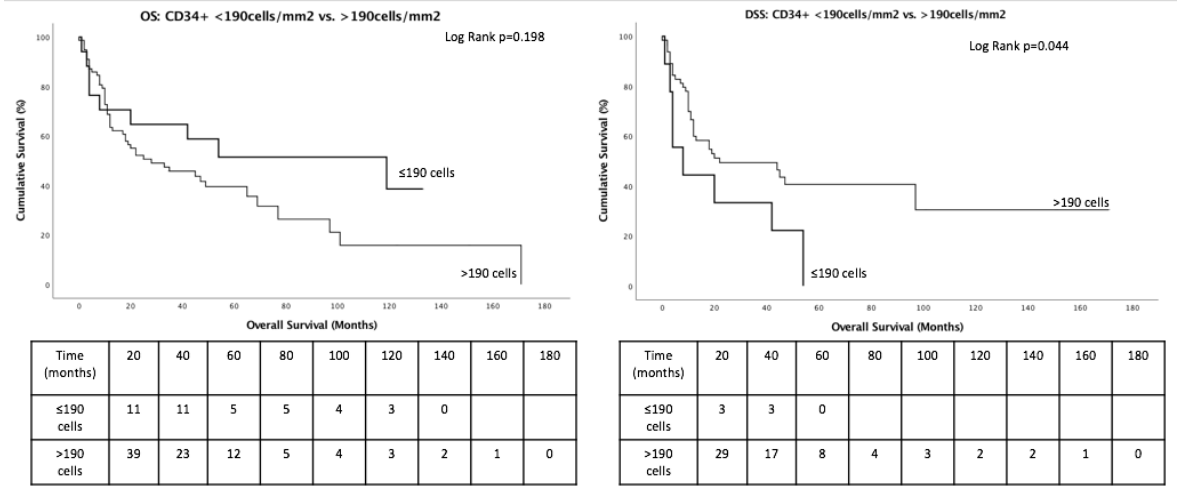
one further positive core. Given the high probability of this being unlikely in a moderately large data set, p53 was not considered for further analysis.

2.6.4.8 CD34⁺ cell count

All 95 cases were available for analysis. CD34⁺ cell counts were scored for mean counts per mm^2. A positive skew to the cell counts was seen, see Figure 5-24.

Survival analysis was performed on the basis of cell counts of ≤190 cell counts/mm^2 and >190 cell counts/mm^2 (see Table 7. Table demonstrating cut-offs for analysis for subsequent testing on included hypopharyngeal tumour cores

). There was a statistically significant difference for counts based on DSS but not OS (HRs: OS 1.89, p=0.206 and DSS 0.47, p=0.05), see Figure 2-26 and Table 8.



Time (months)	20	40	60	80	100	120	140	160	180
≤190 cells	11	11	5	5	4	3	0		
>190 cells	39	23	12	5	4	3	2	1	0

Time (months)	20	40	60	80	100	120	140	160	180
≤190 cells	3	3	0						
>190 cells	29	17	8	4	3	2	2	1	0

Figure 2-26. Kaplan Meier Survival Analysis for any CD34⁺ cell count/mm^2 and survival, left OS (p=0.198) and right DSS (p=0.044).

No association was demonstrated for CD34⁺ cells and treatment failure (OR 0.85 [95% CI 0.16-4.54], p=0.846. There was a significant association demonstrated for decreasing number of CD34⁺ cells and late recurrence (OR 0.23 [95% CI 0.07-0.78], p=0.018). No data was computable for odds of a second primary.

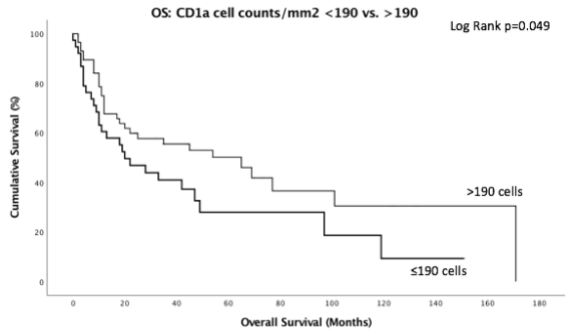
2.6.4.9 CD1a⁺ cell count

58 (61.1 %) of all cases were available for analysis (see Table 7. Table demonstrating cut-offs for analysis for subsequent testing on included hypopharyngeal tumour cores

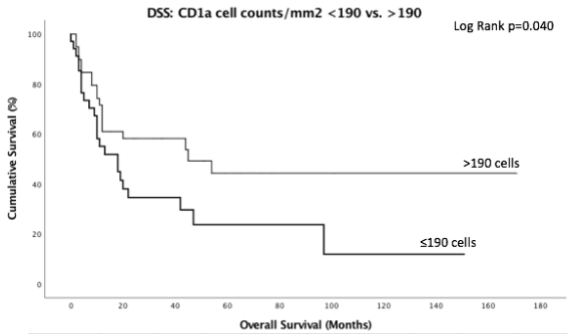
). A positive skew of results was seen on the stem and leaf plot (see Figure 5-25).

There was a significant association for survival based on cell counts above and below 190 cells/mm^2, (HRs: OS 0.59, p=0.054, DSS 0.51, p=0.026), see Table 8 and Figure 2-27. A greater proportion of CD1a$^+$ cells/mm^2 was associated with an increased rate of both OS and DSS.

No association was demonstrated for CD1a$^+$ cells and treatment failure (OR 0.76 [95% CI 0.22-2.61], p=0.667, late recurrence (OR 0.71 [95% CI 0.23-2.15], p=0.018) or presence of a second primary (OR 2.07 [95% CI 0.33-13.18]).



Time (months)	20	40	60	80	100	120	140	160	180
≤190 cells	19	11	3	3	2	0			
>190 cells	32	23	14	7	6	5	1	0	



Time (months)	20	40	60	80	100	120	140	160	180
≤190 cells	11	7	2	2	1	0			
>190 cells	21	13	6	2	2	1	1	0	

Figure 2-27. Kaplan Meier Survival Analysis for any CD1a cell count/mm^2 and survival, left OS (p=0.049) and right DSS (p=0.040).

		Cohort Total (n=95)
		Number (%)
p16	Negative Positive (70%) Unknown	54 (56.8%) 14 (14.7%) 27 (28.4%)
p53	Negative Positive (70%) Unknown	45 (47.3%) 1 (1.1%) 49 (51.6%)
CD3⁺ Rank	≤500 immune cells/mm^2 >500 immune cells/mm^2	50 (52.6%) 45 (47.4%)
CD8⁺ Rank	≤1000 immune cells/mm^2 >1000 immune cells/mm^2 Unknown	86 (90.5%) 8 (8.4%) 1 (1.1%)
CD4⁺ Rank	≤750 immune cells/mm^2 >750 immune cells/mm^2 Unknown	72 (75.8%) 20 (21.1%) 3 (3.20%)
CD4⁺CD25⁺FoxP3⁺	≤30 immune cells/mm^2 >30 immune cells/mm^2	12 (12.6%) 83 (87.4%)
SMA	Negative Positive	38 (40.0%) 57 (60.0%)
CD34⁺ Rank	≤190 fibroblast cells/mm^2 >190 fibroblast cells/mm^2	17 (17.9%) 78 (82.1%)
CD1a Rank	≤150 dendritic cells/mm^2 >150 dendritic cells/mm^2 Unknown	38 (40.0%) 20 (21.1%) 37 (38.9%)

Table 7. Table demonstrating cut-offs for analysis for subsequent testing on included hypopharyngeal tumour cores

		OS		Disease Specific Survival	
		HR (95% CI)	*Significance*	*HR (95% CI)*	*Significance*
p16⁺	*No*	1		1	
	Yes	0.54 (0.24-1.21)	p=0.133	0.60 (0.22-1.61)	p=0.311

		OS		Disease Specific Survival	
		HR (95% CI)	*Significance*	*HR (95% CI)*	*Significance*
p53⁺	*No*	1		1	
	Yes	0.64 (0.37-1.12)	p=0.118	0.87 (0.47-1.61)	p=0.653
CD3⁺ cells/mm²	*≤500 cells*	1		1	
	>500 cells	0.82 (0.48-1.38)	p=0.445	0.69 (0.38-1.28)	p=0.242
CD8⁺ cells/mm²	*≤1000 cells*	1		1	
	>1000 cells	0.47 (0.15-1.49)	p=0.199	0.38 (0.09-1.57)	p=0.181
CD4⁺ cells/mm²	*≤750 cells*	1		1	
	>750 cells	0.81 (0.42-1.57)	p=0.527	0.60 (0.28-1.30)	p=0.197
SMA	*Negative*	1		1	
	Positive	1.39 (0.81-2.41)	p=0.231	2.14 (0.66-6.92)	p=0.205
CD4⁺CD25⁺ FoxP3⁺	*≤30 cells*	1		1	
	>30 cells	1.89 (0.81-4.45)	p=0.143	2.09 (0.64-6.89)	p=0.222
CD34⁺ cells/mm²	*≤190 cells*	1		1	
	>190 cells	1.59 (0.77-3.30)	p=0.206	0.47 (0.22-1.01)	p=*0.05*
CD1a cells/mm²	*≤150 cells*	1		1	
	>150 cells	0.59 (0.36-1.01)	p=*0.054*	0.51 (0.28-0.92)	p=*0.026*

Table 8. Hazard ratios for OS and DSS for potential prognosticators of hypopharyngeal cancer

2.6.5 Associations between Immune cells

Correlations between CD34⁺ cell counts and other immune cells were made and are illustrated in Table 9. Relationships between CD34+ levels and SMA and p16 status, CD3+, CD4+ and CD8+ cells in hypopharyngeal carcinoma (n=95)

. No significant association was drawn for CD4⁺ counts (p=0.186) or CD8⁺ counts (p=0.352) or p16 status (p=0.100). 52.6% of tumours positive for SMA status, were also positive of CD34⁺ status and this approached significance (p=0.07). The association between an increase in CD34⁺ cells and

SMA positivity status was not anticipated, given the tendency in the literature to show a reduction of $CD34^+$ cells with an increase of SMA positivity (188).

		CD34 cell count Number (%)		**Significance**
		≤190 cells	*>190 cells*	
α-SMA Status	*Negative*	10 (10.5%)	28 (29.5%)	*p=0.07*
	Positive	7 (7.4%)	50 (52.6%)	
CD3 Status	*≤500 cells*	12 (12.6%)	38 (40.0%)	p=0.085
	>500 cells	5 (5.3%)	40 (42.1%)	
CD4 Status	*≤750 cells*	15 (17.4%)	71 (82.6%)	p=0.186
	>750 cells	2 (2.1%)	7 (7.4%)	
CD8 Status	*≤1000 cells*	12 (13.8%)	68 78.2%)	p=0.352
	>1000 cells	2 (2.29%)	5 (5.74%)	
p16 Status	*Negative*	6 (8.8%)	48 (70.6%)	p=0.100
	Positive	4 (5.9%)	10 (14.7%)	
p53 status	*Negative*	3 (6.5%)	42 (91.3%)	p=0.789
	Positive	0 (0%)	1 (2.2%)	

Table 9. Relationships between CD34+ levels and SMA and p16 status, CD3+, CD4+ and CD8+ cells in hypopharyngeal carcinoma (n=95)

2.6.6 Immune cells as Prognosticators in Hypopharyngeal Carcinoma

To establish the merits of using immune cell counts as prognosticators in hypopharyngeal carcinoma receiver operating characteristic (ROC) curves were fashioned, using both overall and 3-year survival as the dependent variable (see Figure 5-26). Only CD1a+ positivity demonstrated any degree of association. These are discussed further in Appendix K.

2.6.7 Distribution of Immune cells in hypopharyngeal cancer

For all counts above, no adjustment was made for location of tumour infiltrating lymphocytes within cellular tissue. Importantly, tumoural tissue was not found to be infiltrated with $CD3^+$ cells in our study. More commonly, if present, $CD3^+$ cells were much more likely to be located within

the core stroma as opposed to the tumour (see Figure 2-28), which has been proven to be associated with survival in a myriad of tumours, including oropharyngeal malignancy (145). When looking at a single dataset (Poole), 50% of all cores demonstrated intra-stromal infiltration, with 38.9% demonstrating either no infiltration or an infiltration that appeared non-specific in that it did not follow a particular intra-tumoural or intra-stromal distribution. Only 11.1% demonstrated intra-tumoural infiltration. This was similar for $CD8^+$ cells also (see Figure 2-29). In the study by Ward et al. looking at oropharyngeal TIL, only tumour islands were used for analysis, ignoring staining located within the stroma(22).

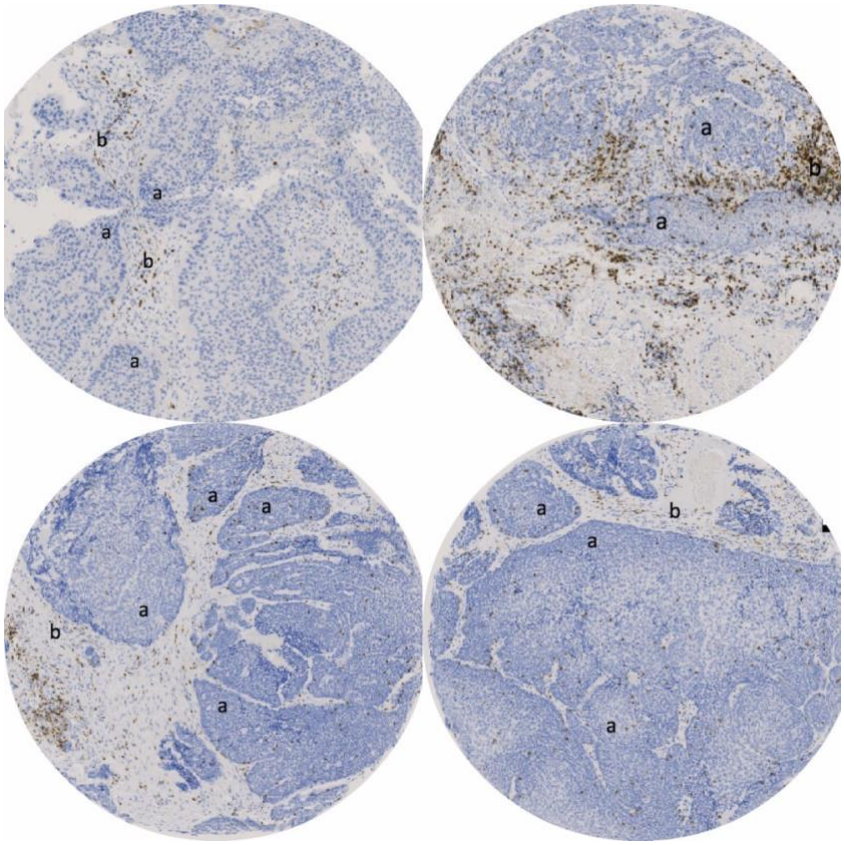


Figure 2-28. TMA images of CD3 cores, demonstrating distribution of immune cells in hypopharyngeal cancer. a: tumour nests enclosing cells with squamous cell carcinoma, b: stroma

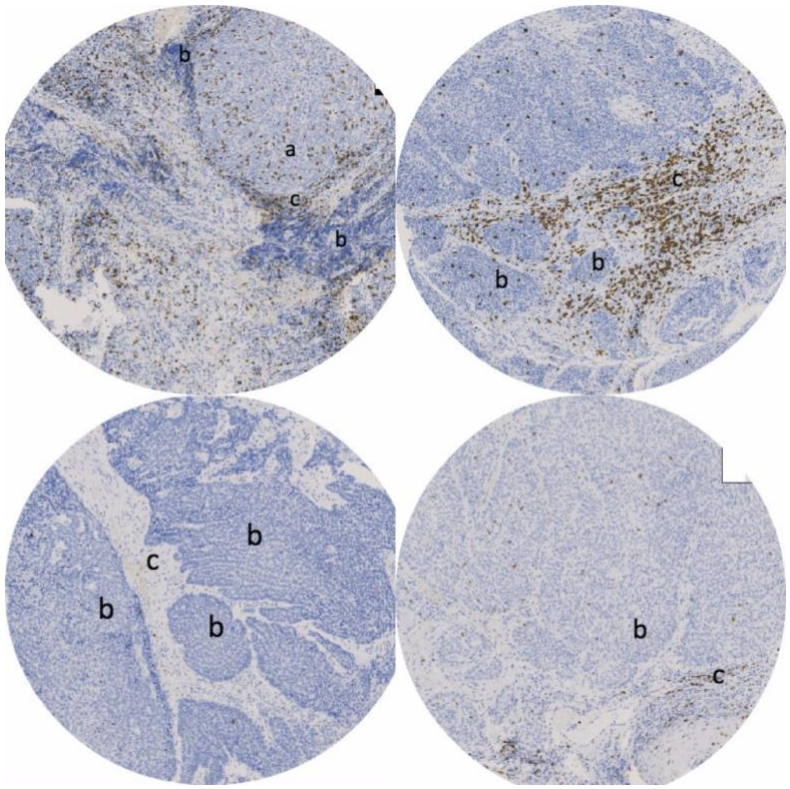


Figure 2-29. TMA images of cores stained for $CD8^{+}$, demonstrating distribution of immune cells in hypopharyngeal cancer. a: immune cells, b: tumour nests enclosing cells with squamous cell carcinoma, c: stroma.

Examples of immune stain distributions are given in the figure below but were suitable only for a qualitative analysis (see Figure 2-30).

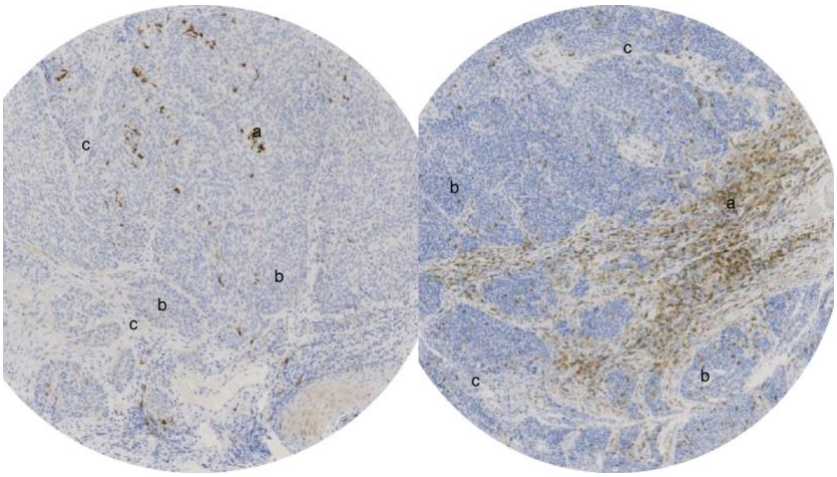


Figure 2-30. Right, CD4 stain. Left CD1a stain. Spatial distribution of immune cells in hypopharyngeal cancer. a: immune cells, b: tumour nests enclosing cells with squamous cell carcinoma, c: stroma.

2.6.7.1 The role of the tumour milieu in hypopharyngeal cancer

A so-called stromal reaction (infiltration with inflammatory cells) is said to hold a significant role in both the diagnosis and prognosis of malignant tumours (211) and a strong stromal reaction was

observed for the greatest majority of our specimens. Cell positivity to SMA was seen to demonstrate some association with poor OS, but this did not approach significance (p=0.205), see Table 8. Hazard ratios for OS and DSS for potential prognosticators of hypopharyngeal cancer

. Examples of negatively staining $CD34^+$ cores are demonstrated in Figure 2-31 with their corresponding negatively staining SMA cores. Only endothelium is seen to test positive (commonly used as a control) and $CD34^+$ expression is lost in the stroma.

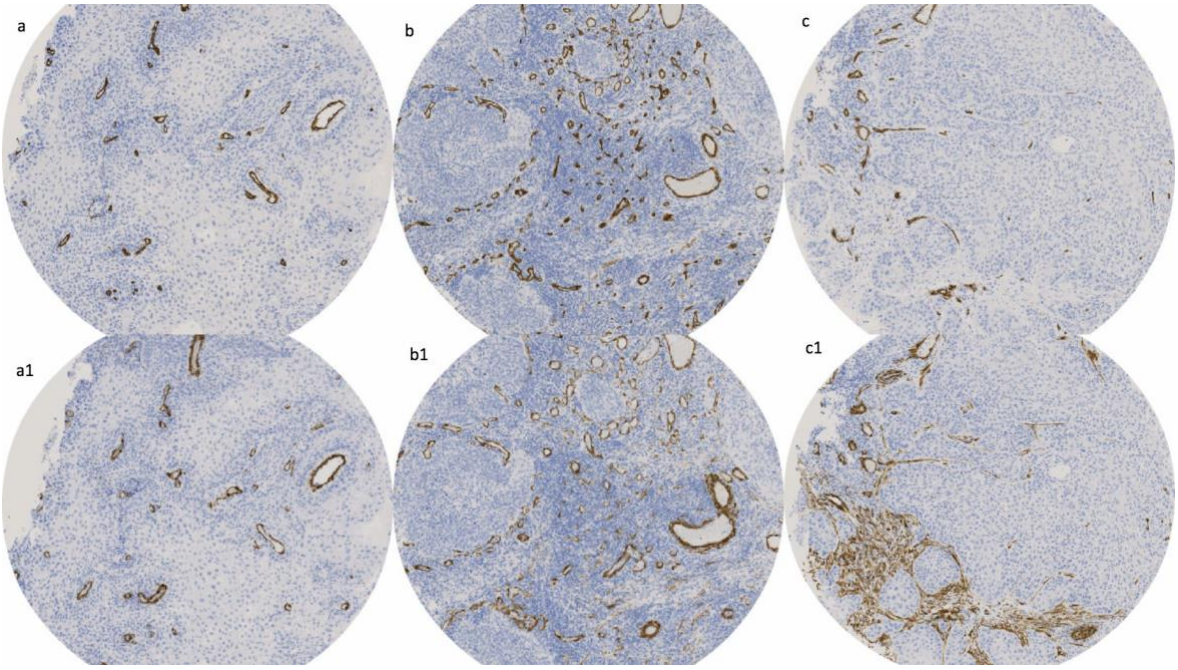


Figure 2-31. Immunohistochemichal images of negatively staining $CD34^+$ and SMA cells. Above - examples of negatively staining $CD34^+$ cores and their corresponding SMA cores below. Largely it followed that negatively staining $CD34^+$ cores would score negatively for SMA infiltration. Exceptions to this are shown in c and c1, demonstrating a negative $CD34^+$ infiltrate in the stroma but a corresponding positive SMA infiltrate.

A single exception was seen in case c and c1 (see Figure 2-31). Although evidence of $CD34^+$ endothelial staining was seen as per the other examples, SMA infiltration was diffusely present on the succeeding stain, possibly supporting the story for desmoplasia – i.e. CD34 rich cores being associated with tumour protection and a loss of CD34 expression (with subsequent replacement by SMA expression) suggesting neoplasia.

Cores that were positive for CD34 demonstrated both SMA positivity (see b and b1 and c and c1, Figure 2-32) and SMA negativity (see a and a1, Figure 2-32). Cross tabulation demonstrated an association between SMA and CD34 (p=0.07), see Table 9. Relationships between CD34+ levels and SMA and p16 status, CD3+, CD4+ and CD8+ cells in hypopharyngeal carcinoma (n=95)

, with the greatest proportion of SMA positive cells also demonstrating a high immunostain for $CD34^+$ cells (52.6%). The single largest observation otherwise was for CD34 positivity and SMA negativity (29.5%), underlining the importance of a high infiltration of $CD34^+$ cells and a higher survival rate (see Figure 2-26).

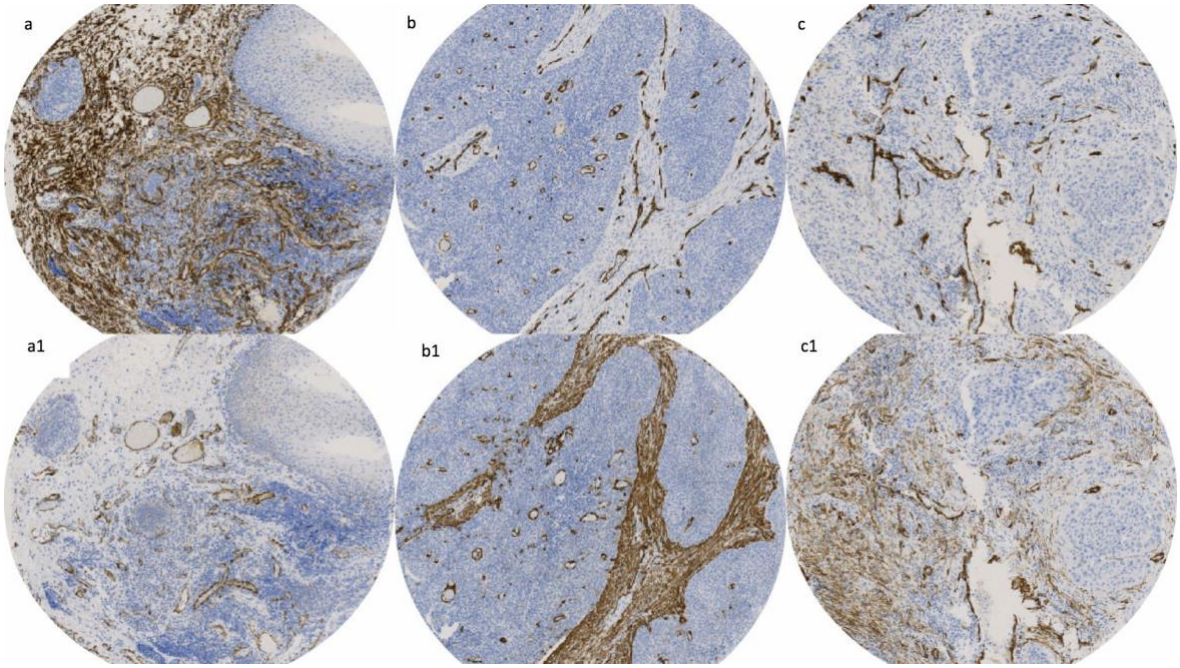


Figure 2-32. Examples of positive $CD34^+$ stains (above) and their corresponding SMA (below) counterstains.

2.7 Discussion

This study aimed to identify the role of the immune system in hypopharyngeal cancers, namely whether immune infiltration into the tumour milieu was a discriminator in predicting patient outcomes.

2.7.1 Hypopharyngeal cancer and $p16^{INK41}$ status

p16, a tumour suppressor protein integral to cell cycle regulation, has been demonstrated as a surrogate for HPV status in cases of OPSCC (208). In our study, patients with hypopharyngeal carcinoma were more commonly found to be p16 negative (56.8%), with no relationship to either treatment or survival outcomes (OS p=0.113, DSS p=0.311). No association was established on multivariate analysis. Although other datasets have also shown similar outcomes, we were not expecting such a large proportion to be p16 positive (200, 212). Of note, it has been shown that patients with hypopharyngeal cancer that are both HPV positive with DNA and p16 status, are associated with a better survival (173).

Furthermore, our dataset showed a significant association between p16 positivity and incidence of second primary tumours (OR 6.43, p=0.008). However, the large confidence interval (95% 1.61-25.65) likely reflects the small sample size and caution should be exercised in its interpretation.

Importantly, due to its retrospective nature, this study was not able to correlate the presence of HPV DNA and p16 over-expression, which in conjunction, can more accurately identify the presence of HPV infection. This was not possible within the context of this study due to the lack of availability of fresh tissue for analysis. It should be noted however; PCR amplification of a positive HPV DNA result will only demonstrate the presence of infection and does not definitively imply its role in carcinogenesis.

2.7.2 Does immune infiltrate affect outcome in hypopharyngeal carcinoma?

It is likely our dataset was not big enough to identify any significant associations between outcome and immune infiltrate. Specifically, total number of T cells ($CD3^+$), did not demonstrate a significant link with survival (OS, p=0.445 and DSS, p=0.242), treatment failure (p=0.180), tumour recurrence (p=0.243) or second primaries (p=0.416). Importantly however, increased numbers of $CD3^+$ cells were linked with a reduced HR (see Table 8. Hazard ratios for OS and DSS for potential prognosticators of hypopharyngeal cancer

), which fits with other cancers biologically; unfortunately, this was not significant as the dataset was too small. This was repeated throughout the dataset, with $CD4^+$, $CD8^+$ and FOXP3+ cells all demonstrating no or at best, a weak association.

There was a link between $CD1a^+$ cells, (dendritic cells) and survival, with an increased proportion of $CD1a^+$ cells associated with a 50% reduction of both OS (p-0.054) and DSS (p=0.026), see Table 8. Hazard ratios for OS and DSS for potential prognosticators of hypopharyngeal cancer

. Again, these data support the fact that hypopharyngeal cancers, consistent with other solid tumours (186, 213) (183) could possibly be linked with an improved survival with a dense immune infiltrate.

Tumour specific responses have been demonstrated in many human cancers (145, 183, 186, 213). Within the head and neck they have been shown peri-tumourally in tongue carcinoma (184) and nasopharyngeal carcinoma (214). Distribution is key and tumours with a greater population of tumour infiltration appear to show a greater tendency for loco-regional metastasis than those with peri-tumoural cells (213). Where dendritic cells have been researched more extensively, e.g. breast cancer, there is considerable evidence to support that not only is invasive neoplasia associated with a frail anti-tumour response but that this may be down to dendritic cell function

and peri-tumoural location (183, 215). Within the context of this study, there did not appear to be a clear and consistent distribution of immune cells. Further, there was no qualitative evidence to suggest a strong intra-tumoural infiltration.

Although no significant association was shown for $FOXP3^+$ cells and hypopharyngeal cancer outcome, there was a relationship between a higher proportion of regulatory cells and worse survival (OS 1.89, p=0.143 and DSS 2.09, p=0.222). As a marker of regulatory immune cell function, this is in keeping with what is known of $FOXP3^+$ cells in human cancers, i.e. increasing expression is associated with immunosuppression and metastatic spread (178, 179, 216).

Of note, smaller populations of $FOXP3^+$ cells were associated with an 80% reduction in late recurrence (p=0.002). This again supports the role of regulatory immune cells in hypopharyngeal cancer. Understanding what the difference is in between the $FOXP3^+$ cell used to regulate autoimmunity and those expressed/mutated in cancer cells is key in the future, particularly if $FOXP3^+$ expression is to be used as an immune-target.

In summary, there is some evidence to suggest a relationship between hypopharyngeal cancer and immune cells. This is most clear in $CD1a^+$ and $FOXP3^+$ cell types, but some associations are demonstrated elsewhere also. The lack of a strong pattern may itself represent the fact that hypopharyngeal cancer is not typically an immunogenic tumour.

2.7.3 A prognostic model for hypopharyngeal carcinoma

Likelihood ratios were calculated to indicate whether there were any markers in the dataset that might indicate means of stratifying hypopharyngeal patients, namely, those who were more likely to die overall and at 3 years. For OS and 3-year models, only one test approached significance and this was for $CD1a^+$ (p=0.083).

$CD1a^+$, a confirmed marker for dendritic cell activity has not been demonstrated as an immune marker in the literature, in the context of hypopharyngeal cancer. $CD1a^+$ cells are central in antigen presentation and have been shown to correlate with outcome in many different sub-types of human cancer, including but not limited to breast, colon, cervix, oesophagus, thyroid and salivary gland. In nearly all of these, although the mechanism is unclear, an increase in the number of dendritic cells (indicated by $CD1a^+$ count) have been associated with an increase in rate of survival.

2.7.4 Limitations of Study

The study originally aimed to accrue approximately 300 patients, with allowances for the loss of 50-100 patients secondary to failure to collect data, failure to gather tissue or problems with post inclusion analysis. The final dataset however listed 95 patients, somewhat lower than expected. A larger study may have contributed a more meaningful dataset to identify the presence or absence of clinic-pathological associations and a statistically meaningful result.

It is noteworthy that there was a very poor immunohistochemical response for p53 positivity in this study (only 1 was found to be positive) and this may represent the underlying method of antibody concentration used and subsequent antigen retrieval. It is acknowledged that, in the case of head and neck cancer, while p53 mutations are common and may be useful in tailoring therapeutic approaches, there is contention as to whether p53 immunohistochemistry accurately reflects true p53 mutations and certainly may under or over play the mutational p53 load in studies(217).

For the purposes of immune cell distribution, a multiplex immunolabelling analysis may have been useful to objectively identify specific cells types and make more meaningful inferences regarding tumour cell patterns in hypopharyngeal cancer. Unfortunately, in the context of this study, there was no access to spectrometry, microscopy and immunofluorescence expertise and so this was therefore not undertaken. In the future use of multi-spectral imaging to identify T-cell sub-populations and their potential interactions with the mesenchymal stroma of hypopharyngeal cancer would be prudent.

2.8 Conclusion

Hypopharyngeal cancer shows some evidence of being a tumour influenced by immune cell infiltration. Increasing proportions of immune cells were shown to show some positive relationship for survival and this was particularly the case for $CD1a^+$ cells which demonstrated significance; patients with higher $CD1a^+$ counts demonstrating improved survival, patients with higher $FOXP3^+$ infiltration, demonstrating reduced survival.

In the future, further studies could be performed to address some of the more noteworthy outcomes found within this study, namely; the association between patients with low $FOXP3^+$ expression and higher rate of tumour recurrence and the stromal relationship between $CD34^+$ expression and subsequent SMA, in that the hypopharyngeal outcomes, may possibly be decided based upon stromal reaction entirely and not in tumour islands .

A prospective study would allow for accurate selection of patients and data acquisition. Further work is required to elucidate whether contemporary immunotherapies may have a role in the management of recurrent or metastatic hypopharyngeal carcinoma.

Chapter 3: The prognostic impact of nodal metastasis in the HPV era of Head and Neck cancer

3.1 Introduction and Hypothesis

Cancer is thought to be a systemic disease and the TNM scale is used to indicate a patient's prognosis, with higher tumour staging corresponding to a poorer prognosis. Fisher's *systemic* suggests that a primary tumour exists independently, after which point it cedes tumour, which can spread regionally or distally, with no predilection for either (218). Treatment of cancer locally therefore is thought to only control local disease, with distant seeding of tumour having already occurred.

This however does not support what is understood about cancer treatment clinically, in that loco-regional control is known to prolong both DFS and OS. In the VA Laryngeal Cancer Study a multi-centre randomised trial described in Section 1.5.3, patients with Stage III and IV laryngeal disease were randomised to ablative laryngectomy with post-operative RT or 3 cycles of cisplatin and 5-FU chemotherapy, succeeded by RT (115). On three years of follow up, 68% of patients were seen to survive in both groups, suggesting that local control was as efficacious as systemic treatment (115).

Hellman's spectrum theory contrarily combines the two above theories, with an idea of indefinitely circulating tumour cells. In this theory, lymph node metastasis *is* of prognostic importance (219). Cervical lymphatic disease, not only acts as a second source of potential metastasis, but also alludes to the underlying tumour biology, i.e. its aggressiveness. This follows contemporary management – cancer must be ablated entirely, both at the primary site and loco-regionally so to prevent further metastasis and prolong survival overall (220).

Lymph node metastasis in HNSCC is common, seen in approximately 50% of patients at the time of presentation (221, 222). Its presence, in keeping with other solid body tumours, is usually indicative of a poor prognosis for the patient (223, 224).

By contrast, distant metastasis is rare (see Figure 3-1). Of 73,247 patients reviewed from the SEER database, only 2,066 (2.82%) were found to have distant metastasis on diagnosis (225). The rate of metastasis was highest for tumours of the hypopharynx at 6.17% and lowest for the lip, being only 0.33% (225). Risk of metastases at first presentation was correlated to size of primary

tumour, with most T4 tumours (of all sub-sites) having an increased risk of distant metastasis (225).

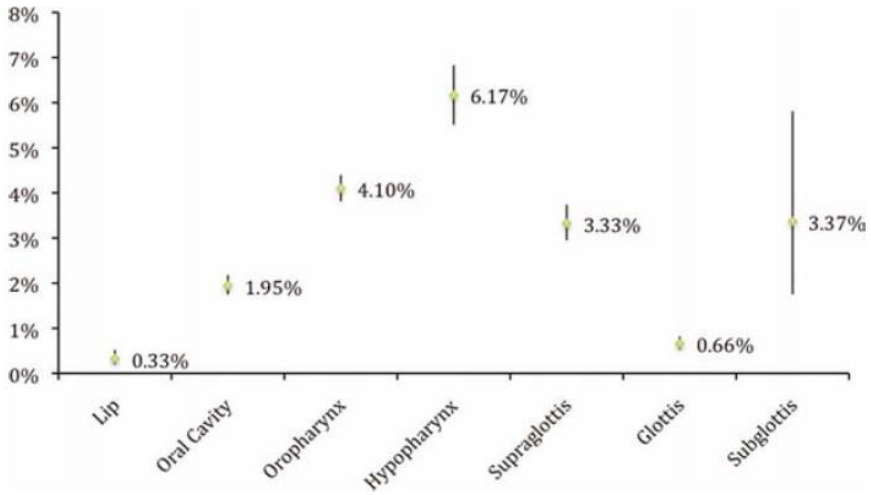


Figure 3-1. Primary site and rate of distant metastasis at time of presentation. Taken from (225)

Today, around 30% of HNSCC cases are caused by human papillomavirus (HPV), with the majority of these tumours arising in the oropharynx. These virally driven tumours demonstrate a natural history unique to that of HPV-independent disease (16, 224, 226). HPV(+) patients often present with smaller primary tumours but advanced nodal status (224). Although the UICC TNM system categorises these cases as advanced stage (based on nodal metastasis as opposed to tumour size), most HPV(+) patients respond well to treatment, with a 5-year survival of around 85% (227). This contrasts with a 5-year survival of approximately 50% in HPV(-) cases (10).

Extranodal extension (ENE) is also a negative prognosticator in HPV(-) disease (154). In current practice, its presence typically triggers intensification of adjuvant treatment in both HPV(+) and HPV(-) disease (117, 228). Although there are data supporting additional treatment in HPV(-) cases(229), few datasets have suitably examined the importance of either node number or ENE in HPV(+) tumours (230, 231).

Overall, it is now clear that patients with HPV(+) oropharyngeal disease have a demonstrably better oncological outcome than those with HPV(-) (22). This is despite the natural history of the disease, which clinically presents with large bulky loco-regional disease. Based upon the previous TNM v.7 clinical staging system, this would be “up” staged, to confer a more aggressive tumour, therefore requiring a more aggressive or invasive treatment course. Today however, it is understood that HPV(+) oropharyngeal disease is a distinct entity from its HPV(-) counterpart. We hypothesise that the TNM version 7 staging system will be inappropriate for stratifying patients with HPV(+) disease.

This study is a retrospective review of HNSCC cases undergoing surgical neck dissection (ND) and thereby using pathology data. It includes HPV(+) and (-) disease and assesses the differences between these two cohorts with regard to pathological stratifiers linked with survival.

3.2 Materials and Methods

3.2.1 Collection of Cohort

Ethical approval was obtained as part of a previous study by Ward et al (22); (UKCRN 8130; ISRCTN 71276356; and REC references 09/H0501/90 and 07/Q0405/1). This existing database, based on patients treated for HNSCC, was expanded to include the relevant clinical information required for completion of this study. Information from Ward et al. (22) that pre-existed and new information sought is detailed further in Appendix D.

This database was a cohort of patients with a diagnosis of OPSCC who were treated between 1st January 2000 and 31st December 2010. Patients were identified using MDT lists of HNSCC diagnoses from Poole Hospital Foundation Trust and University Hospital NHS Foundation Trust. This was accessed via two methods; from locally based MDT Co-ordinators with accompanying assistance from nursing practitioners at each site respectively and via retrospective hospital archival case note reviews. In all cases, patient case notes were reviewed, alongside confirmation using Electronic Patient Records (EPR), and pathology and imaging reporting systems; eQUEST and PACS (Picture Archiving and Communication Systems).

662 patients diagnosed with HNSCC in South England between 2000-2010 were retrospectively identified using this approach. 532 (80.4%) of these patients were treated at Southampton General Hospital and 130 (19.6%) at Poole Hospital Foundation Trust. HPV-status was determined using a combination of p16 immunohistochemistry (based on strong staining in >70% of tumour cells) and HPV *in-situ* hybridisation (based on nuclear staining in tumour cells). Tumours positive for both were confirmed as HPV(+).

131 patients were given the status of HPV(+) and 531 HPV(-). Of these, 63 and 262 patients did *not* undergo ND in the HPV(+) and HPV(-) group respectively and were excluded from further analysis. Lymph nodes status was taken retrospectively from pathology reports of ND.

3.2.2 Ethical Approval and Patient Anonymity

As described, ethical approval was sought and approved within the context of a previous study by Ward et al. ((UKCRN 8130; ISRCTN 71276356; and REC references 09/H0501/90 and

07/Q0405/1)(22). Within the auspices of this work, the same pathology reports that were accessed for the original work were re-accessed and included no additional or unnecessary information. In keeping with the previous work, the patients remained link anonymised, in that once included in the study, there was no patient specific information. A study ID was used to correlate patient and clinical information to additional pathological data.

3.2.3 Statistical Analysis

The primary end point was OS (OS), defined as time from initial diagnosis of malignancy to death, related to any cause. Disease specific survival (DSS) was also used and defined as time from date of diagnosis to date of death from malignant disease. Patients still alive at date of last contact were censored at this point.

Categorical data were cross-tabulated and assessed using Fisher's exact test (for 2x2 tables) or Chi-squared test (for all other tables) as appropriate. For continuous data, an independent *t* test or Mann-Whitney test was used depending on whether data was normally distributed (e.g. age at diagnosis) or not (e.g. length of follow up).

Survival analysis was plotted used Kaplan Meier charts with log-rank analysis. Cox's proportional hazards was used to predict hazard ratios. For HPV(-) cases, multivariate analysis controlled for UICC disease stage, smoking status and age. For HPV(+) disease only TNM tumour stage (T stage), smoking status and age was used contrarily. This was in response to data published previously demonstrating that while overall disease stage on the previous staging system of TNMv7 does not adversely reflect HPV(+) prognosis, T stage does have an effect (224). The contemporary TNMv8 however does make this differentiation and can distinguish between Stage I-II and II-IV.

Smoking status was attributed a binary stratum, being 0 (non-smoker, ex-smoker, smoker <10 pack years) and 1 (smoker of >10 pack years), given categories to be related significantly to OS (118).

All statistical tests were two sided and a significance of p=0.05 was assumed. Analysis was performed by the author, using SPSS 23.0 for Macintosh (IBM, Armonk, NY, USA).

3.3 Results

Patient clinical characteristics are given in Table 10 and Table 21. The former represents cases where neck dissection was performed as a primary measure (Table 10) and the latter for salvage cases only (Table 21, seen in Appendix L). This differentiation was made to acknowledge the likely differing natural histories of patients where ND formed part of their initial treatment and for

cases where patients had failed primary treatment or recurred. However, for all subsequent analyses, both primary and salvage cases were considered together, to preserve patient numbers for statistical analysis.

	HPV Positive Patients (n=41)	**HPV Negative Patients (n=218)**	**Statistical Significance**
Gender			
Male	31 (75.6%)	140 (64.2%)	*0.158*
Female	10 (24.4%)	78 (35.8%)	(Chi-Sq.)
Age at Diagnosis			
<50	10 (24.4%)	41 (18.8%)	***0.018***
50-69	29 (70.7%)	122 (56.0%)	(Kruskall-Wallis)
>70	2 (4.9%)	55 (25.2%)	
Median ± SD	54 ± 14.6 years	56 ± 26.3 years	
Tumour Site*			
Oral Cavity	0	152 (69.7%)	***<0.001***
Oropharynx	39 (95.1%)	49 (22.3%)	(Kruskall-Wallis)
Hypopharynx	2 (4.9%)	14 (6.4%)	
Larynx	0	3 (1.4%)	
Disease Stage (Based on TNM v7)			
I/II	1 (2.4%)	98 (45.0%)	***<0.001***
III/IV	39 (95.1%)	165 (54.6%)	(Kruskall-Wallis)
Unknown	1 (2.4%)	1 (0.5%)	
Type of Neck Dissection			
Radical	08 (19.5%)	016 (7.3%)	***<0.001***
Modified Radical	26 (63.4%)	45 (20.6%)	(Kruskall-Wallis)
Selective	5 (12.2%)	152 (69.7%)	
Unknown	2 (4.9%)	5 (2.3%)	
Number of Lymph Nodes *Mean± Standard Deviation*			

Total *Positive*	35.15 ± 16.2 5.35 ± 11.99	33.70 ± 18.4 1.78 ± 4.6	*0.678* ***<0.001*** (2 sample unpaired T Test)
Size of Positive Lymph Nodes (mm)			
Mean± SD	35.97 ± 11.3	27.45 ± 14.9	*0.179* (2 sample unpaired T Test)
Nodal Extra-Capsular Spread			
Yes *No* *Unknown*	15 (36.6%) 20 (48.8%) 6 (14.6%)	37 (17.0%) 62 (28.4%) 119 (45.4%)	*0.567* (Chi-Sq.)

Table 10. Patients undergoing primary neck dissection only. Clinical and Pathological Characteristics. *It is of note that the HPV(-) cohort comprised a mix of tumour sub-types, most being derived from the oral cavity, whereas HPV(+) tumours were almost exclusively oropharyngeal in origin. This could subsequently effect results and outcomes.

333 patients were included in the final analysis. 230 (69.1%) patients were male. Median follow up was 33 months. 64 (19.2%) of all cases were HPV(+). Consistent with other studies (224), patients with HPV(+) disease had better OS and DSS than those with HPV(-) disease (OS and DSS log rank p=0.001) overall, see Figure 3-2.

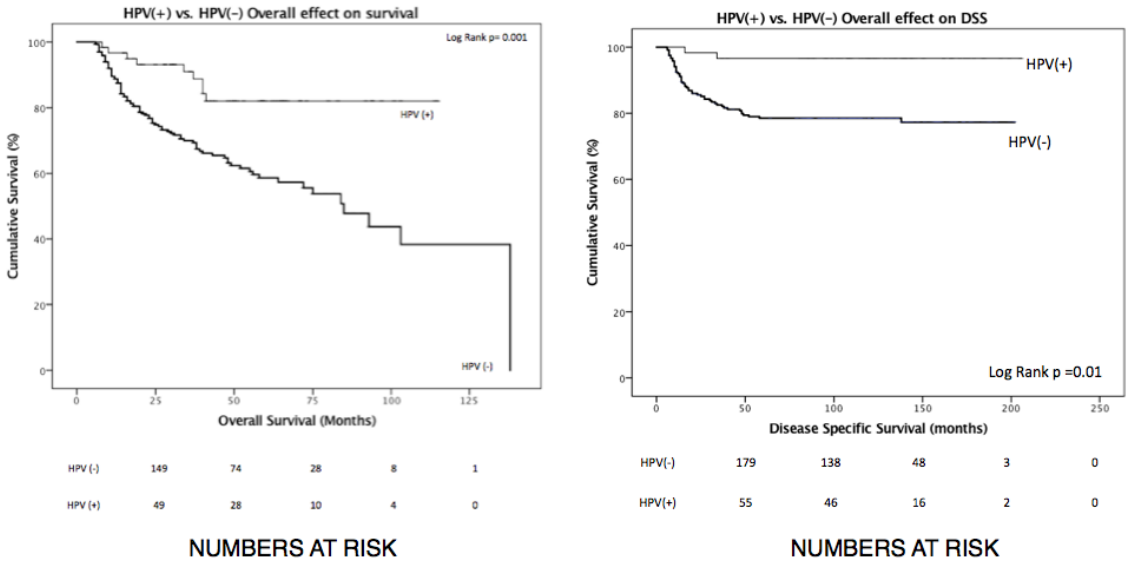


Figure 3-2 Impact of HPV status on OSS and DSS. Kaplan Meier survival curves and log-rank values. OS; p=0.001 and DSS; p=0.01.

3.3.1 Total Lymph Node Yield

There was no evidence of a significant difference in mean numbers of lymph nodes when comparing HPV(-) (n=31.8) and HPV(+) (n=32.3), (p=0.373). For comparative purposes, the total numbers of nodes were separated into <20, 21-40 and ≥41, based on a cadaveric study of 98 necks, where an average of 24 nodes were harvested for dissections of Levels I-V and 19 for levels II-IV (232).

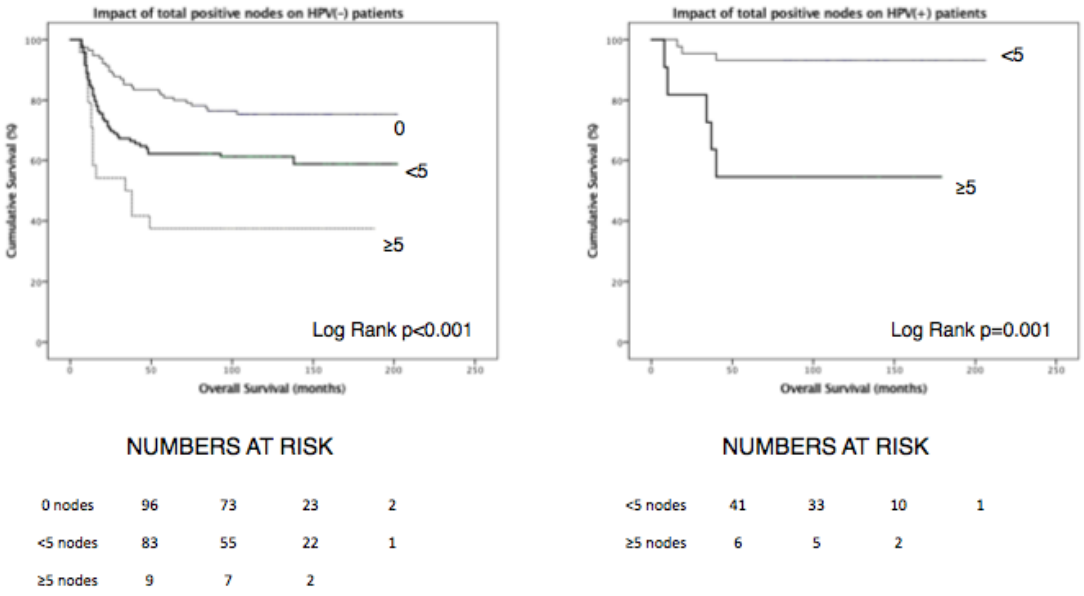
The total numbers of nodes did not influence OS in the HPV(+) cohort, or HPV(-) cohorts for any of the nodal groups (log rank, p=0.937 and p=0.239 respectively; see Figure 5-27, Appendix M).

3.3.2 Total Positive Lymph Node Yield

The total number of positive LNs were also compared. More positive nodes were seen in the HPV(+) than HPV(-) groups (4.05 nodes in HPV[+] and 1.87 nodes in HPV[-] cohorts [p=0.005]).

The effect of total positive nodes on survival was significant for HPV(+) and HPV(-) groups. For analytic purposes, total numbers of positive nodes were divided in two different ways. The first groups were for 0, 1-2, 3-4 and >5 nodes and the results are demonstrated in Figure 5-28 (Appendix M). Patients in both groups were noted to have an increasingly frequent rate of death as the number of positive nodes increased, with a significant loss of survival above 5 nodes for both HPV(+) and (-) disease (Figure 5-28).

Analysis was repeated for nodal groups including 0, <5 and ≥5, demonstrated in Figure 3-3 below. Strength of significance was increased for numbers of positive nodes over 5, which showed an increase in the rate of death for both HPV(+) and HPV(-) groups (Figure 3-3).



NUMBERS AT RISK

0 nodes	96	73	23	2
<5 nodes	83	55	22	1
≥5 nodes	9	7	2	

NUMBERS AT RISK

<5 nodes	41	33	10	1
≥5 nodes	6	5	2	

Figure 3-3. 2nd Analysis. Impact of total positive nodes on OS; Left HPV(-) log-rank p<0.001, Right HPV(+) log-rank p=0.001

Of note, as mentioned in Table 10 above, there is a clear difference in tumour sub-type origin when comparing HPV(+) and HPV(-) disease. Where HPV(+) is almost exclusively served by oropharyngeal tumours alone, HPV(-) is a mixture of different sub-types. The natural history of these individual tumours are known to encompass different loco-regional extensions and this may be what is reflected here.

3.3.3 Size of Positive Nodes

Lymph nodes were found to be larger in the HPV(+) group, with a mean size of 35.5mm in the HPV(+)and 28.5mm in the HPV(-) group, but this was not significant p=0.348. Groups were created for sizes of nodes <30mm, 31-59mm and >60mm. No association was identified for size of lymph node and survival outcome (Figure 5-29, Appendix M).

3.3.4 Lymph Node Ratio

LN ratio was defined by number of positive nodes against total number of nodes. A higher mean ratio was seen for HPV(-) nodes, 0.71 ± 0.15 in comparison to 0.19± 0.30 for HPV(+). On univariate logistic regression, every unit increase of lymph node ratio reduced the relative risk of death by 0.006 in the HPV(-) group (p<0.001) and 0.24 in the HPV(+) group (p=0.239). When correcting for

male sex, smoking and T Stage, HPV(-) disease was associated with a 0.008 reduction in relative risk (p=0.006) and 0.037 for HPV(+), (p=0.037).

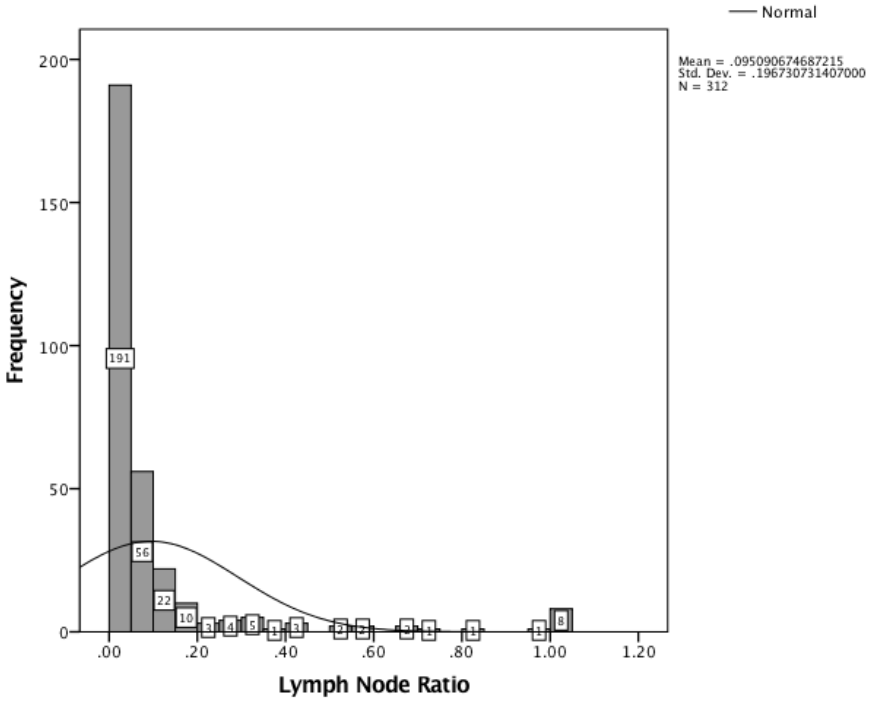


Figure 3-4. Histogram of Lymph Node Ratios

All groups were pooled for analysis of DSS and OS. Two groups were created based on the median of the histogram (0.03 ± 0.06) demonstrated above in Figure 3-4 being; <0.03 and ≥0.03. On survival analysis, there was a reduction in survival as the ratio of positive nodes to total nodes approached 1, for both OS (p=0.001) and DSS (p=0.001) (see Figure 3-5). This effect persisted when considering both HPV(-)disease OS and DSS independently, but not for HPV(+) disease, see Figure 5-30, Appendix M.

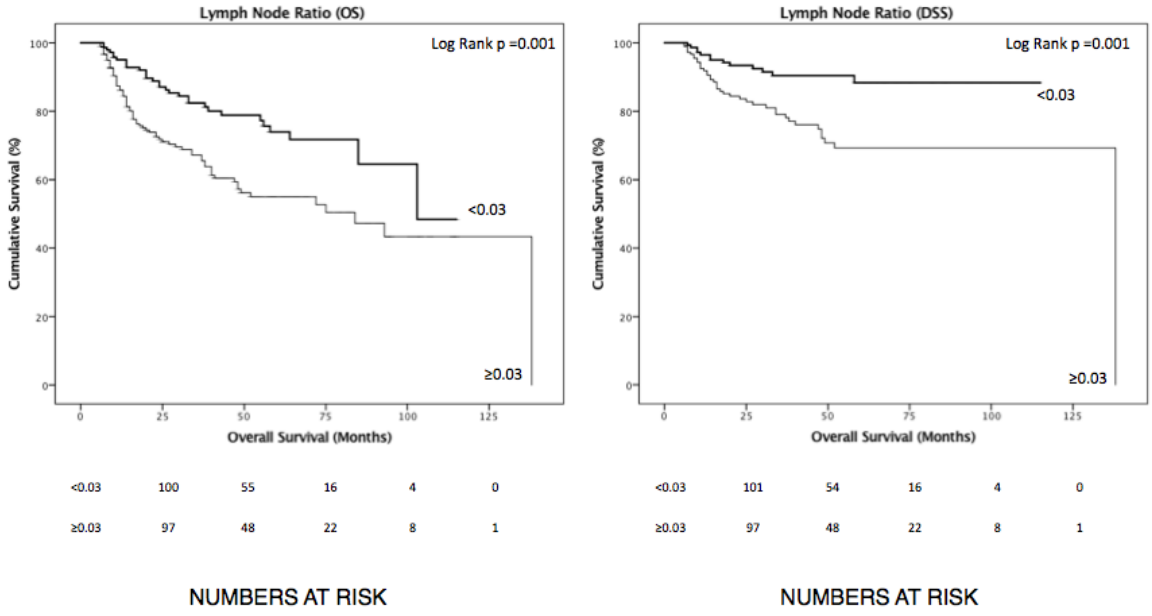


Figure 3-5. Lymph node ratio and effect on survival of *all* tumours. Left OS, p=0.001 and Right DSS, p=0.001.

3.3.5 Extranodal Extension

There was no significant demonstrable difference in the incidence of ENE in either cohort of patients (p=0.242). ENE was seen in 34.4% of HPV(+) cohort, but it had no significant effect on OS (p=0.269; Figure 3-6), or DSS (log rank p=0.162; Figure 3-7) with incalculable HRs (see Table 11).

By contrast, only 17.4% of the HPV(-) cohort had evidence of ENE, but where present it had a significant effect on multivariate analysis for both OS (HR 3.16 [1.45-6.87], p=0.004) see Figure 3-6 and Table 11) and DSS (HR 4.74 (1.62-13.9), p=0.005) see Figure 3-7 and Table 11.

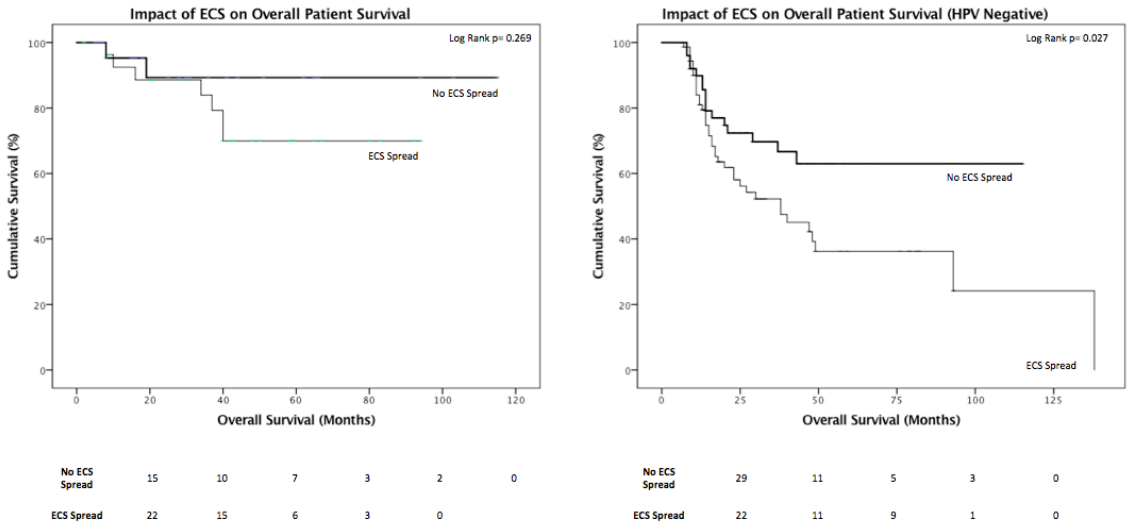


Figure 3-6. Impact of ECS on OS; Left HPV(+) log-rank p=0.269, Right HPV(-) log-rank p=0.027

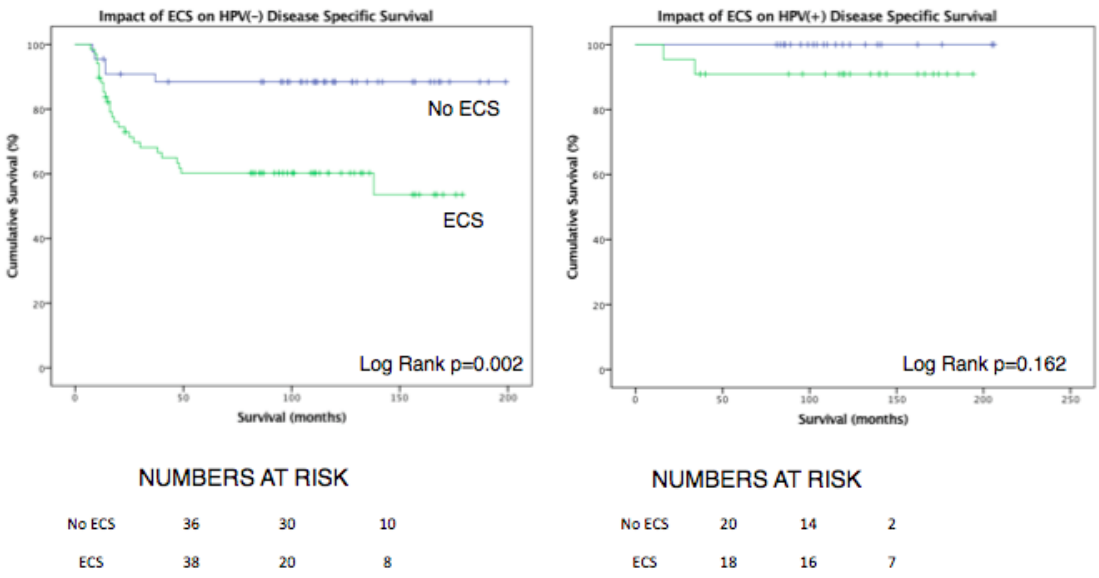


Figure 3-7. Impact of ECS on DSS; Left HPV(-) log-rank p=0.002, Right HPV(+) log-rank p=0.162

3.3.6 Multivariate Analysis

Looking at all cases together, analysis via multivariate Cox regression (adjusted as described in Section 3.2.3) demonstrated an improved survival in HPV(+) tumours (HR 0.29 [0.14-0.59, p=0.001]) and a negative effect of late stage diagnosis (HR 1.56 [1.0-2.4, p=0.05]) on DSS, see Table 11. For HPV(-) and (+) cumulatively, ENE (HR 3.66 [1.76-7.51, p=0.001]), positive lymph nodes greater than 5 (HR 2.62 [1.23-5.59, p=0.013]) and age of <70 at original diagnosis (HR 2.9 [1.5-5.7,p=0.001]) were negative prognostic markers for OS. Cases were further stratified for HPV status but due to low patient numbers in the HPV(+) cohort, results were either incalculable or insignificant (see Table 11).

For all primary and salvage cases, patients with HPV(+) tumours had significantly more positive nodes than those with HPV(-) disease (mean positive nodes: HPV(+) = 4, HPV(-) = 1.5, p=0.005). Five or more positive nodes were found to be significantly correlated with poor OS in HPV(+) disease, irrespective of the statistical stratification [0, 1-2, 3-4 and ≥5 nodes; p=0.049 and ≤4 and ≥5 nodes; p<0.001 (see Figure 5-28 and Figure 3-3).

	Univariate HR OS (95% CI)	Significance	Multivariate HR OS (95% CI)	Significance	Univariate HR DSS (95% CI)	Significance	Multivariate HR DSS (95% CI)	Significance
Age								
HPV(+) <50	1		*Not calculable*		*Not calculable*		*Not calculable*	
HPV(+) 50-69	0.89 (0.22-3.56)	p=0.878						
HPV(+) >70	*NC*							
HPV(-) <50	1		1		1		1	
HPV(-) 50-69	1.31 (0.72-2.41)	p=0.38	1.63 (0.81-3.23)	p=0.165	1.1 (0.5-2.43)	p=0.806	1.78 (0.66-4.75)	p=0.250
HPV(-) >70	1.96 (1.01-3.79)	**p=0.046**	3.52 (1.62-7.62)	**p=0.001**	1.99 (0.85-4.66)	p=0.111	4.43 (1.52-12.8)	**p=0.006**
Gender								
HPV(+) Female	1		1		*Not calculable*		*Numbers too small*	
HPV(+) Male	1.41 (0.29-6.77)	p=0.671	4.75 (0.46-49.1)	p=0.192				
HPV(-) Female	1		1		1		0.95 (0.49-1.85)	p=0.89
HPV(-) Male	1.15 (0.74-1.79)	p=0.540	0.95 (0.94-1.57)	p=0.833	1.20 (0.66-2.19)	p=0.554		
Smoking Status								

	Univariate HR OS (95% CI)	Significance	Multivariate HR OS (95% CI)	Significance	Univariate HR DSS (95% CI)	Significance	Multivariate HR DSS (95% CI)	Significance
(+) Non/Ex/ Smoker <10 pys	1		1		*Not calculable*		*Not calculable*	
(+) Smoker >10 pys	12.5 (1.46-107.5)	**p=0.021**	9.31 (1.05-82.4)	**p=0.045**				
(-) Non/Ex/Smoker <10 pys	1		1		1		1	
(-) Smoker >10 pys	1.45 (0.92-2.23)	p=0.108	0.91 (0.64-1.29)	p=0.596	1.12 (0.61-2.04)	p=0.708	1.19 (0.65-2.17)	p=0.576
Stage of Disease (TNM v7)								
(+)Early (I/II)	1		*Not calculable*		*Not calculable*		*Not calculable*	
(+)Late (III/IV)	0.43 (0.54-3.43)	p =0.424						
(-) Early (I/II)	1				1		1	
(-) Late (III/IV)	2.01 (1.28-3.17)	**p=0.002**	1	**p=0.001**	3.91 (1.84-8.32)	**p<0.001**	4.07 (1.79-9.19)	**p=0.001**
			2.33 (1.38-3.90)					

	Univariate HR OS (95% CI)	Significance	Multivariate HR OS (95% CI)	Significance	Univariate HR DSS (95% CI)	Significance	Multivariate HR DSS (95% CI)	Significance
Primary Mode of Treatment								
(+)Surgery (+)Radiotherapy	1 0.20 (0.25-1.61)	p=0.130	*Not calculable*		Not calculable		*Not calculable*	
(-) Surgery (-)Radiotherapy	1 0.82 (0.47-1.43)	p=0.482	1 0.62 (0.34-1.13)	p=0.109	1 0.88 (0.43-1.81)	p=0.729	1 0.59 (0.28-1.26)	p=0.175
Extranodal Extension								
(+) Not Present	1		1		*Not calculable*		*Not calculable*	
(+)Present	6.83 (0.84-55.5)	**p=0.072**	5.84 (0.54-62.7)	p=0.145				
(-) Not Present	1		1		1		1	
(-) Present	2.77 (1.42-5.39)	**p=0.003**	3.16 (1.45-6.87)	**p=0.004**	4.13 (1.59-10.76)	**p=0.004**	4.74 (1.62-13.9)	**p=0.005**
Total No. of Positive Lymph Nodes								
HPV(+) <5	1		*Not calculable*		1		*Not calculable*	
HPV (+) ≥5	8.27 (1.97 -	**p=0.004**			4.61 (0.28-	p=0.280		

	Univariate HR OS (95% CI)	**Significance**	**Multivariate HR OS (95% CI)**	**Significance**	**Univariate HR DSS (95% CI)**	**Significance**	**Multivariate HR DSS (95% CI)**	**Significance**
	34.7)				73.7)			
HPV(-) 0	1		1		1		1	
HPV(-) <5	1.93 (1.21-3.08)	**p=0.006**	1.36 (0.75-2.46)	p=0.308	3.20 (1.56-6.57)	**p=0.002**	2.04 (0.86-4.87)	p=0.107
HPV(-) ≥5	3.78 (2.01-7.09)	**p<0.001**	3.11 (1.41-6.87)	**p=0.005**	7.44 (3.09-17.95)	**p<0.001**	4.95 (1.68-14.6)	**p=0.004**

Table 11. Cox Regression Model for OS and DSS, Univariate and Multivariate (adjusted for age, stage and smoking status for HPV[-] and age, T stage and smoking status for HPV[+]).

Abbreviations: CI = confidence interval, NC = not calculable.

3.4 Discussion

As previously demonstrated, HNSCC can be stratified by its underlying aetiology, i.e. HPV positive vs. HPV negative disease (see Figure 3-2) (224). Further, it has been shown that stage of disease in the previous TNMv7 staging system, for patients with HPV(+) cancer, does not correlate with the poor outcomes traditionally associated with HPV(-) cancer of similar stage (Table 11) (224, 233, 234)

Historical literature reports ENE in 60% of HNSCC cases with positive nodes (233). In our dataset, 34.4% HPV(+) nodes were reported with ENE, compared to 17.4% of HPV(-) nodes. Importantly, the presence of ENE was significantly associated with poor OS and DSS in the HPV(-) group (log rank p=0.027 and log rank p=0.002), but not in the HPV(+) group. This suggests that historical data linking ENE to poor prognosis may require revision in the age of HPV driven disease. Future trials may clarify this association with greater patient numbers (117).

Lymph node density (positive nodes/total nodes) has in the past been proposed as an important adverse prognostic marker (235), but to date a numerical value has not been consistently defined as predictive of poor outcome. In a previous study, lymph node ratios were given for three groups; <0.06, 0.06-0.17 and >0.17 (236). In this scenario, increasing ratios were associated with a reduction in OS at 3 years, with a difference of >50% between highest and lowest groups (77.8% vs. 23.5%, p=0.003)(236). No allowance was made for HPV status.

In our corresponding tumour set, 3-year OS was associated with an almost negligible discrepancy of 5% for the two defined groups (49% vs. 44%) only. This incongruence could be explained with note of the very polar groupings in the study by Chen et al., and the contrasting median split (<0.03 and ≥0.03) of this present investigation.

In any case, the idea of lymph node ratio lacks a consistency to allow its introduction into routine clinical practice, particularly given that the specified lymph node ratio for comparison can vary from trial to trial, e.g. 0.06 in Gil et al. (235) and 0.09 in Sayed et al. (237).

Our data demonstrate a discrepancy in disease burden when looking at metastasis in the neck, with an average of 5 positive nodes in HPV(+) disease, in comparison to 2 in HPV(-) (p<0.001). This reinforces the concept that despite multi-focal regional disease, HPV(+) disease is associated with better survival outcomes. Importantly however, we have also shown a remarkable difference in reduction in survival in both HPV(+) and (-) patients with ≥5 positive nodes (log rank p=0.001 and p<0.001), see Figure 3-3. Therefore, evidence suggests HPV(+) patients with ≥5 positive nodes should not be considered for de-intensification trials, given a potentially poor outcome. This has

been corroborated by large clinical studies looking into oropharyngeal HPV(+) disease where it has been demonstrated, similar to the work here, that total number of positive nodes, specifically 5 nodes or greater were significant for signs of disease recurrence, OR 3.12 (95% CI 1.1-8.83, p=0.032) (238).

During the course of this work, the 8th edition of AJCC Staging Manual of 2018 was released and is now cognizant of HPV(+) disease as a separate entity (106). The changes in this newer edition are listed and compared in Appendix N. As part of this, the new pathological staging system makes note of the relevance of nodal number, but not size or site of nodes as previously. 1-4 nodes are now graded as pN1 and >5 as pN2. The previous ENE score has been removed. Size and laterality remains a part of the clinical staging (see Appendix N), but nodal number is not described.

In the 7th staging edition, HPV(+) Stage IV disease (diagnosed on the basis of multiple ipsilateral nodes, regardless of T stage), would be treated with the expectation of a poor 5-year outcome, despite increasing knowledge to contrary. Inevitably this would mandate over-treatment of patients and a subsequent reduction in quality of life outcomes. Using our dataset to exemplify this, Stage IV HPV(+) disease was associated with a 5-yr survival of 35.6%, in comparison to HPV(-) which only demonstrated only 15% (Log-rank p<0.001).

To investigate this, a small additional study was performed to ascertain whether previously treated HPV(+) disease would be down-staged appropriately in the new classification. This review is detailed in Appendix N and demonstrates the success of this revised iteration.

The findings from this review are reinforced by single institution retrospective study from Toronto, looking at refining the AJCC criteria for oropharyngeal cancer (239). 810 patients were included with 573 defined as HPV(+). As illustrated in Appendix O although clear heterogeneity of outcomes was demonstrated for HPV(-) stages (Log-rank p=0.004), this was not the same for HPV(+).

The revised AJCC 8th edition TNM was released on 1st January 2018.

3.5 Conclusion

The identification of HPV in HNSCC offers an opportunity to refine oncological practice and patient stratification. We show that the presence of ENE is only linked with a poor outcome in patients who are HPV(-), with no relationship in HPV(+) patients. Our data also demonstrate that number of positive nodes are linked to survival and five or more positive nodes is a significant threshold for HPV(+) patients. This data supports the current implementation of number of lymph

nodes into pathological nodal staging in TNM v8 and may be a significant tool for differentiating patients into high and low risk treatment groups.

Chapter 4: Impact of social deprivation on major head and neck cancer surgery outcomes in England: a national analysis

4.1 Introduction

Socioeconomic status plays an important role in both the incidence and prognosis of cancer, from entire global regions to local communities and neighbourhoods (160, 161, 164, 165). In a recent publication from the National Cancer Intelligence Network (NCIN), incidence and mortality of all cancers combined were notably higher in the most deprived regions of society, compared to the least (166). If cancer incidence rates were adjusted for deprivation group, 15,300 fewer cancers would have been diagnosed in a period between 2006-2010 (166). In a similar period (2007-2011) there was instead a yearly excess of 19,200 deaths (166).

The changing epidemiology of HNC (25, 240) is a balance between a significant fall in the rates of tobacco and excess alcohol consumption, particularly in high-income countries (226, 241), and a concurrent increase in the incidence of OPSCC (226). This rise is associated with Human Papilloma Virus (HPV) infection (25), a biologically and phenotypically distinct type of HNC (226, 240). It is projected that by 2020, HPV-associated OPSCC will be more prevalent than cervical cancer (17).

Whilst as yet there are no studies to directly link HPV subtype 16 and social deprivation, there is evidence to suggest associations between reduced education level and household income and HPV driven cancers of the penis, vagina, oral cavity and oropharynx (161).

Furthermore, whilst the incidence of smoking continues to decline in both sexes, tobacco consumption in the UK appears to have a relationship with socio-economic deprivation. Persons seeking employment demonstrate higher rates of smoking than those currently employment (241). Individuals with an annual income of less than £10,000 (23%) are twice as likely to smoke than those with an income over £40,000 (11%) (241). Those with the lowest incomes are less likely to have quit smoking (242). When accounting for areas of socio-economic deprivation, both men and women demonstrated a linear relationship between increasing deprivation and tobacco consumption (243). Using smoking as a surrogate for health behaviours in general, whilst epidemiological data is reassuring overall, there is an incongruity between the highest and lowest deprivation groups and this could explain the differences in incidence and outcomes for HNC (167).

In patients undergoing major surgery, factors related to socioeconomic status have been suggested as influential, not only to cancer presentation, but also to outcomes of surgery (25). This can be significant, affecting parameters such as UICC TNM stage (244), length of hospital stay (163), number of post-operative complications (163, 245) and OS (163, 246, 247).

The aforementioned associations between socioeconomic status, surgical outcomes and risks factors for HNC suggest a relationship may exist (247, 248) and in turn, there may be a notable health gradient outcome when applied to HNC also. To look into this in more detail, a large retrospective population based observational study was performed, looking at patients undergoing major head and neck cancer operations in the UK. We sought to investigate this relationship between social deprivation and clinical outcomes in patients undergoing primary surgery for HNC. Primary outcomes were predominantly based on inpatient death and OS. Secondary outcomes were based upon length of inpatient stay, number of complications and rate of re-admission following discharge.

The following study was accepted for publication in Head and Neck Journal (Mirza, A. H. et al. *Head & Neck* 2018; 1-9).

4.2 Materials and Methods

4.2.1 Case Identification

All patients over the age of 18 who underwent major head and neck cancer surgery in England between April 2002 and March 2012 for oral cavity, oropharynx, and larynx and hypopharynx cancers were included. WHO ICD-10 codes and NHS OPCS codes were used to interrogate the English Hospital Episodes Statistics (HES) dataset for head and neck cancer diagnoses, morbidities, resection codes and complications. These are detailed entirely in Appendix Q. The derived dataset was first published in an informatics study by Nouraei et al. (249).

Consent was obtained in the original study under the senior author and was valid for further study based on the NHS Act 2006, Section 251. This said, no identifiable or confidential patient information was sought or required within the context of this study and therefore full use of section 251 was not required. This was depersonalised to the standard required by the Information Commissioners Office (2012) on receipt from NHS Digital.

The final dataset concerned only patients who had undergone their first major head and neck cancer operation within this period. No patients were excluded due to insufficient or missing information.

4.2.2 Resection Stratification

Six of the seven Resection Strata (RS) previously correlated with an increased length of stay (see Figure 4-1) and numbers of complications were used to divide major subtypes of head and neck surgery (249). These were oral glossectomy, maxillectomy, oral vault excisions (RS1), pharyngeal glossectomy and palatal resections (RS2), floor-of-mouth resections and mandibulectomies (RS3), combined oral-pharyngeal resections (RS4), laryngectomy (RS5) and pharyngo-laryngectomy (RS6) (250).

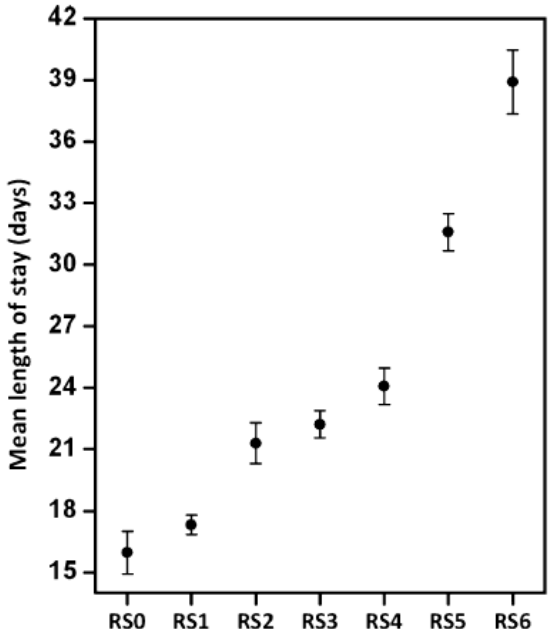


Figure 4-1. Relationship between length of stay and resection strata (Kruskall Wallis $p<0.05$). Taken from (249).

Individual RS and data from Length of Stay (LOS) was used to calculate Failure to rescue LOS threshold (FTRL), referring to the 75th centile of LOS for each individual RS. This was a measure introduced to specifically identify patients seen to undergo unusually long hospital stays, i.e. 75% of their comparable group. FTRL thresholds for RS1-RS6 were 19, 25, 26, 27, 37 and 48 days respectively (249). Effects of primary surgery, vs. emergency surgery were also measured.

4.2.3 Burden of Morbidities

The ICD-10-based *Charlson Co-Morbidity Index* was used to assign morbidity scores (251, 252). Originally validated on a cohort of women with breast cancer, the Index allows for a prediction of one-year mortality for patients who have a range of morbid conditions (252). Each additional patient decade adds a further point to the patient's morbidity score. An amended score as based on NHS HES codes was used as specified by Bottle & Aylin 2011 (251). Levels were categorised as

follows: <5, 5-10, >10-20, >20-30 and >30 based on the amended scores given below (see Table 12).

Co-morbidity Value	Original Charlson Weighting	Amended Charlson codes based on HES weights
Cancer	2	7
Connective Tissue Disorder	1	0
Cerebro-vascular Accident	1	14
Dementia	1	12
Diabetes with complications	0	2
Diabetes without complications	1	0
Congestive Heart Failure	1	9
HIV	6	0
Mild-Moderate Liver Disease	1	0
Pulmonary Disease	1	1
Metastatic Cancer	3	7
Acute Myocardial Infarction	1	2
Paraplegia	2	-3
Peptic Ulcer	1	5
Peripheral Vascular Disease	1	2
Renal Disease	2	9
Severe Liver Disease	3	27

Table 12. Amendments from original Charlson Classification. Taken from (251)

4.2.4 Social Deprivation

Social deprivation was derived from the English Index of Multiple Deprivation (IMD) 2015, a composite score based upon seven independent variables; income, employment, education, skills and training, health and disability (comprised of indices including years of potential life lost, comparative illness and disability ratio, acute morbidity and incidence of mood and anxiety disorders), crime, barriers to housing and services and living environment (253).

These weighted scores were used to rank 32,844 so called Lower-layer Super output areas (LSOA) [each with an average of 1,500 residents and 650 households] from least to most deprived areas (159). Number of LSOAs vary from region to region, dependent on size and density of population. The city of Southampton as an example has 148 LSOAs, with 19 within the most deprived and 0 in

the least deprived in England (254). Of 326 local authorities, on average rank of LSOAs, Southampton rates as 54th on the most deprived in England (254).

This indicator has been used extensively, not only within government publications and socio-economic studies, but also in clinical data in the past. From this ranking 5 quintiles were formed, with 1 being the least deprived to 5 being most. For the purposes of this study, quintiles 1-2 (Less Socially Deprived; LSD) and 4-5 (More Socially Deprived; MSD) were compared as two groups respectively. Patients in quintile 3 (n=2,932) were thought to not lend themselves easily to either a more or less socially deprived group. Therefore, in order to allow for a better dichotomy, they were not considered for further analysis and were removed from the dataset.

4.2.5 Statistics

Categorical data were cross-tabulated and assessed using Chi-squared testing. For continuous data, an independent two-tailed *t* test or Mann-Whitney test was used, depending on normality of distribution. Kruskall-Wallis testing was used to determine the presence of significant differences between multiple strata. A two-way ANOVA was used to compare mean differences between two independent variables. Bonferroni error corrections were made for multiple comparisons. Binary logistical regression models were performed for FTRL, complications (0, $\geq$1) and in-hospital death (death during an inpatient admission). OS was an actuarial variable and was analysed using Cox regression. For multivariate analysis, corrections were made for age, sex, smoking status and alcohol consumption and site of disease. Survival analysis was plotted used Kaplan Meier charts with log-rank analysis. All statistical analysis was performed using SPSS for Macintosh (IBM Corp. Armonk, NY, USA, Version 24).

4.2.6 Outcome Measures

The main outcome measures were inpatient death and OS. Inpatient death was any death that occurred during the period of the patient's index admission. OS was defined as the period from original diagnosis to patient death. Otherwise survival was calculated to last point of contact, whereupon the patient was censored thereafter.

Complications were based on binary variables from patient outcome codes. Length of stay was defined as total time of inpatient admission, from date of admission to date of discharge.

4.3 Results

Between 2003 and 2012, 12,333 patients in the social deprivation quintiles 1, 2, 4 and 5, with cancers of the oral cavity, oropharynx, and hypopharynx-larynx underwent major head and neck surgery. There were 5,051 procedures performed in the LSD groups and 7,282 procedures performed in the MSD group. Median follow up was 30.1 months (SD ±33.5 months), with a range of 0-133 months. Male patients made up 67% of the LSD group but 74% of the MSD group respectively.

Oral cavity was the most common site of cancer in both groups (LSD; 52% and MSD; 43%, $p<0.000001$). Hypertension was the most common morbidity overall seen in 24.7% of patients, with comparable incidences in both LSD and MSD groups. There was a significant difference in smoking habits, with 26% of the MSD cohort smoking in comparison to 14% of the LSD ($p<0.000001$). Table 22 in Appendix Q provides further details.

4.4 Differences between LSD and MSD groups at Baseline

4.4.1 Differences in Age

Mean age at surgery was higher in the LSD group (63 ± 12) compared with the MSD group (61 ± 11; $p<0.000001$). Distributions of age were largely comparable for both groups, with predispositions to ages between 55 and 74 ($p<0.000001$).

4.4.2 Difference in Morbidity

Distribution of morbidity scores suggested an increased burden of morbidity in MSD groups, in particular for strata 4 and 5 ($p<0.000001$). MSD patients were more likely to smoke (25.8% vs. 13.6%, $p<0.000001$), (see Table 22).

4.4.3 Differences in HNC Location

Distribution between the two groups demonstrated an increased proportion of hypopharyngeal-laryngeal cancer subtype in the MSD group (41% vs. 30%, $p<0.000001$), Table 22. There was however a preponderance of oropharyngeal cancers in the LSD group.

4.5 Differences in Patient Treatment

A total of 7,687 patients underwent primary surgical procedures (LSD = 3130 and MSD = 4557). The most common procedure in the LSD group was Oral Glossectomy (27%), followed by Laryngectomy (23%). Overall, 77% of patients underwent a Neck Dissection (ND) and 50%, a Tracheostomy. 72% of patients required reconstructive surgery and 13% were fed via a gastrostomy tube.

The most common procedure in the MSD group was Laryngectomy (RS5 [30%]) followed by Mandibulectomy (RS3 [20%]). More MSD patients underwent both ND and Tracheostomy (76.6% and 51%) but this was not significant. Fewer patients required reconstruction (64.8%) and this was significant ($p<0.00001$) and possibly reflected the fact that a higher proportion of MSD patients undergo laryngectomies and pharyngo-laryngectomies, which do not routinely require reconstructive surgery.

Fewer patients in the MSD group were also seen to require gastrostomy feeding (12.7%). MSD patients were more likely to undergo surgery during an emergency admission (OR 1.57 [95% 1.40-1.77]; $p<0.000001$).

4.6 Differences in Post-operative course

Overall, 4,670 (37.9%) patients suffered at least one complication (Table 13). MSD patients had significantly higher rates of complications on bivariate analysis, including myocardial infarctions (OR 1.58 [95% CI 1.29-1.93], $p<0.000001$), respiratory tract infections (OR 1.54 [95% CI 1.34-1.77], $p<0.000001$) and surgical site complications (OR 1.28 [95% CI 1.15-1.42], $p<0.000001$; Table 13).

Both tracheostomy and PEG malfunctions were also more common in MSD group ($p<0.000001$ for both respectively); (OR 1.05 [95% CI 0.98-1.13]. $p=0.179$) and (OR 1.49 [95% CI 1.15-1.89], $p=0.02$) see Table 13. Increasing resection strata type from RS1 to RS6 was also associated with an increasing number of complications. MSD status was independently associated with failure to rescue length of stay. Table 13 provides further information about the nature and incidence of complications.

	Social Deprivation Quintile 1-2 (n=5,051)	**Social Deprivation Quintile 4-5 (n=7,282)**	**Significance**
Complications			

Abdominal	212 (4.2%)	332 (4.6%)	p=0.336
*MACE	201 (4.0%	379 (5.2%)	**p=0.002**
Myocardial Infarction	159 (3.1%)	321 (4.4%)	**p<0.000001**
Cardio-respiratory Arrest	49 (1.0%)	100 (1.4%)	**p=0.044**
Lower Respiratory Tract	324 (6.4%)	695 (9.5%)	**p<0.000001**
Acute Renal Failure	31 (0.6%)	49 (0.7%)	p=0.687
Sepsis	43 (0.9%)	70 (1.0%)	p=0.529
Stroke	26 (0.5%)	42 (0.6%)	p=0.647
Any Other Infectio	356 (7.0%)	709 (9.7%)	**p<0.000001**
Venous Thrombo-	44 (0.9%)	61 (0.8%)	p=0.842
Urinary Tract Infection	72 (1.4%	99 (1.4%)	p=0.758
Psychiatric	69 (1.4%	136 (1.9%)	**p=0.032**
Skin Ulceration	55 (1.1%)	93 (1.3%)	p=0.345
Haemorrhage	377 (7.5%)	549 (7.5%)	p=0.876
Surgical Site Infections	625 (12.4%)	1146 (15.7%)	**p<0.000001**
Iatrogenic	181 (3.6%	289 (4.0%)	p=0.272
Tracheostomy	139 (2.8%)	299 (4.1%)	**p<0.000001**
PEG malfunction	99 (2.0%)	222 (3.0%)	**p<0.000001**
Reconstruction failure	138 (2.7%)	205 (2.8%)	p=0.783
Number of Complications			
0 1 2 ≥3	3338 (66.1%) 743 (14.7%) 518 (10.3%) 452 (8.9%)	4325 (59.4%) 1164 (16.0%) 939 (12.9%) 854 (11.7%)	**p<0.000001** (Kruskal-Wallis)
Length of Stay (Mean ± SD [range])			
	16 ± 18 days (0-258)	26 days ± 23 days (0-350)	**p<0.000001 (T-Test)**
Emergency Admission within 30 days			
	1393 (27.6%)	2522 (34.6%)	**p<0.000001** (Chi-Sq.)

Recurrence of Disease			
	2096 (41.5%)	3345 (45.9%)	**p<0.000001** (Chi-Sq.)
Site of Recurrence			
Primary Site Regional Distant	1951 (93.1%) 427 (20.4%) 204 (9.7%)	3158 (94.4%) 604 (18.1%) 380 (11.4%)	**p=0.047** **p=0.034** p=0.059
Inpatient Death			
	147 (2.9%)	266 (3.7%)	**p=0.024 (Chi-Sq.)**

Table 13. Patient Outcomes by Deprivation. (*MACE – Major Acute Cardiovascular Events)

4.6.1 Length of Stay

MSD patients spent 10 days longer in hospital than LSD patients (p<0.000001, see Table 13) and this was significant on bivariate analysis also, OR 1.72 [95% CI 1.57-1.88] (see Table 13). Failure to rescue length of stay (FTRL) was used to assess factors impacting upon length of stay (see 4.2.5). Tobacco consumption (OR 1.13 [95% CI 1.02-1.25], p=0.002), alcohol (OR 1.77 [95% CI 1.51-1.94], p<0.000001), insertion of tracheostomy (OR 1.96 [95% CI 1.82-2.13], p<0.000001), emergency surgery (OR 4.53 [95% CI 3.99-5.14], p<0.000001) and social deprivation (OR 1.72 [95% CI 1.57-1.88], p<0.000001) were all significant on multivariate analysis and seen to independently increase length of stay (see Table 14).

	Binary Logistic Regression [95% CI]			Cox's Proportional Hazards [95% CI]	
	Failure to rescue LOS threshold	**Complications (0, ≥1)**	**In-hospital Mortality**	**Recurrence**	**OS**
Age (continuous variable)	**1.04 [1.02-1.05]**	**1.03 [1.021-1.029]**	**1.06 [1.05-1.07]**	**1.00 [1.003-1.009]**	**1.03 [1.03-1.04]**
Male Gender	0.92 [0.85-1.00]	1.39 [1.25-1.55]	1.01 [0.82-1.25]	1.01 [0.94-1.07]	1.19 [1.10-1.29]
Smoker	**1.13 [1.02-1.25]**	**1.22 [1.08-1.37]**	0.79 [0.60-1.05]	0.98 [0.91-1.06]	0.94 [0.85-1.03]
Alcohol	**1.71 [1.51-1.94]**	**1.46 [1.26-1.69]**	**1.94 [1.41-2.65]**	**1.22 [1.16-1.38]**	**1.42 [1.26-1.60]**
Resection Strata *Oral Cavity* *Oropharynx* *Hypopharynx-Larynx*	1 **1.18 [1.06-1.31]** 1.08 [0.99-1.22]	1 0.98 [0.86-1.12] **1.21 [1.09-1.34]**	1 0.96 [0.71-1.28] **1.25 [1.02-1.53]**	1 1.27 [0.71-1.38] **1.65 [1.55-1.76]**	1 **1.13 [1.03-1.25]** **1.24 [1.15-1.33]**
[+]Resection Strata *RS1* *RS2* *RS3* *RS4* *RS5* *RS6*	 1 0.99 [0.81-1.21] 0.98 [0.86-1.13] 1.03 [0.89-1.18] **0.71 [0.89-1.18]** 0.75 [0.56-1.01]	 1 1.19 [0.91-1.56] 1.15 [0.98-1.36] **1.40 [1.16-1.68]** **1.19 [0.80-1.76]** **1.66 [1.13-2.43]**	 **1** **1.87 [1.08-3.24]** **1.96 [1.41-2.72]** **2.41 [1.69-3.43]** 1.61 [0.77-3.33] **3.38 [1.68-6.81]**	 **1** **1.23 [1.06-1.45]** 1.08 [0.97-1.19] **1.17 [1.04-1.31]** **1.53 [1.21-1.92]** **1.92 [1.53-2.39]**	 **1** 1.17 [1.97-1.41] 1.08 [0.97-1.19] **1.37 [1.21-1.56]** **1.42 [1.09-1.84]** **2.38 [1.85-3.05]**
Neck Dissection	**1.16 [1.05-1.27]**	**1.26 [1.11-1.42]**	0.92 [0.74-1.13]	1.05 [0.98-1.13]	1.03 [0.95-1.12]
Tracheostomy	**1.96 [1.82-2.13]**	**1.47 [1.33-1.63]**	**1.61 [1.33-1.94]**	**1.08 [1.02-1.14]**	**1.22 [1.14-1.31]**

Primary Surgery	1.02 [0.94-1.10]	**0.95 [0.86-1.05]**	**0.82 [0.68-0.99]**	**0.93 [0.87-0.98]**	**0.88 [0.83-0.96]**
Emergency Surgery	**4.53 [3.99-5.14]**	**1.31 [1.11-1.54]**	**2.39 [1.86-3.07]**	**1.31 [1.19-1.69]**	**1.59 [1.43-1.78]**
Social Deprivation	**1.72 [1.57-1.88]**	**1.23 [1.10-1.36]**	**1.47 [1.19-1.82]**	**1.20 [1.12-1.29]**	**1.34 [1.24-1.45]**

Table 14. Binary Logistic Regression Models and Cox's Proportional Hazards [95% CI]. **Bold** indicates significant results. All results represent multivariate analyses, corrected for age, male gender, smoking and alcohol consumption and site of disease.

4.6.2 Emergency Re-admission

More MSD patients re-presented as an emergency within 60 days than LSD patients on bivariate analysis (35% vs. 28%, p<0.000001), Table 13 and Figure 4-2. Primary surgery was associated with a lower risk of emergency readmission (OR 0.93 [95% CI 0.87-0.98], p<0.000001), see Table 14. Alcohol consumption (OR 1.22 [95% CI 1.16-1.38], p=0.004), site of cancer (hypopharynx-larynx OR 1.65 [95% CI 1.55-1.76], p<0.000001) increasing invasiveness of resection, emergency surgery (OR 1.31 [1.19-1.69], p<0.000001) and social deprivation status (OR 1.20 [95% CI 1.12-1.29], p<0.000001) were all associated with an increased risk of emergency re-admission, on multivariate analysis (see Table 13).

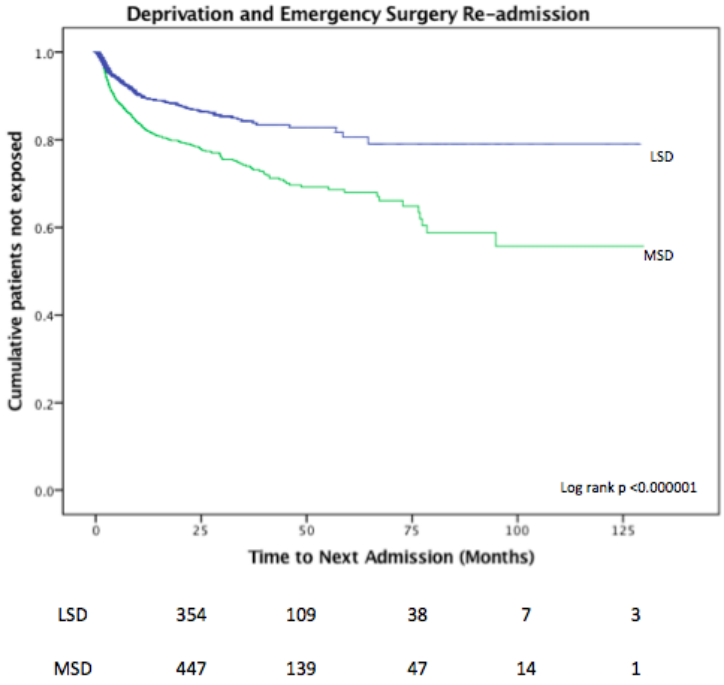
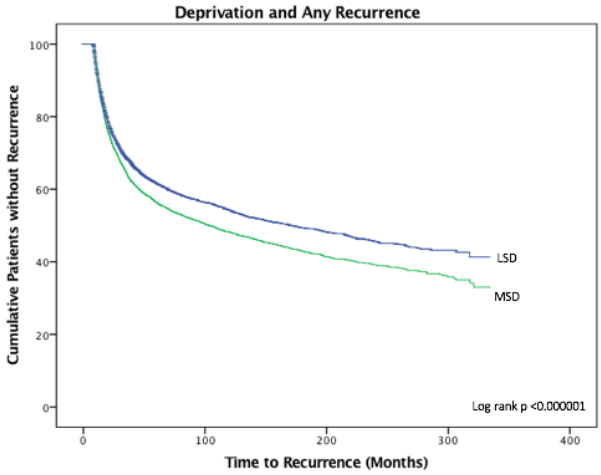


Figure 4-2. Survival chart comparing re-admission outcomes for LSD and MSD patients (Log rank p<0.0000010.

4.6.3 Recurrence of Disease

Recurrence of disease was higher in patients in the MSD cohort (45.9%) than the LSD cohort (41.5%) and this was significant (Chi Sq. p<0.000001), see Table 13 and Figure 4-3. This difference was reinforced and significant for primary disease, (93.1% vs. 94.4%, p=0.047), but not for distant metastasis (9.7% vs. 11.4%, p=0.059). Incidence of regional disease was more common contrarily in LSD patients (20.4% vs. 18.1%, p=0.034) and this may have reflected the likely underlying predisposition of LSD patients to HPV(+) disease and hence greater cervical disease burden (226).

There was no significant difference between the groups on Cox's proportional Hazard's testing (0.97 [95% CI 0.92-1.03]), see Table 14.



LSD	1611	567	84	0
MSD	1974	664	107	0

Figure 4-3. Survival chart comparing any recurrence of cancer for MSD and LSD patients (Log rank p<0.000001).

4.7 Mortality

MSD patients had a significantly higher incidence of in-hospital death compared with the LSD group (p=0.024), (OR 1.47 [95% CI 1.19-1.82], see Table 14 and Figure 4-4). In-hospital mortality was also more significantly associated with alcohol consumption (OR 1.94 [95% CI 1.41-2.65], p<0.000001), cancers of the hypopharynx-larynx (OR 1.25 [95% CI 1.02-1.53], p=0.031), increasing invasiveness of resection and those undergoing emergency surgery (OR 2.39 [95% CI 1.86-3.07], p<0.000001) on multivariate analysis, see Table 14.

Comparing OS based on deprivation, there was a clear distinction between patients in the LSD and MSD groups, with a reduced rate of survival in the MSD group (p<0.000001, Figure 4-4), (OR 1.34 [95% CI 1.24-1.45], p<0.000001) see Table 14. Alcohol consumption (OR 1.42 [95% CI 1.26-1.60]) and emergency surgery (1.59 [95% CI 1.43-1.78], p<0.000001) were also notably and significantly associated with a reduced rate of OS. Primary surgery was seen to deliver a protective effective upon OS on multivariate analysis (OR 0.88 [95% CI 0.83-0.96], p<0.0000001), see Table 14.

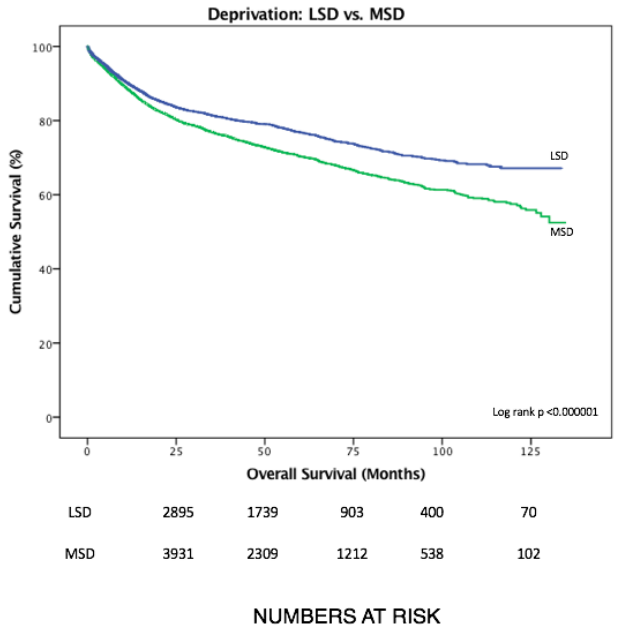


Figure 4-4. The effect of deprivation on OS (Log rank p<0.000001)

4.8 Discussion

4.8.1 Background to Study

Social determinants of health are complex and exert a significant effect upon health outcomes in patients with head and neck cancer (25, 165). Contemporary rates of disease in the UK suggest approximately 1 in 42 men and 1 in 97 women will develop a HNC in their lifetime (247). A notable proportion (28%) are seen in the most deprived quintile (247).

In the most deprived population, net survival assessed at 1 and 5 years following diagnosis, is only 69% and 43% for men and 74% and 47% for women, respectively (247). In men, this represents a net survival benefit in favour of less social deprivation of 11% and 17% at 1 and 5 years (247). Both surgical and oncological outcomes are susceptible to influence from a patient's socio-economic status at time of presentation and this appears independent of factors which might otherwise be thought to contribute to survival outcomes (247).

In England, life expectancy has increased to 78.3 years and 82.3 years for men and women in the years 2007-2009(255). In the more affluent wards of Kensington and Chelsea, life expectancies are 6.1 years and 6.7 years above the English average, for men and women respectively. In more deprived areas, life expectancy was as low as 72.5 years in Manchester (males) and 78 years in Liverpool (females)(255); reinforcing the importance of social circumstances in all-cause mortality.

For many, the cumulative effect of a lifetime of social health discriminators is difficult to avoid and manifests itself via disease incidence, timing of patient presentation and final outcome.

4.8.2 Synopsis of Key Findings

Our study assessed the impact of social deprivation on 12,333 patients undergoing major head and neck cancer operations between 2003 and 2012 in England. MSD patients were younger and were more likely to present with hypopharyngeal-laryngeal cancers. They had higher burdens of morbidity and more frequently required surgery during an emergency admission. Following surgery, MSD status was an independent risk factor for increased length of inpatient stay.

MSD patients were more likely to die than their LSD counterparts, both in hospital (OR 1.47 [95% CI 1.19-1.82]) and overall (OR 1.34 [95% CI 1.24-1.45]). MSD patients spent approximately 10 days longer as inpatients, which persisted when correcting for confounding factors on multivariate analyses (OR 1.72 [95% CI 1.57-1.88]), see Table 14.

This might be explained by the increased proportion of hypopharynx and larynx cancers within this cohort; with hypopharyngeal cancer known to present in advanced stages, necessitating invasive and major operations. Of note, outcomes were adjusted for disease site in the multivariate analysis and so differences here, statistically speaking, would have been accounted for.

More oropharyngeal cancers were seen in the LSD group and hypopharyngeal-laryngeal cancers in the MSD groups, perhaps reflecting the underlying aetiology (HPV vs. tobacco/alcohol consumption).

A previous study of all-cause emergency admissions, stratifying along comparable SD quintiles (LSD =1-3, MSD 4-5) did not demonstrate any difference on LOS, with an average of 5 days ($p<0.001$) in each group (256). Re-admission however was associated with lower socioeconomic status, with a greater risk of re-admission by 60% for the lowest relative to the highest groups (256). This effect was corroborated by Tsai et al. (with a surgical population), where risk adjusted re-admission rates were notably higher in deprived populations (OR 1.29, 95% CI 1.16-1.22, $p<0.001$) (257). In our study, increased odds of 19% for readmission in the MSD group were observed on multivariate analysis (OR 1.19 [95% CI 1.11-1.27]), see Table 14.

Patients in the MSD group had significantly higher rates of in-hospital (OR 1.47 [95% CI 1.19-1.82]) and subsequent mortality (OR 1.31 [95% CI 1.21-1.42]) following major head and neck cancer surgery, see Table 14. Combining all cancers (except non-melanoma skin cancer), Public Health England demonstrated an age-standardised risk (ASR) ratio for OS of 1.6 for men and 1.5 for

females on comparing the least to the most deprived quintiles (258). This was seen to dramatically increase for oropharyngeal, oral cavity and laryngeal cancers (ASR 3.7 and 3.6, ASR 3.2 and 1.6 and ASR 3.9 and 3.0, respectively) (258).

Smoking status, as expected, was negatively correlated with clinical outcome. In our study, MSD patients were twice as likely to smoke than patients in LSD populations (26% vs. 14%, p<0000001). This said, when correcting for smoking, and other confounding factors, socially deprived patients fared poorly in nearly all categories including, length of stay, complications, in-hospital survival and OS.

Whilst MSD may smoke in greater number, there are additional factors of importance when considering this outcome disparity. In other studies, time to presentation (i.e. delayed presentation), patient education level, presence of social support and unemployment, are purported to be significant factors, possibly associated with this effect (160). It was not possible to stratify our data to comment on this.

Socio-economic status is a complex state, the product of a series of composite parameters of differing weighting. This condition creates a gradient of health outcomes and a state of living that combined; reinforce the individual's situation in a cyclical fashion, often compounding their position.

4.8.3 Strengths and Limitations

Information was extracted via coded data from HES, beginning at the translation of clinical information into administrative code and back once again into clinical information for the purposes of this study. This can introduce coding-related subjectivity, variability and errors (250). Additionally, although LSOAs provide a reasonable means of addressing the socio-economic state of people's lives, they do not provide the most accurate means of ecological analysis. There is evidence however, that some correlation exists between domestic postcodes and self-reported data (259, 260).

Furthermore, while it is known that patients with HPV(+) OPSCC demonstrate an improved survival relative to other HNSCC subsites (106, 234), it was not possible to correct for the presence of HPV from our dataset. For most datasets of this nature however, it is not possible to correct for information related to HPV status. Finally, no provision could be made for AJCC/UICC TNM stage, which would have provided valuable clinical information and would have been paramount to control for within the multivariate analysis.

4.9 Conclusions

There is a discrepancy in health outcomes in patients with increasing social deprivation and this is also evident in patients with head and neck cancer undergoing major surgical procedures. Socially deprived patients are more likely to die as inpatients and overall as outpatients. These patients are associated with an increased number of complications, a prolonged hospital stay and increased probability of recurrence. The strength of these associations and persistence of their effect warrant further exploration to ascertain why this might be the case. Further information, by the way of independent patient interviews, is required to further elucidate the socio-economic demography of each patient group. It is important for similar datasets in the future to not only control for tumour stage, but also for HPV status

Chapter 5: Overall Conclusions

The work presented, highlights the complexity of the pathology at hand; a malady that is defined not only at genotypic and phenotypic levels, but by subsequent re-moulding within the environment in which we live. Head and neck cancer, is more complex still, the result of a disease that etches itself onto the individual's identity and person, altering the way we eat, speak and even breathe. As clinicians the way we respond to and manage these complexities will be continually challenged in the years to come.

5.1 Summary of Findings

5.1.1 The role of immune cell infiltration in Hypopharyngeal Cancer

Overall, there did not appear to be a trend for statistically significant relationships was shown between immune cells survival and outcomes in hypopharyngeal cancer. Although there were results suggestive of an underlying association in certain cases, e.g. $CD4^{+}CD25^{+}FOXP3+$ cells (see Section **Error! Reference source not found.**) and CD1a+ (see Section 2.6.4.9).

Looking at the associations overall, without context of their statistical significance, there did appear to be a story to support a role for increased immune cells in hypopharyngeal cancer but this did not approach significance. It is likely that although having some form of immune response is beneficial to patients overall, it does not carry the same clear improvement of prognosis that is seen in other solid cancers, e.g. oropharyngeal carcinoma.

$CD1a^{+}$ cells were associated with an improved survival with *higher* proportions and this is in keeping with what has proven about hyper-methylation and antigenic presentation in the literature to date (183). In our study increased proportions of $CD1a^{+}$ cells were associated with an improved survival outcome, both overall (HR 0.59, p=0.05) and specific to head and neck cancer (HR 0.51, p=0026). Clearly dendritic cells, with their powerful ability to present antigens are key in anti-tumour immunity. It is also known that specific $CD1a^{+}$ subsites are particularly important in anti-tumour responses and stimulating the host tumour micro-environment and harbour a particular ability to further differentiate, with additional suppressive functions.

Interestingly although, larger populations of $FOXP3^{+}$ cells appeared to show a relationship with better outcome, lower numbers of $FOXP3^{+}$ cells were associated, contrarily with late recurrence

The location of immune cells in the tumour milieu, appeared strictly stromal with a limited permeation of immune cells within the tumour itself. There was support for an increase of SMA

positive cells, with loss of corresponding $CD34^+$ cells, suggesting a stromal reaction perhaps requisite of invasive carcinoma. Survival outcomes were supportive of this also, supporting improved odds of survival in SMA negative patients, though this did not reach significance.

5.1.2 The prognostic impact of nodal metastasis on the outcome of Head and Neck Cancer

The fundamental differences between HPV(+) and HPV(-) disease of the oropharynx were confirmed once again. Overall HPV(+) patients were younger with a predilection to presentation in late AJCC/UICC stages, based on a since retired 7th version of the TNM classification. The total number of positive nodes was greater in the HPV(+) group and this was significant. There was no association to lymph node size.

On survival analysis, there was a strongly significant effect relative to total numbers of positive nodes. Worst outcomes were seen specifically in total positive nodes of 5 or greater and this was seen in both HPV(+) patients and HPV(-). The ratio of positive nodes to total nodes was shown to be significant, namely for the HPV(-) population, where a higher ratio was associated with an increased rate of mortality.

Very interestingly, no statistically significant effect was demonstrated for the presence of ECS and HPV(+) patients, in stark contrast to HPV(-) patients, questioning its role in the management of these patients.

The revamped TNM staging classification (version 8) takes note of this latter effect, with ECS excluded as a prognosticator in HPV(+) positive disease and acting as an immediate indicator of highly aggressive loco-regional disease (N3b – see Appendix N). A short study was performed to confirm the utility of the 8th edition of TNM in down-staging HPV(+) OPSCC within our dataset, based on clinical staging only, and this too was confirmed (see Appendix O).

5.1.3 Social deprivation and its effect upon patients undergoing Major Head and Neck Cancer operations

The importance and effect of social deprivation upon healthcare outcomes is significant and correlation between the two has been proven in the past. Quality of life however has undeniably improved in the preceding decades and alongside this patient choices reflect a changing public attitude towards health and active measures to preserve it; from reduction in smoking incidence (and the subsequent rise of vaporised cigarettes) to fixation on diets and super-foods with greater emphasis placed upon what is consumed and what is experienced by patients on an individual basis. A greater number of students attend higher academic institutions, with emphasis on

professional employments on graduation and a greater proportion of the eligible workforce are in current active employment. Access to healthcare although not universal and entirely comparable across the UK, is fundamentally free and openly available.

Contrary then, to what might be expected, our study demonstrates that social deprivation remains a significant factor in determining presentation and outcomes in patients undergoing major head and neck surgery in the UK. Patients in MSD groups are younger at time of operation that their LSD counterparts. At baseline, they were found to have an increasing burden of morbidity and were much more likely to exhibit laryngeal-hypopharyngeal disease, ordinarily associated with alcohol and tobacco consumption and *less* likely to present with oropharyngeal disease, the latter increasingly related to HPV driven disease (an indicator of higher socio-economic class).

Additionally, patients with lower socio-economic status were found to require relatively more emergency surgery on presentation (an indirect marker of poor patient education, aggressiveness of disease and inability to present symptoms in a timely manner).

Following surgery, MSD patients were more likely to suffer at least one complication and indeed more total complications overall. Said patients were also associated with relatively more incidences of mechanical complications, failures of tracheostomy and PEG tubes. Finally, MSD patients were also associated with a longer inpatient stay. They were much more likely to be re-admitted as an emergency in the 60 days succeeding discharge and more likely to die, both during admission and following discharge.

5.2 Limitations of Studies

5.2.1 The role of immune cell infiltration in Hypopharyngeal Cancer

As described in section 2.7.4, there was a limitation in that the original intended number of included patients required to populate the study was not met. Given the design of this study, there was no defined number to target to attain statistical power, but a larger population would very likely deliver a more meaningful result.

Further to this, included cases with accessible human tissue were subsequently removed due to missing data, precluding accurate if any analysis. From the original drafted total, a further 16% of cases were lost this way.

The methodology also offers limitations in that close to the entirety of the cell-counting and analysis was performed by the lead author, leading to a possibility for observer and assessor bias.

This confounder might have been excluded by the addition of a further assessor who might confirm or refute the findings, thereby adding some degree of validation.

5.2.2 The prognostic impact of nodal metastasis in the HPV era of Head and Neck Cancer

This retrospective study could not be controlled sufficiently to ask a singular question and lacked the accuracy of a prospective study. Retrospective data was based on third party notes and analysis, which although not necessarily false, could be associated with a lack of accuracy. There was an association of selection bias based on the preformed cohort and using this patient list to design a study. Identifying consecutive cases within a prospective trial may have been more useful.

One of the most important outcomes that subsequently became clear in the latter period of this study was the effect of positive nodes greater than 5 in total, for both HPV(+) and HPV(-) cancer. This has been demonstrated elsewhere in the literature (238) and indeed forms an important part of the contemporaneous TNM staging, version 8, where for cases of HPV positivity, 4 or more nodes are used to differentiate pN1 (≤4 giving an N1 staging and >4 grading as N2).

5.2.3 Impact of social deprivation on the outcome of major head and neck cancer in Surgery

The greatest limitation of this study was based upon the English Index of Multiple Deprivation 2015 score. Being a relative measure, it could not quantify exactly how deprived a particular area was and reflect real time changes in deprivation. It acted merely as a single snapshot of an area in time.

Furthermore, its results do not pertain to individual people, instead extrapolating results to a singular geographic area within a country. Given that LSOA contains at least 1,500 residents, although certain assumptions could be made about its domestic population, individual and discrete measurements are impossible and even the former are implied as opposed to defined. Finally, as comprehensive as the 7 comprising parameters of the index are, there are likely to be measures that influence socio-economic standing that cannot be (easily) measured. Supplemental benefits represent one such predicament, whereby families who might be considered as of “normal” socio-economic status may still claim benefits.

Additionally, coded data can be generic and approximate in itself and healthcare outcomes can be imprecisely forced into the aforementioned codes with subsequent irretrievable loss of clinical information. Whilst Hospital Episode Statistics serve as an invaluable resource in improving

patient care, there are certain shortcomings. HES for now is limited largely to demographic, diagnostic and procedural information and does not accurately collect enough information to allow full disclosure of a patients journey before hospital, in primary care, to discharge and their return.

Follow up data is lacking, particularly in outpatient departments and accident and emergency units. Most importantly, there is no data that reliably marries primary to secondary care data. A death in the community exemplifies this most clearly in our dataset above – where a patient not coded as dead in HES (but given the natural history of disease, is highly likely to be so), must be censored to follow up as there is no primary care data to confirm or refute this premise. On an individual level this may be inconsequential but statistically speaking, this can considerably alter conclusions on management, outcome and natural history of disease.

Despite these limitations, the study overall corroborates what was suspected in the original hypothesis and also what has been defined elsewhere, that socio-economic status is an independent risk factor to adverse health. Most importantly, socio-economic status, although perceived as an obsolete parameter of modern life, clearly creates a discrepancy in healthcare outcomes and this persists in patients undergoing head and neck surgery.

In order to deliver uniform healthcare to a socially diverse population, resources need to be apportioned such that uniform outcomes are seen across the UK. Specific public health policies targeting socially deprived populations may be imperative, in order to represent such groups better in risk-adjustment models. This study may harbour implications for head and neck cancer units that cater to more socially deprived populations and therefore potentially reflect a need for a proportionate degree of funding and investment due to a greater burden upon resources.

5.3 Final Key Messages and Implications for patients

1. Hypopharyngeal cancer should be considered an “immune cold” cancer where changes in immune infiltrate do not necessarily cause any effect in survival outcomes, in the same way as Oropharyngeal cancer.
2. Hypopharyngeal cancer is therefore unlikely to be appropriate for future systemic immunotherapies, where signalling pathways are specifically targeted for treatment.
3. Hypopharyngeal tumour stroma appeared to be important in tumorigenesis, with some evidence for a conversion from $CD34^{+}$ labelled fibroblasts, to α-SMA so called Cancer Associated Fibroblasts. This may require further investigation in the future as it may possibly lend information to the pathophysiology of hypopharyngeal cancer.

4. The current implementation of number of lymph nodes into pathological nodal staging in TNM v8 and may be a significant tool for differentiating patients into high and low risk treatment groups.
5. Using revised staging systems for treating patients will allow downstaging of cancer and subsequently a lower intensity regimen of treatment, the implication being oncological control with a reduced incidence of post-treatment complications.
6. Patients undergoing major head and neck surgery are affected by their pre-existing socio-economic status.
7. This affects patients not only at point of diagnosis and treatment outcomes during inpatient stay, but also affects long term outcomes with patients more likely to suffer cancer recurrence and to die.
8. Future studies should identify explore reasons for these differences in outcome; i.e. why does socio-economic state affect healthcare outcomes in the way it does and also address means by which pre-operative care can be optimised so to homogenise care.

Appendices

Appendix A Potential Therapeutic Applications

Immunotherapies can be sub-divided into monoclonal antibodies, adoptive cell transfer, cytokines and vaccines. In the US, anti-PD1 is FDA approved for second line HNSCC treatment. Recently, the UK has NICE approval for anti-PD1 in the same setting(261, 262).

Monoclonal antibodies

Monoclonal antibodies are highly specific, associated with limited side effects and relatively easy to produce (263). These biologically active agents are synthetically created to recognise target molecules that either block an immune response (e.g. PD-1, PD-L1 or CTLA-4) or an over-expression in malignant cells (e.g. EGFR).

The first monoclonal antibody to show significant clinical effect in cancer was *Ipilimumab* (YERVOY®, Bristol-Myers Squibb), a monoclonal antibody designed to block the CLTA-4 protein to promote continued cytotoxic T cell activity. Since March 2011, FDA approval was secured when published data showed significantly improved OS in patients with late-stage metastatic melanoma (264). In late stage melanoma, it has also demonstrated a significant OS benefit as postsurgical adjuvant treatment(265). Early phase trials of anti CTLA-4 have begun in HNSCC, for late stage disease, in the USA (NCT03019003, NCT03162731), France (NCT03212469) and Korea (NCT03292250).

In the USA, *Pembrolizumab* (anti PD-1; KEYTRUDA®, Merck) has already gained accelerated FDA approval for clinical use in HNSCC (266) given its promising performance in PD-L1 positive patients (267). In the KEYNOTE- 012 trial (recurrent and/or metastatic HNSCC) overall response rate was 18% (95% CI, 12 -26%) in 132 patients. PD-L1 positive patients (tumour *and* immune cell positive) had a statistically significant increase in overall response rate (22% v 4%; P = .021), measured by the response evaluation criteria in solid tumours (ReCIST) (268). Data reported separately showed that the response to *Pembrolizumab* was greater in patients who were both PD-L1 and PD-L2 (27.5%) positive, than those positive only for PD-L1. PD-L2 expression was a significant predictor of progression-free survival (PFS), independent of PD-L1 status (269). Of importance however, particularly in the pooled analysis subsequently, was the finding that *Pembrolizumab* was effective regardless of PD-L1 status(270).

The Checkmate 141 study, (NCT02105636) tested the use of *Nivolumab (*anti PD-1*;* YERVOY®, Bristol-Myers Squibb) in patients with HNSCC at progression following platinum-based therapy (154). Median follow up was 5.1 months. It demonstrated that *Nivolumab* reduced the risk of death by 30% (7.5 months vs. 5.1 months OS) compared with standard (investigator's choice) treatment(154) and more patients reached a 1-year progression free survival (36.0% vs. 16.6%)(154). Overall response rate and partial response rate was 13.3% and 10.8% for *Nivolumab* and 5.8% and 5.0% for standard therapy(154).

Cetuximab is an anti-epidermal-derived growth factor receptor (EGFR) chimeric (human/mural derived) IgG1 isotype monoclonal antibody. It has been studied within the context of induction regimes, combination therapies and advanced and recurrent HNSCC cases (271). EGFR is often over-expressed in HNSCC and associated with a reduced progression free survival (101, 272). *Cetuximab* has demonstrated down-regulation of EGFR expression and subsequent radio-sensitisation, improved locoregional control and OS (273). Although 90% HNSCC over-express EGFR (274), clinical responses have only been seen in 10-15% of patients (131, 275) and response with *Cetuximab* does not bear any relationship with clinical outcome when stratifying for levels of tumour EGFR (276).

Combination therapies are also currently being explored in cases of recurrent and/or metastatic HNSCC. KESTREL (NCT02551159) is a phase III open label study, randomising patients into one of three arms; Durvalumab alone (anti-PD-L1 antibody), Durvalumab and Tremlimumab (anti-CTLA-4 antibody) combined and current standard of care. Outcomes include OS at 2 years and trial recruitment completes in December 2018(277), see Table 1. Similarly, the EAGLE study (NCT02368974) tests the same drug combinations with a different recruitment ratio (1:1:1 ratio as opposed to KESTREL's 2:1:1 in favour of the combination treatment, see Table 1(278).

Durvalumab has also been investigated with Tremelimumab in combination with the oligonucleotide STAT3 inhibitor AZD9150 and the CXCR2 inhibitor AZD5069 in a phase I trial. Initial results are promising for safety and efficacy, showing benefit to anti-tumoural activity with a 45% disease control rate in a PD-L1 treatment naïve arm and 20% in those pre-treated with PD-L1 at 12 weeks(279).

Although the p53 genetic deletion remains the most common mutation associated with HNSCC, other common mutations have also been targeted therapeutically. The phosphoinositude 3-kinase (PI3K)-PTEN-AKT has been seen in up to 20% of HNSCC cases(280). This pathway has been countered via various targeted therapies which are out of scope of this article (281).

Adoptive cell transfer

There are several types of adoptive cell transfer including TILs, TCRs (T cell receptors) and CARs (chimeric antigen receptors). This treatment expands the T cell population (with or without modification) from the patient *ex vivo,* and then re-introduces them often following immuno-depletion. The most clinically advanced (i.e. ready for use in the clinical setting) cell transfer is CAR-T where the T cells are genetically engineered to produce chimeric antigen receptors. In the US, CAR-T was approved in August 2017 for paediatric acute lymphoblastic leukaemia.

Within head and neck cancer, adoptive cell transfer of NK T cells has demonstrated some promise. In one study, including 10 patients treated for locally recurrent but operable HNSCC, five were associated with tumour regression and seven with an increase in NK T cell volume within the tumour (282). There are currently 4 trials recruiting for adoptive cell transfer in HNSCC; two in the US and two in Japan. The US trials include TILs (NCT03083873) and TCR (NCT03247309).The Japanese trials are recruiting for TCR in patients with MAGE-A4-expressing tumours (NCT02096614) or NY-ESO-1 tumours (NCT02366546). Melanoma antigen E (MAGE), are quiescent antigens in normal tissue, but up-regulated in HNSCC tissue in up to 70% of patients (283) making it a good target for immunotherapy treatment.

Cytokines

Cytokine therapy is one of the older means of co-ordinating immuno-modulation and has met limited success, at least as a monotherapy in HNSCC (284). A host of cytokines have been investigated, including GM-CSF, IL-1, IL-12 and Interferon-α (263) but have commonly been associated with a degree of toxicity, not tolerated by patients (285). No approval for patient use exists currently, but Phase I-III trials are ongoing (286).

Vaccines

HPV(+) tumours offer an exciting potential for the development of therapeutic cancer vaccines as they are likely to express viral antigens. As with many immunotherapies, studies into melanoma have refined this subject area considerably. RNA-lipoplexes administered intravenously prime APCs (and specifically DCs) in lymphoid organs, stimulating an *in situ* inflammatory response (287). The subsequent interferon response has been shown to be integral to the control of progressive melanoma tumours (287). This delivery system is currently being investigated in an early phase trial for HNSCC patients, using HPV-16 E6 and E7 mRNA (HARE-40: CPMS Trial ID 30900).

DNA vaccines have been used successfully in pre-malignant HPV induced cervical lesions(288). VGX-3100 was shown to induce strong E6/E7-specific CD8 T cell responses and has been tested in a large randomized placebo-controlled trial where clearance of CIN2-3 was increased from 30% to

50% with vaccination, and clinical response was linked with a vaccine-induced immune response(289, 290). Although other DNA vaccines (GX-188E) have also shown a good clinical effect (lesion regression in 7 out of 9 patients with CIN3)(291) this is not the case with all DNA vaccines (pnGVL4a-CRT/E7 DNA) (292).

Peptide vaccines have met with mixed success in treating HPV(+) cervical lesions. GLBL101c is a bacterial vector vaccine administered orally, that expresses HPV-16 E7 protein. 70% of CIN3 patients were downgraded to CIN 2 on this peptide vaccine and HPV-specific T-cells were detected in the cervix(293). Despite this encouraging evidence in cervical pre-malignant disease, it is likely that more established cancers may require combination treatments to overcome immune evasion(294). A synthetic long-peptide was used in 20 women with HPV-16(+) high-grade vulvar intraepithelial neoplasia. At 12 months almost 50% of trial patients had a complete response, which correlated with induction of HPV-16-specific immunity(295).

MAGE-A3 is highly expressed and associated with a poor prognosis in HNSCC (296), forming an ideal study marker for vaccination. Phase I studies have begun and recently a dose escalation study of patients (n=16) with recurrent HPV(+)/MAGE-A3^{+} disease demonstrated no toxicity at tested doses and was positively associated with both T cell and antibody responses (296).

DC based vaccines are produced from DCs harvested by a leukopheresis from individual patients. The cells are exposed or transfected *in vitro* to the relevant tumour antigen and re-introduced *in vivo* to stimulate a specific T cell response (263). Of all the genetic mutations, p53 is the most common in HPV(-) disease (seen in up to 62%) (297). Following DC vaccination T cell response in vitro could be stimulated in both normal and HNSCC populations (298). A recent phase I study (n=16) randomised 3 groups of patients (HLA Class p53 peptide arm, tetanus toxoid arm and additional wild type p53 specific peptide arm) and addressed disease free survival (DFS) at two and three years, using controls as patients treated at the authors' institution. A favourable DFS was seen in the two DC vaccinated cohorts (88% and 80% respectively vs. 70%) (299).

Appendix B UICC TNM Classification version 7

Adapted from TNM Classification of Malignant Tumours (300)

T-Primary Tumour	
Oral Cavity	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour ≤2cm in greatest dimension
T2	Tumour >2cm but ≤4cm in greatest dimension
T3	Tumour >4cm in greatest dimension
T4a (*lip*)	Tumour invades cortical bone, inferior alveolar nerve, floor of mouth or skin (chin or nose)
T4a (*oral cavity*)	Tumour invades cortical bone, deep/extrinsic muscle of the tongue, maxillary sinus or skin of face
T4b (*lip and oral cavity*)	Tumour invades masticator space, ptertygoid plates, or skull base, or encases internal carotid artery
Oropharynx	
T1	Tumour ≤2cm in greatest dimension
T2	Tumour >2cm but ≤4cm in greatest dimension
T3	Tumour >4cm in greatest dimension or extension to lingual surface of epiglottis
T4a	Tumour invades any of the following: larynx, deep/extrinsic muscle of tongue, medial pterygoid, hard palate or mandible
T4b	Tumour invades any of the following: lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base; or encases carotid artery

Hypopharynx	
T1	Tumour ≤2cm in greatest dimension and/or limited to one subsite of hypopharynx
T2	Tumour >2cm but ≤4cm in greatest dimension or invades more than one subsite of hypopharynx or adjacent site, *without* fixation of hemi-larynx
T3	Tumour >4cm in greatest dimension or *with* fixation of hemi-larynx or extension to oesophagus
T4a	Tumour invades any of the following: thyroid, cricoid cartilage, hyoid bone, thyroid gland, oesophagus or central compartment soft tissue
T4b	Tumour invades prevertebral fascia, encases carotid artery or invades mediastinal structures
N-Regional Nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, ≤3cm in greatest dimension
N2	
N2a	Metastasis in a single ipsilateral lymph node >3cm but ≤6cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, ≤6cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes ≤6cm in greatest dimension
N3	Metastasis in a lymph node more than 6cm in greatest dimension
M-Distant Metastasis	

M0	No distant metastasis
M1	Distant metastasis

Appendix C Window of Opportunity Trials in Head and Neck Cancer

Materials and Methods

887 patients were retrospectively identified from patients diagnosed with HNSCC and treated from 2000-2010. Patients were excluded from further analysis if; time to treatment information was incomplete, cause of death was unknown, follow up was less than six months and time to treatment was greater than 60 days. A total of 662 patients remained (University Hospital Southampton n=532, Poole NHS Foundation Trust n=130). Ethical approval was obtained (UKCRN 8130; ISRCTN 71276356; and REC references 09/H0501/90 and 07/Q0405/1).

In keeping with current clinical practice, patient groups were stratified according to HPV status and tumour stage. Three categories of covariate variables were defined based on time to surgical treatment; <15 days, 15-30 days and 31-60 days respectively and were chosen to reflect current clinical practice guidelines.

Time to treatment was defined as date of first contact to definitive medical treatment. From our group's previous experience, we have assumed that trial enrolment would have the potential to delay definitive surgical treatment by up to 14 days. Therefore, baseline hypothesis deems no significant difference on patient mortality outcomes, when treatment is delayed by 1-2 weeks.

Statistics

The primary endpoint defined was disease specific survival, i.e. death from HNSCC. Survival time (months) was defined as the time from date of original diagnosis to either date of death from HNSCC, or date of last known living consultation. Patients who died of other causes were censored at the time of death. Kaplan Meier plots with log rank tests were used alongside Cox's proportional hazards model to annotate survival data.

Appendix D

Results

Of the 662 patients enrolled into the study, 116 (17.5%) were HPV positive. There were 433 deaths from disease in all patients in the study period, to a maximum follow up of 135 months (median follow-up 54 months). In keeping with the literature, patients with HPV^{+} tumours were found to have a significantly improved disease specific survival than those with HPV^{-} disease (HR 0.56 [95% CI 0.38-0.84}, p=0.004).

Survival analyses on the defined groups were performed and are documented in Figure 1. Analysis was performed for all patients, differentiating for HPV positivity, disease stage and mode of treatment. When considering the length of time to treatment, no significant difference was demonstrated in any given circumstance on Kaplan-Meier survival curves. Patient outcome was unaffected by time to treatment, whether within the specified 31-day period. An effect was noted at greater than 60 days.

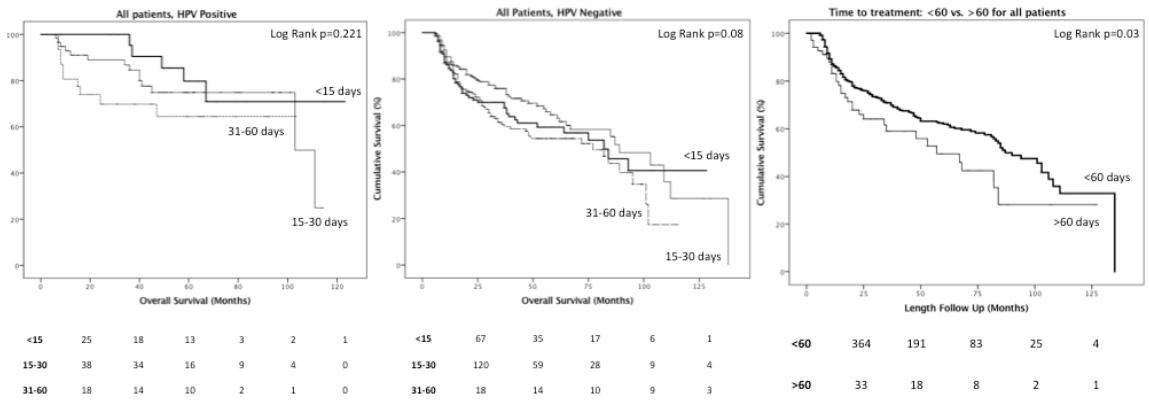


Figure 1. Survival charts for HPV(+) patients (left), HPV(-) patients (middle) and all patients (right).

Discussion

This data demonstrates no impact on patient survival when considering time to delivery of definitive treatment, up to 60 days. This would support a move toward a controlled adjournment of traditional treatment so to allow for institution of window studies. An instinctive reticence to delaying first line treatment will always prevail and in many cases, particularly where diagnosis presents a clinical challenge, an accelerated effort to begin patient treatment is understandable. This is supported in the literature where pronounced delays (greater than 3 months) confer an undeniable disadvantage to patient outcomes.[i] In one systematic review, there were no negative associations between treatment delay and patient outcome in patients with HNSCC.[ii] In other cancers, findings were mostly mixed and only breast, colorectal melanoma consistently suggesting that a delay in patient treatment resulted in negative patient outcomes.

Our data found a similar negative effect on outcome, but only when initiation of treatment was delayed beyond 60 days, as demonstrated in Figure 1 (p=0.03). In our data, median time to treatment was 26 days and even accounting for delays for up to one month, this would still be within 60 days and statistically confer no effect upon OS.

This short correspondence goes some way into informing the window of opportunity debate, affording clinicians an opportunity to allow for delivery of experimental molecular treatments. On the basis of ease of access to anatomical site and patient turnover, HNSCC patients provide an ideal population upon which to institute window of opportunity studies.

Understandably there are limitations to retrospective reviews of this nature. Our outcomes are difficult to generalise due to a relatively small cohort of patients and limited diversity of the subject population. Nevertheless, in relevant patients, a postponing of definitive treatment so to allow for assessment of experimental therapy could be feasible. NTA can be assessed in the absence of a likely attrition rate due to advanced stage tumour burden and treatment resistance. New treatments can be instituted and monitored and hopefully developed without erroneous early discarding. If performed correctly, they may allow for further informing of a robust and successful Phase II and/or Phase III study

Appendix D Data Parameters for Chapter 3

Information from Ward et al (22).

Patient name	
Patient hospital number	
Patient date of birth	Date
Alcohol Status	0 = Non-drinker, 1 = Drinker, 2 = Ex drinker, 4 = Unknown
Date of Diagnosis	Date
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 = tonsillar-lingual sulcus, 15 = pyriform fossa, 16 = post-cricoid, 17 = supraglottis, 18 = glottis, 19 = vallecula, 20 = lung, 21 = other
Primary Side	0 = Unknown, 1 = Right, 2 = Left, 3 =Bilateral
Final T Stage	0 = T0, 1 = T1, 2 = T2, 3 = T3, 4 = T4, 5 = Tx, 6 = Tis, 7 = T1a, 8 = T1b, 9 =T4a, 10 = T4b
Final N Stage	0 = N0, 1 = N1, 2 = N2a, 3 = N2b, 4 = N2c, 5 =
Distant Metastases at presentation?	0 = No, 1 = Yes
HPV Status	0 = Negative, 1 = Positive

Appendix D

Primary Treatment	0 = Surgery, 1 = Radiotherapy, 2 = Chemoradiotherapy, 3 = None/Palliative treatment, 4 = Neoadjuvant/CRT, 5 = Chemotherapy, 7 = Neoadjuvant chemoradiotherapy
Cause of Death	0 – Still alive/died of other cause, 2 – Died of disease
Living Status	0 = Dead, 1 = Alive

Information collected additionally to above parameters

Smoking Status	0 = ≤ 10 pack years, 1 = >10 pack years
Neck Dissection	0 = No, 1 = Yes
Neck Dissection Side	1 = Right, 2 = Left, 3 = Bilateral
Total Number of Nodes	Number
Total Number of Positive Nodes	Number
Size of Positive Nodes (mm)	Number
Presence of Extra-Capsular Spread	0 = No, 1 = Yes
Last Known Contact Date	Date
Date of Death	Date

Appendix E Groovy Computer Script for CD8 cells

```
setImageType('BRIGHTFIELD_H_DAB');

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"Background" : " 255 255 255 ")');

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"darkBackground": false,  "smoothImage": true,  "medianCleanup": true,  "dilateBoundaries":
false,  "smoothCoordinates": true,  "excludeOnBoundary": true,  "singleAnnotation": true}fe');

selectAnnotations() //

runPlugin('qupath.imagej.detect.nuclei.PositiveCellDetection', '{"detectionImageBrightfield":
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"medianRadiusMicrons": 0.0,  "sigmaMicrons": 1.5,  "minAreaMicrons": 10.0,  "maxAreaMicrons":
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"makeMeasurements": true,  "thresholdCompartment": "Cell: DAB OD mean",
"thresholdPositive1": 0.2,  "thresholdPositive2": 0.4,  "thresholdPositive3": 0.6,
"singleThreshold": true}');

def shortName = getCurrentImageData().getServer().getShortServerName()

def outputName = shortName + '.txt'

def pathExport = buildFilePath(PROJECT_BASE_DIR, 'results')

mkdirs(pathExport) // Just to be sure

pathExport = buildFilePath(pathExport, outputName)

saveAnnotationMeasurements(pathExport)
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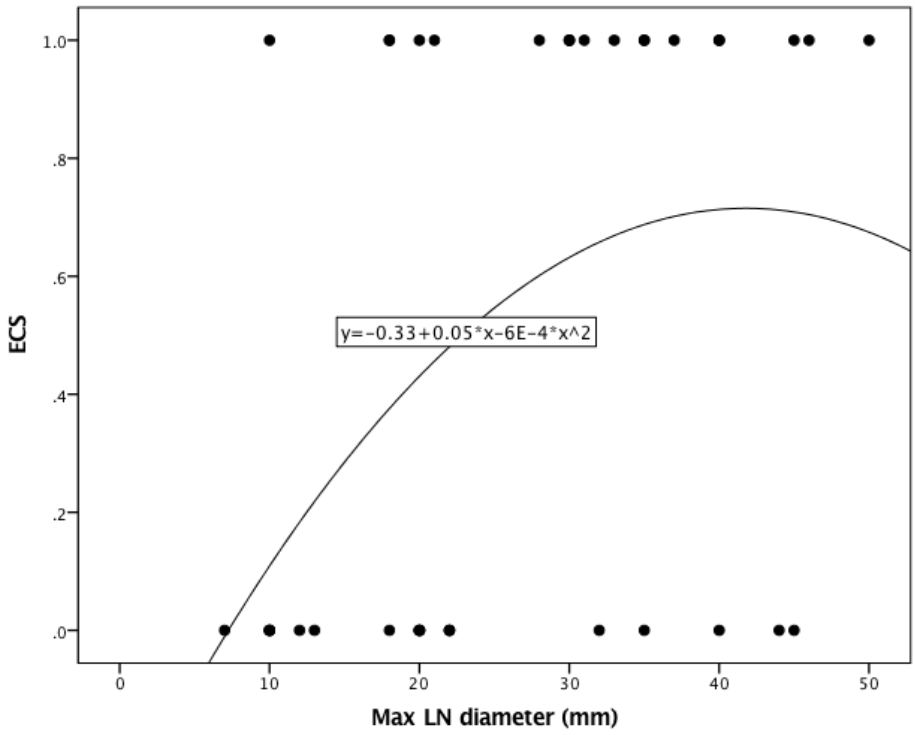
Appendix F Regression Analysis ECS vs. LN diameter

Simple logistic regression detailing the relationship between LN diameter and ECS. No significant association was made for lymph node ratio (OR 0.72 [95% CI 0.13-4.06], p=0.711) or number of positive nodes (OR 1.55 [0.92-2.62], p=0.102) though perhaps the latter lacked numbers sufficient enough to power the calculation.



Appendix G Relationship of Radiotherapy to OS

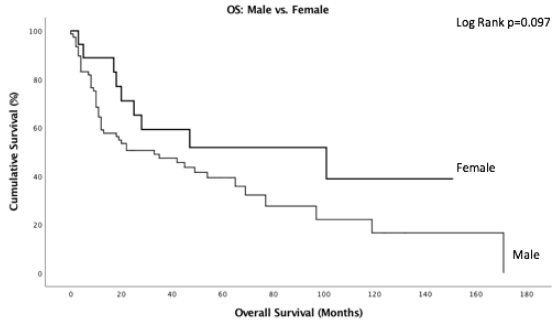
A table demonstrating relationship of radiotherapy to survival outcomes. No change to this association was demonstrated on exclusion of the outlying quartile of survival (p=0.144 and p=0.026).

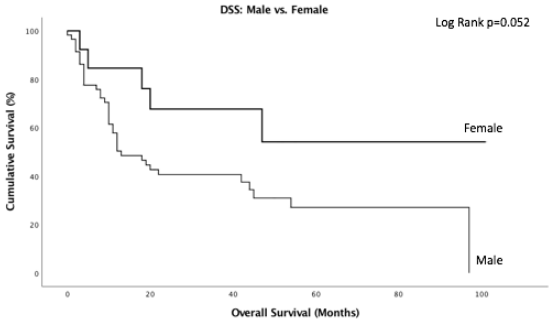
Patient Outcome	Odds Ratio	Significance
No Radiotherapy	1	
Primary Radiotherapy	2.73 (95% CI 0.74-10.07)	p=0.130
Post-operative Radiotherapy	4.80 (95% CI 1.21-19.08)	p=0.026

Appendix H Relationship of patient demographics, tumour characteristics, histology and cancer treatment to survival outcomes

5.1 Gender

Patient gender was found to be indicative of poor survival, with male patients faring worse than female (HRs: OS 1.72, p=0.139, DSS 1.75, p=0.047), see Figure 5-1 and Table 15.





Time (months)	20	40	60	80	100	120	140	160
Male	38	26	13	6	4	3	1	0
Female	12	8	4	4	4	2	1	0

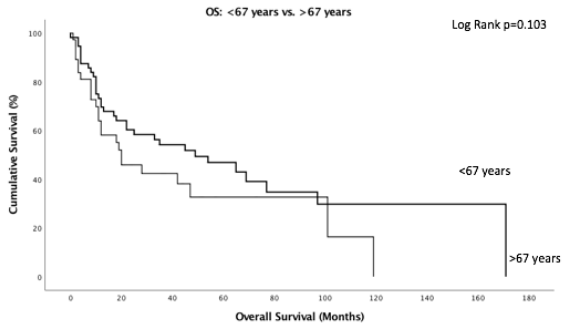
Time (months)	20	40	60	80	100
Male	22	13	5	1	0
Female	8	5	1	0	4

Figure 5-1. Kaplan Meier Survival Analysis for all patients and Gender, left OS (p=0.097) and right DSS (p=0.052)

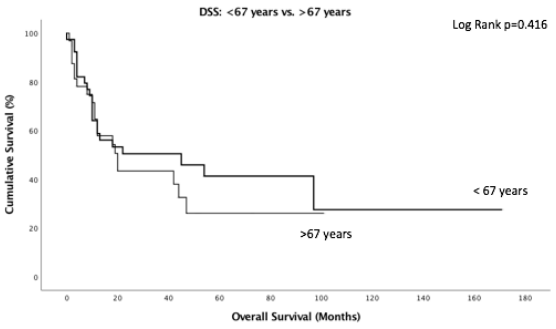
5.2 Age

There appeared to be a trend for increasing age and poor outcome (HRs: OS 1.02, p=0.08, DSS 1.02 p=0.165), see Figure 5-2 and Table 9. Relationships between CD34+ levels and SMA and p16 status, CD3+, CD4+ and CD8+ cells in hypopharyngeal carcinoma (n=95)

.



Time (months)	20	40	60	80	100	120	140	160
<67 years	34	23	14	8	6	6	2	1
>67 years	16	10	3	2	2	0		



Time (months)	20	40	60	80	100	120	140	160
<67 years	19	11	6	3	2	2	2	1
>67 years	12	8	2	1	0			

Figure 5-2. Kaplan Meier Survival Analysis for all patients and Age, left OS (p=0.103) and right DSS (p=0.416)

5.3 Weight

There was no significant association between weight and outcomes of patients with hypopharyngeal carcinoma (HRs: OS 0.99, p=0.991, DSS 0.98, p=0.517). However, there was a trend supporting that increasing BMI was related to improved survival (HRs: OS 0.96, p=0.339, DSS 0.85, p=0.09), see Table 15.

5.4 Tobacco Consumption

High vs. low/no tobacco consumption demonstrated a negative association with those in the former group (HRs: OS 1.19, p=0.627, DSS 1.44, p=0.387), see and Figure 5-11. A significant negative association on DSS was seen on consumption of alcohol alone (HR: OS 1.68, p=0.113, DSS 2.65, p=0.013), see Table 15.

Combining patients with both high alcohol consumption and heavy tobacco use, demonstrated poor survival and this was significant, (HRs: OS 1.81, p=0.108, DSS 2.33, p=0.056), see Table 9. Relationships between CD34+ levels and SMA and p16 status, CD3+, CD4+ and CD8+ cells in hypopharyngeal carcinoma (n=95)

and Figure 5-12.

.

		OS		Disease Specific Survival	
		HR (95% CI)	*Significance*	*HR (95% CI)*	*Significance*
Gender	*Female*	1		1	
	Male	1.72 (0.84-3.5)	p=0.139	2.59 (1.01-6.6)	**p=0.047**
Age	*Cont. variable*	1.02 (0.99-1.05)	**p=0.08**	1.02 (0.99-1.05)	p=0.165
Weight	*Cont. variable*	0.99 (0.96-1.04)	p=0.911	0.98 (0.94-1.03)	p=0.517
BMI	*Cont. variable*	0.96 (0.89-1.04)	p=0.339	0.85 (0.86-1.01)	**p=0.09**
Smoking Status	*Non/Ex/ ≤10py smoker*	1		1	
	>10py smoker	1.19 (0.59-2.43)	p=0.627	1.44 (0.63-3.32)	p=0.387
Alcohol	*Non/Ex/≤20u alcohol*	1		1	
	>20u	1.68 (0.88-3.19)	p=0.113	2.65 (1.22-5.73)	**p=0.013**
Smoker and Alcohol	*Control+*	1		1	
	*Heavy smoker or alcohol**	1.81 (0.88-3.75)	p=0.108	2.33 (0.98-5.55)	**p=0.056**

Table 15. Hazard ratios for OS and DSS for each demographic factor in patients with hypopharyngeal SCC. +Control = Non/Ex-smoker, Non/Ex alcohol, ≤10py smoking or ≤10u alcohol. *>10py smoking or >10u alcohol

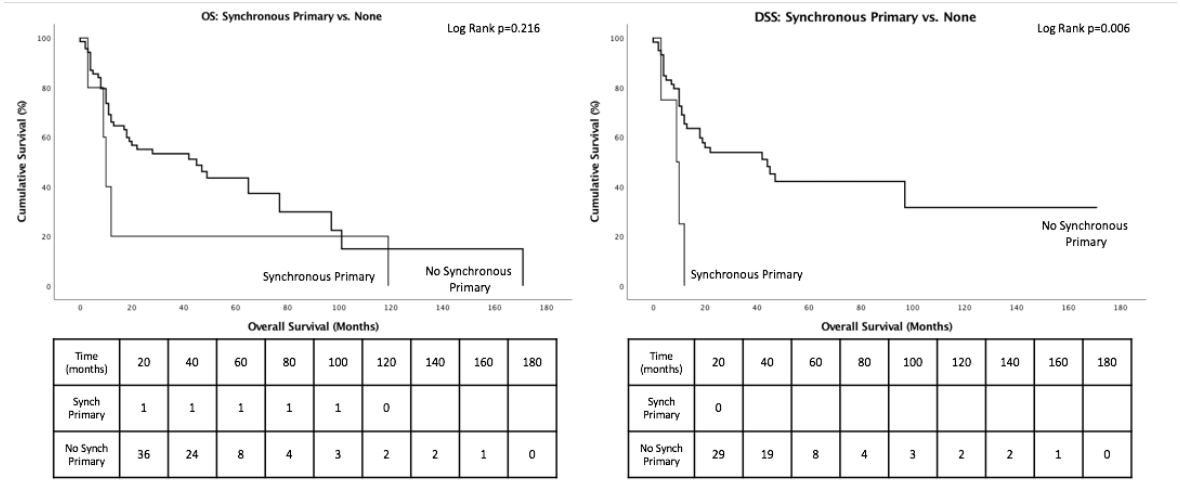
5.5 Relationship of Tumour Characteristics and Survival Outcomes

5.5.1 Tumour Site

Lateral and posterior pharyngeal wall tumours were combined to make a single 'pharyngeal wall" group. A single patient with supraglottic cancer was removed from analysis (erroneously coded as a hypopharyngeal cancer). There was no association between sub-site of hypopharyngeal cancer and survival (see Table 10 and Figure 5-13).

5.5.2 Presence of synchronous primary tumour

The presence of a synchronous primary was significantly associated with a poor survival outcome (HRs: OS 1.82, p=0.227, DSS 3.98, p=0.012), see Figure 5-3 and Table 16. This may be related to field-change and will be discussed in detail later.



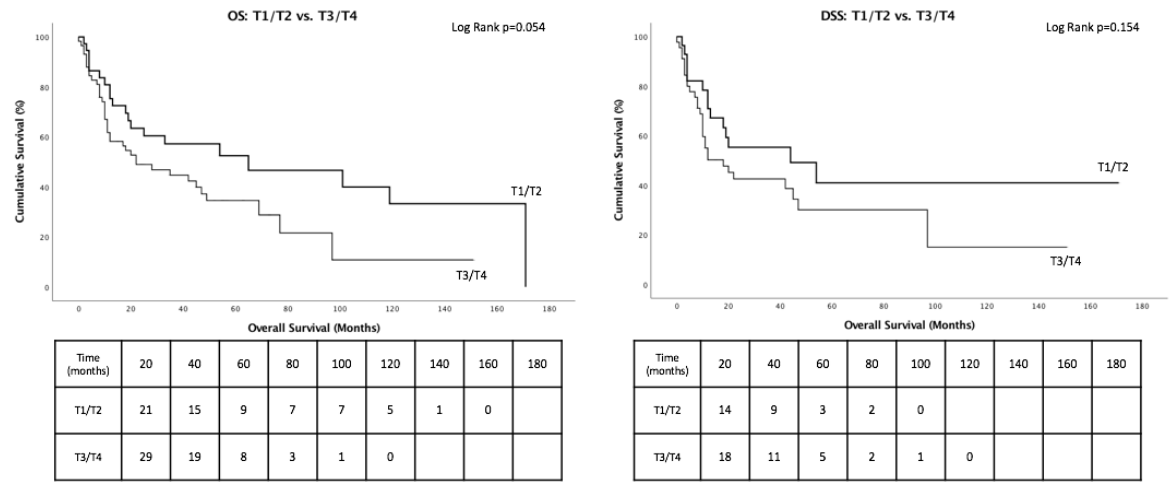
Time (months)	20	40	60	80	100	120	140	160	180
Synch Primary	1	1	1	1	1	0			
No Synch Primary	36	24	8	4	3	2	2	1	0

Time (months)	20	40	60	80	100	120	140	160	180
Synch Primary	0								
No Synch Primary	29	19	8	4	3	2	2	1	0

Figure 5-3. Kaplan Meier Survival Analysis for presence of synchronous primary and survival in patients with hypopharyngeal SCC, left OS (p=0.216) and right DSS (p=0.006).

5.5.3 Tumour stage and survival outcomes

Tumour stage was divided into groups comparing 'T1 and T2' and 'T3 and T4' tumours. Increasing tumour size was associated with a poorer outcome, though this did not achieve significance (HRs: OS 1.72, p=0.059, DSS 1.57, p=0.165), see Table 16 and Figure 5-4.



Time (months)	20	40	60	80	100	120	140	160	180
T1/T2	21	15	9	7	7	5	1	0	
T3/T4	29	19	8	3	1	0			

Time (months)	20	40	60	80	100	120	140	160	180
T1/T2	14	9	3	2	0				
T3/T4	18	11	5	2	1	0			

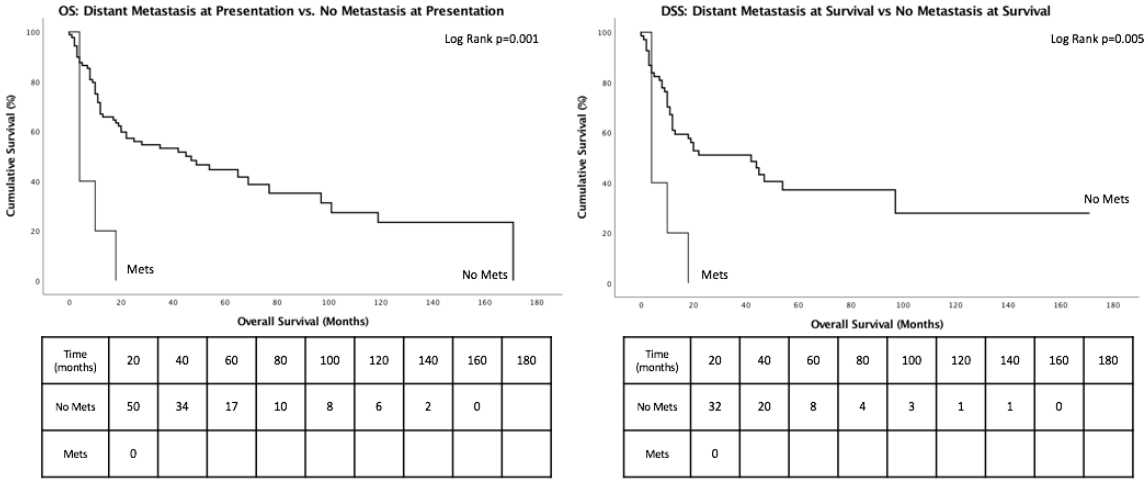
Figure 5-4. Kaplan Meier Survival Analysis for TNM T stage and survival in patients with hypopharyngeal SCC, left OS (p=0.054) and right DSS (p=0.154).

5.5.4 Nodal stage and survival outcomes

Nodal groups were created for N0/N1 and N2/N3. No difference was demonstrated for survival between N0/N1 and N2/N3 groups from the hypopharyngeal cohort, see Table 10 and Figure 5-14.

5.5.5 Distant Metastasis at presentation and survival outcomes

The presence of metastasis at presentation was associated with worse survival and this was significant (HRs: OS 4.43, p=0.002, DSS 3.55, p=0.010), see Table 16 and Figure 5-5.



Time (months)	20	40	60	80	100	120	140	160	180
No Mets	50	34	17	10	8	6	2	0	
Mets	0								

Time (months)	20	40	60	80	100	120	140	160	180
No Mets	32	20	8	4	3	1	1	0	
Mets	0								

Figure 5-5. Kaplan Meier Survival Analysis for metastasis at presentation and survival in patients with hypopharyngeal SCC, left OS (p=0.001) and right DSS (p=0.005).

		OS		Disease Specific Survival	
		HR (95% CI)	*Significance*	*HR (95% CI)*	*Significance*
Primary Site	*Pharyngeal Wall*	1		1	
	Pyriform Fossa	0.64 (0.23-1.83)	p=0.644	0.52 (0.19-1.35)	p=0.179
	Post-cricoid	0.73 (0.21-2.53)	p=0.624	0.74 (0.21-2.59)	p=0.642
Synchronous Primary	*Not present*	1		1	
	Present	1.82 (0.69-4.78)	p=0.227	3.98 (1.35-11.74)	**p=0.012**
Distant Metastasis	*Not present*	1		1	
	Present	4.43 (1.7-11.54)	**p=0.002**	3.55 (1.35-9.32)	**p=0.010**
AJCC/UICC Tumour Stage	*Stage I*	1		1	
	Stage II	0.93 (0.22-3.93)	p=0.604	0.73 (0.12-4.45)	p=0.730
	Stage III⁺	0.47 (0.09-2.33)	p=0.467	0.78 (0.13-4.69)	p=0.785
	Stage IV	1.00 (0.31-3.26)	p=0.756	1.00 (0.24-4.26)	p=0.999
T Stage	*T1/T2*	1		1	
	T3/T4	1.72 (0.98-3.03)	**p=0.059**	1.57 (0.83-2.98)	p=0.165
Nodal Stage	*N0*	1		1	
	N1	1.34 (0.54-3.49)	p=0.503	1.49 (0.51-4.39)	p=0.461
	N2	1.09 (0.61-1.98)	p=0.763	1.25 (0.59-2.59)	p=0.555
	N3	3.49 (0.45-29.93)	p=0.229	3.09 (0.39 - 24.74)	p=0.287

Table 16. Hazard ratios for OS and DSS for each tumour characteristics. Significant results are marked in bold.

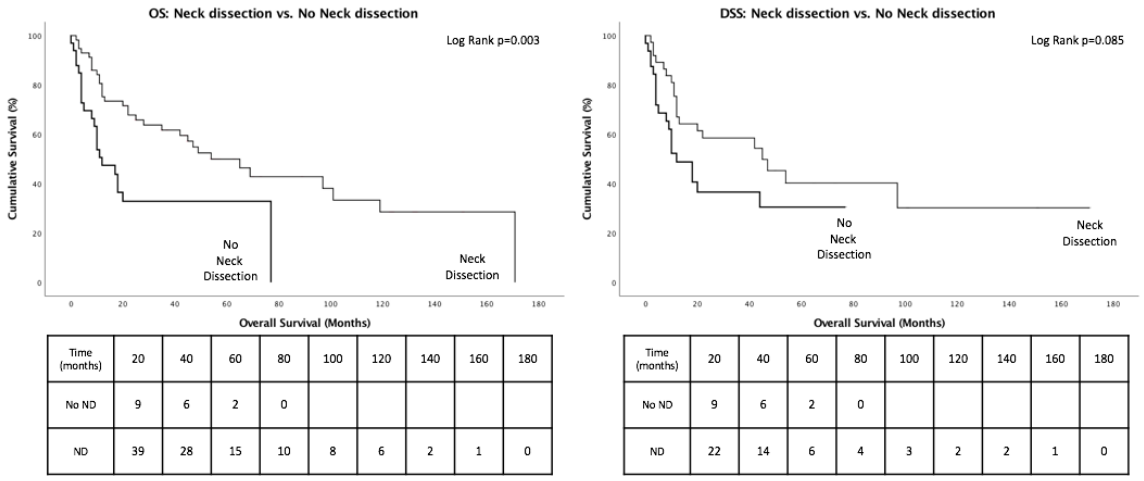
5.6 Relationship between tumour histology and survival outcomes

5.6.1 Tumour grade

No association was seen for grade of tumour at diagnosis and survival outcome, see Figure 5-15 and Table 11.

5.6.2 Neck Dissection

Patients who did not undergo a deck dissection, at any stage of their treatment, had a better survival (HRs: OS 0.44, p=0.004, DSS 0.58, p=0.094), see Table 17 and Figure 5-6. This was confirmed on adjusted multivariate analysis (HRs: OS 0.36, p=0.031, DSS 0.45, p=0.110), see Table 19. This is likely to reflect the patient stage of disease and will be discussed later.



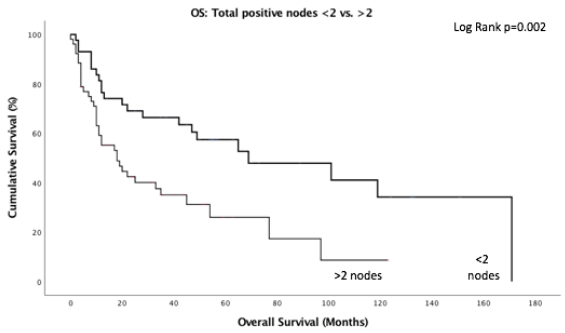
Time (months)	20	40	60	80	100	120	140	160	180
No ND	9	6	2	0					
ND	39	28	15	10	8	6	2	1	0

Time (months)	20	40	60	80	100	120	140	160	180
No ND	9	6	2	0					
ND	22	14	6	4	3	2	2	1	0

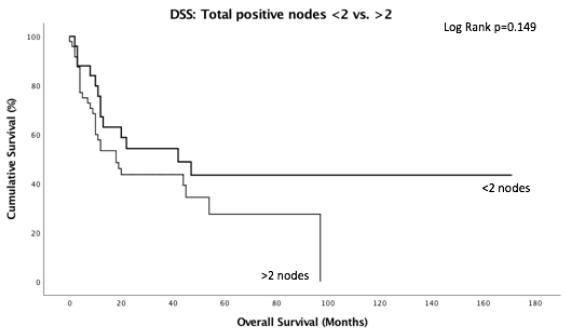
Figure 5-6. Kaplan Meier Survival Analysis for neck dissection at any stage and survival in patients with hypopharyngeal SCC, left OS (p=0.003) and right DSS (p=0.085).

5.6.3 Number of positive nodes

For comparative purposes, total numbers of positive nodes were categorised into groups of ≤2 and >2 nodes. Patients with >2 positive nodes were found to have a significantly worse outcome, based on OS (HRs: OS 2.31, p=0.003, DSS 1.61, p=0.160), see Table 17 and Figure 5-7 and this was confirmed on adjusted analysis (HRs: OS 1.99, p=0.008, DSS 1.38, p=0.500), see Table 19.



Time (months)	20	40	60	80	100	120	140	160	180
<2 nodes	29	23	13	8	7	5	2	1	0
>2 nodes	21	11	4	2	0				

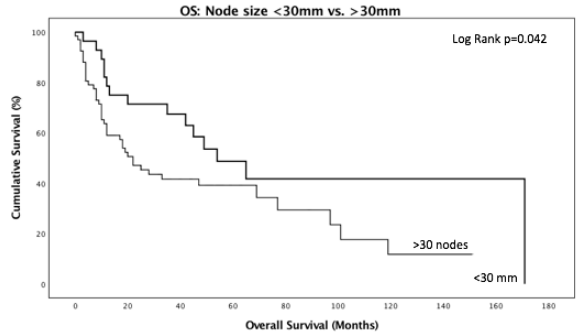


Time (months)	20	40	60	80	100	120	140	160	180
<2 nodes	14	10	5	3	3	2	2	1	0
>2 nodes	18	10	3	1	0				

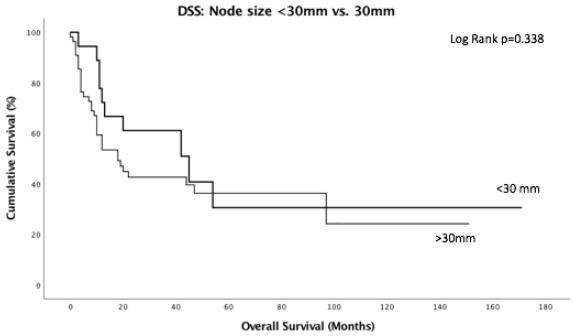
Figure 5-7. Kaplan Meier Survival Analysis for total number of positive nodes and survival in patients with hypopharyngeal SCC, left OS (p=0.002) and right DSS (p=0.149).

5.6.4 Size of lymph nodes

For comparative purposes, the size of lymph nodes was divided into those nodes ≤30mm and those that were greater than 30mm. Patients with larger nodes were associated with an increased rate of death (HR 1.85, p=0.048, DSS 1.40, p=0.348), see Figure 5-8 and Table 17. This association was not observed in the multivariate analysis, as per **Error! Reference source not found.** (HRs OS 1.65, p=0.244, DSS 2.13, p=0.145).



Time (months)	20	40	60	80	100	120	140	160	180
<30 mm	20	15	8	4	4	4	1	0	
>30 mm	30	19	9	6	4	2	1	0	



Time (months)	20	40	60	80	100	120	140	160	180
<30 mm	11	6	3	1	0				
>30 mm	21	14	5	3	2	1	0		

Figure 5-8. Kaplan Meier Survival Analysis for size of lymph nodes at dissection and survival in patients with hypopharyngeal SCC, left OS (p=0.042) and right DSS (p=0.338).

5.6.5 Extranodal Extension

There was no association between presence of ENE and survival based on either, OS or DSS (HRs: 0.99, p=0.977, DSS 0.61, p=0.387), see Figure 5-16 and Table 11.

		OS		Disease Specific Survival	
		HR (95% CI)	*Significance*	*HR (95% CI)*	*Significance*
Tumour Grade	*Well*	1		1	
	Moderate	0.37 (0.11-1.26)	p=0.111	0.57 (0.16-1.99)	p=0.570
	Poor	0.29 (0.09-0.99)	**p=0.048**	0.36 (0.11-1.24)	p=0.362
Neck Dissection	*No*	1		1	
	Yes	0.44 (0.25-0.77)	**p=0.004**	0.58 (0.31-1.09)	p=0.094
Number of Nodes	*Cont. variable*	0.99 (0.98-1.01)	p=0.668	0.98 (0.96-1.01)	p=0.171
Number of positive nodes	*≤2 nodes*	1		1	
	>2 nodes	2.31 (1.33-4.03)	**p=0.003**	1.61 (0.83-3.10)	p=0.160
Size of positive lymph nodes (mm)	*≤30mm*	1		1	
	>30mm	1.85 (1.01-3.39)	**p=0.048**	1.40 (0.69-2.85)	p=0.348
ECS	*No*	1		1	
	Yes	0.99 (0.44-2.24)	p=0.977	0.61 (0.20-1.86)	p=0.387

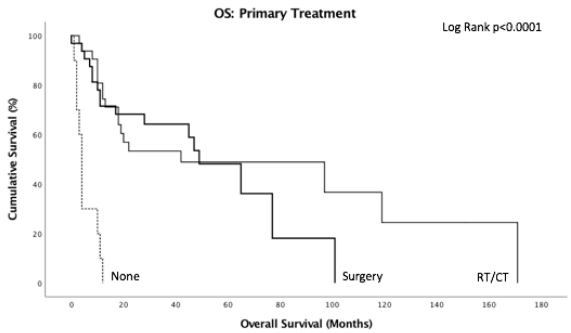
Table 17. Univariate Hazard ratios for OS and DSS for tumour histology and patient outcomes. Bold values indicate significant results. There was insufficient information from pathology reports to accurately include for assessment and analysis in this table.

5.7 Relationship between patient treatment and survival outcomes

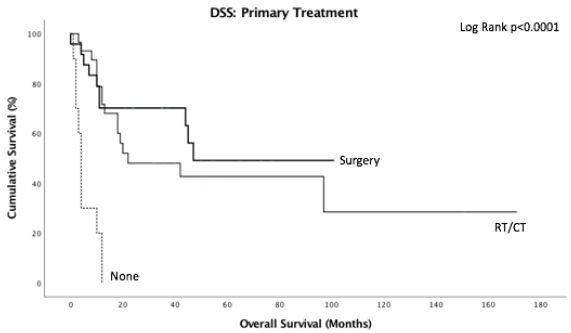
The relationship of patient and treatment and outcomes within the database are summarised in Table 12 and expanded upon below.

5.7.1 Primary mode of treatment and survival outcomes

For all cases, worst outcomes were seen for those patients given palliation only (see Table 18 and Figure 5-9).



Time (months)	20	40	60	80	100	120	140	160	180
Surgery	21	13	4	1	0				
RT/CT	16	12	5	40	4	2	1	0	
None	0								

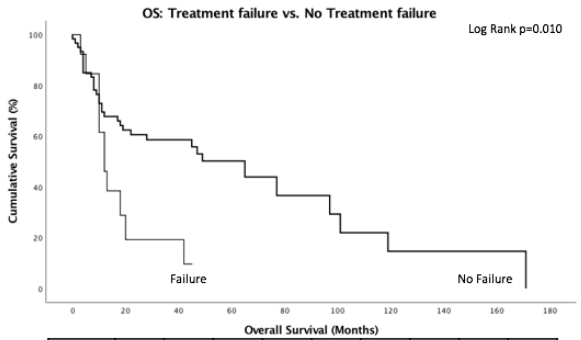


Time (months)	20	40	60	80	100	120	140	160	180
Surgery	16	10	4	1	0				
RT/CT	13	9	4	4	1	0			
None	0								

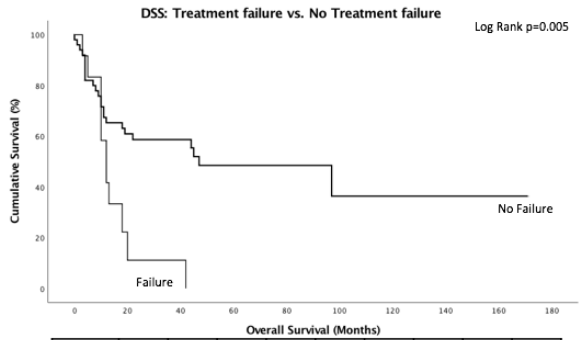
Figure 5-9. Kaplan Meier Survival Analysis for primary mode of treatment and survival in patients with hypopharyngeal SCC, left OS (p<0.0001) and right DSS (p<0.0001).

5.7.2 Treatment failure and survival outcomes

The failure of treatment, being residual and active disease following treatment (either in the form of surgery or chemo/radiotherapy) was associated with a significantly worse survival (HRs: OS 2.46, p=0.014, DSS 2.75, p=0.008), see Table 18 and Figure 5-10.



Time (months)	20	40	60	80	100	120	140	160	180
No Failure	35	23	9	5	4	2	1	1	0
Failure	2	2	0						



Time (months)	20	40	60	80	100	120	140	160	180
No Failure	28	18	8	4	3	2	1	1	0
Failure	1	0							

Figure 5-10. Kaplan Meier Survival Analysis for treatment failure and survival in patients with hypopharyngeal SCC, left OS (p=0.010) and right DSS (p=0.005).

Kaplan Meier analyses were not created for the parameters of 'late recurrence', 'second primary' and 'late distant metastasis' as these failed to reach significance on testing via Cox's proportional hazards (see Table 18).

		OS		Disease Specific Survival	
		HR (95% CI)	*Significance*	*HR (95% CI)*	*Significance*
Primary Treatment	*Surgery*	1		1	
	Chemo/Radio-therapy	0.87 (0.44-1.74)	p=0.874	1.38 (0.62-3.06)	p=0.425
	None/Palliative	8.14 (3.40-19.5)	**p<0.0001**	8.50 (3.14-22.9)	**p<0.0001**
Radiotherapy	*None*	1		1	
	Primary Treatment	0.42 (0.21-0.82)	**p=0.012**	0.47 (0.23-0.96)	**p=0.038**
	Post-operative Treatment	0.36 (0.16-0.79)	**p=0.012**	0.18 (0.06-0.54)	**p=0.002**
Treatment Failure	*No*	1		1	
	Yes	2.46 (1.19-5.07)	**p=0.014**	2.75 (1.29-5.84)	**p=0.008**
Late Recurrence	*No*	1		1	
	Yes	0.92 (0.46-1.85)	p=0.818	1.08 (0.51-2.30)	p=0.843
Second Primary Tumour	*No*	1		1	
	Yes	0.34 (0.82-1.45)	p=0.146	0.41 (0.09-1.74)	p=0.224
Late Distant Metastasis	*No*	1		1	
	Yes	1.70 (0.78-3.72)	p=0.186	1.81 (0.84-3.89)	p=0.130
Treatment Failure, Recurrence or Metastasis	*No*	1		1	
	Yes	1.40 (0.76-2.58)	p=0.280	2.19 (1.09-4.43)	**p=0.027**

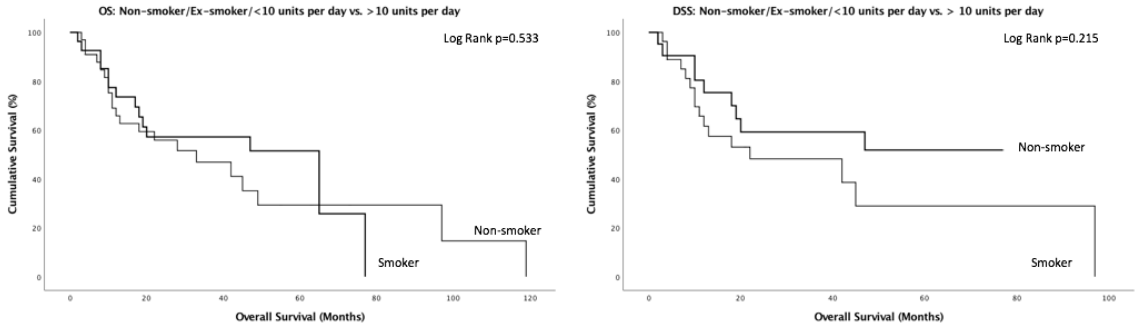
Table 18. Hazard ratios for OS and DSS for treatment outcomes and survival analyses. Significant results are highlighted in bold.

All hazards with significant results were adjusted for potential confounding factors, being male sex, age and smoking and/or alcohol status. The results are demonstrated in Table 19 below. All results were as previously described, with the exception of neck dissection, which on adjustment demonstrated a predilection to survival in the dissection arm (HRs: OS 0.53, p=0.116, DSS 0.69, p=0.433), see Table 19**Error! Reference source not found.**.

		OS		**Disease Specific Survival**	
		Adjusted HR (95% CI)	*Significance*	*Adjusted HR (95% CI)*	*Significance*
Synchronous Primary	*No*	1		1	
	Yes	0.59 (0.13-2.61)	p=0.483	7.31 (1.75-30.5)	*p=0.006*
Distant Metastasis	*No*	1		1	
	Yes	4.88 (1.02-23.5)	**p=0.048**	5.36 (1.02-28.0)	**p=0.047**
Neck Dissection	*No*	1		1	
	Yes	0.53 (0.24-1.17)	p=0.116	0.69 (0.29-1.71)	p=0.433
No. of Positive Nodes	*≤2 nodes*	1		1	
	>2 nodes	1.99 (0.93-4.26)	*p=0.08*	1.38 (0.54-3.53)	p=0.500
Size of Positive Nodes	*No*	1		1	
	Yes	1.65 (0.71-3.84)	p=0.244	2.13 (0.77-5.91)	p=0.145
Primary Treatment	*Surgery*	1		1	
	Chemo-radiotherapy	0.97 (0.41-2.33)	p=0.973	1.90 (0.59-6.13)	p=0.283
	None	9.11 (2.38-34.9)	**p=0.001**	9.61 (1.79-51.4)	**p=0.008**
Treatment Failure	*No*	1		1	
	Yes	2.42 (1.02-5.71)	**p=0.045**	2.38 (0.94-6.08)	p=0.069

Table 19. Adjusted hazard ratios for significant results demonstrated above. Adjusted for male sex, age and smoking and/or alcohol status. Significant results are indicated in bold.

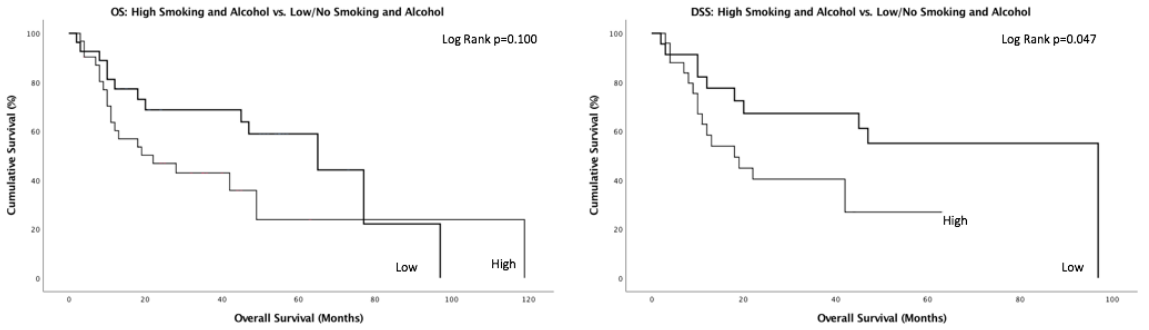
Appendix I Survival charts for Chapter 2



Time (months)	20	40	60	80	100	120
Non-smoker	34	23	14	8	6	6
Smoker	16	10	3	2	2	0

Time (months)	20	40	60	80	100
Non-smoker	11	9	2	0	
Smoker	12	5	3	1	0

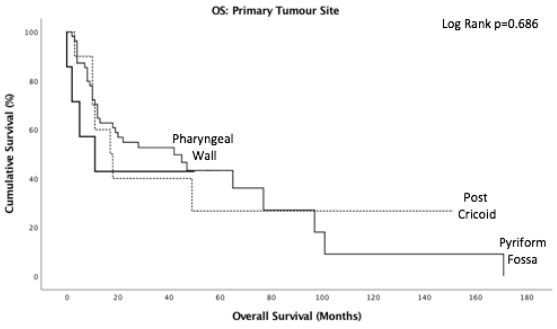
Figure 5-11. Kaplan Meier Survival Analysis for Smoking status, left OS (p=0.533) and right DSS (p=0.215).



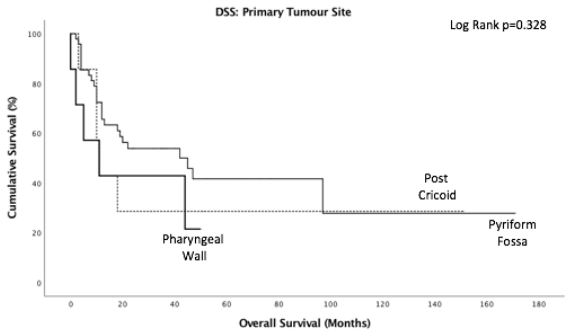
Time (months)	20	40	60	80	100	120
Low	16	14	4	1	0	
High	15	6	2	1	1	0

Time (months)	20	40	60	80	100
Low	13	11	4	1	0
High	10	3	1	0	

Figure 5-12. Kaplan Meier Survival Analysis for High smoking and alcohol consumption status, left OS (p=0.100) and right DSS (p=0.047). See legend beneath **Error! Reference source not found.** for explanation of high smoking and alcohol consumption

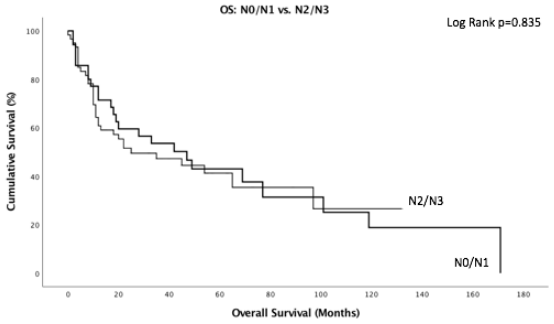


Time (months)	20	40	60	80	100	120	140	160	180
Pharyngeal Wall	2	0							
Pyriform Fossa	29	18	7	3	1	0			
Post-Cricoid	4	3	1	0					

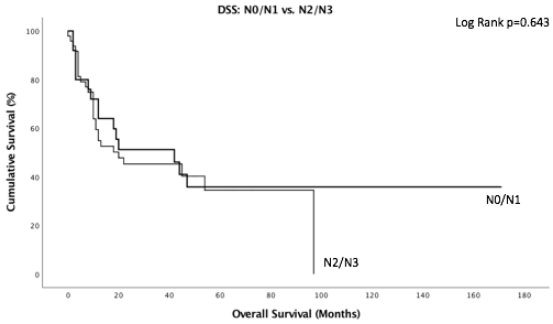


Time (months)	20	40	60	80	100	120	140	160	180
Pharyngeal Wall	2	2	1	0					
Pyriform Fossa	24	14	7	3	2	0			
Post-Cricoid	2	2	1	0					

Figure 5-13. Kaplan Meier Survival Analysis for location of subsite tumour and survival, left OS (p=0.686) and right DSS (p=0.328).

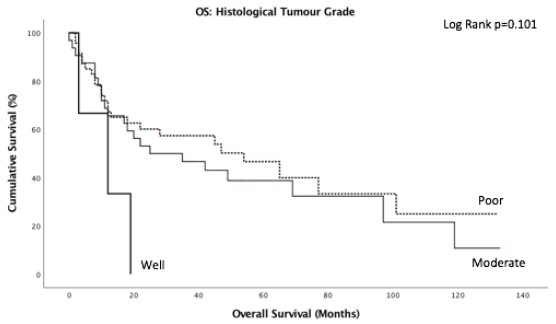


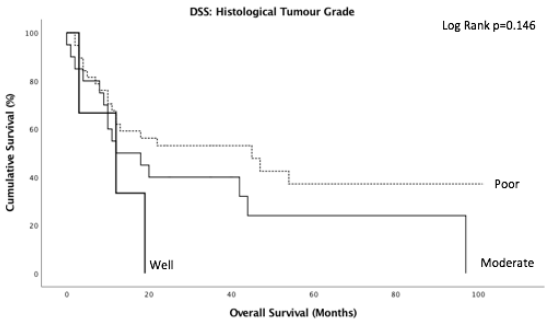
Time (months)	20	40	60	80	100	120	140	160	180
N0/N1	20	16	8	5	3	1	0		
N2/N3	30	18	9	3	3	0	0		



Time (months)	20	40	60	80	100	120	140	160	180
N0/N1	20	10	4	1	3	2	1	0	
N2/N3	12	8	2	1	0				

Figure 5-14. Kaplan Meier Survival Analysis for TNM N stage and survival, left OS (p=0.835) and right DSS (p=0.643).

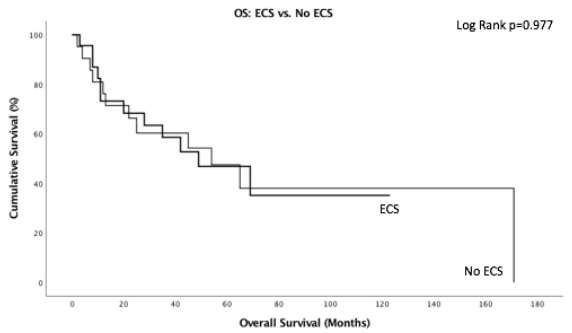


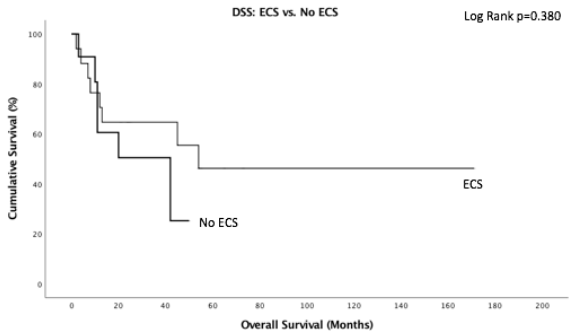


Time (months)	20	40	60	80	100	120	140
Well	0						
Moderate	18	13	7	3	2	1	0
Poor	26	17	8	5	4	3	0

Time (months)	20	40	60	80	100	120	140
Well	0						
Moderate	8	5	2	1	0		
Poor	19	11	4	1	1	0	

Figure 5-15. Kaplan Meier Survival Analysis for tumour grade at diagnosis and survival, left OS (p=0.101) and right DSS (p=0.146).

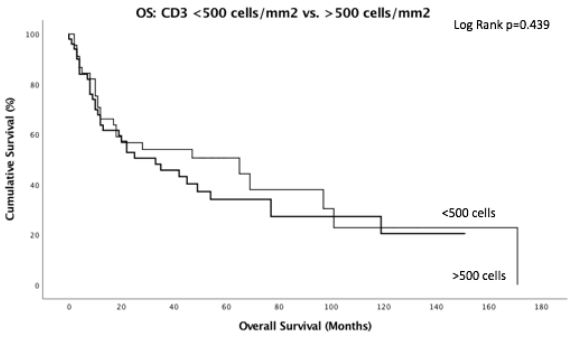




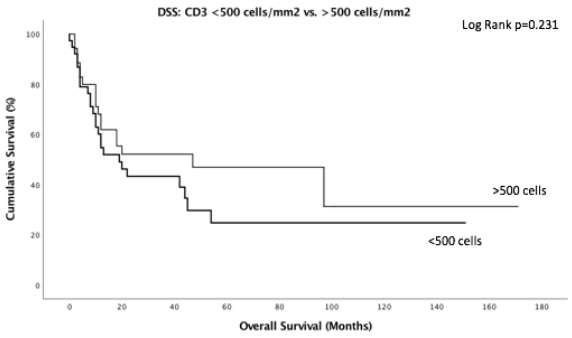
Time (months)	20	40	60	80	100	120	140
ECS	15	10	5	4	2	2	1
No ECS	14	10	5	2	1	1	0

Time (months)	20	40	60	80	100	120	140
ECS	15	10	5	4	2	2	1
No ECS	14	10	5	2	1	1	0

Figure 5-16. Kaplan Meier Survival Analysis for ENE and survival, left OS (p=0.977) and right DSS (p=0.380).

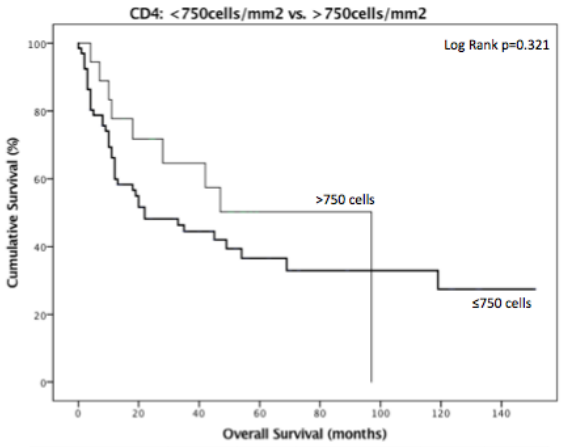


Time (months)	20	40	60	80	100	120	140	160	180
<500 cells	26	18	9	4	4	3	1	0	
>500 cells	24	16	8	6	4	3	1	0	

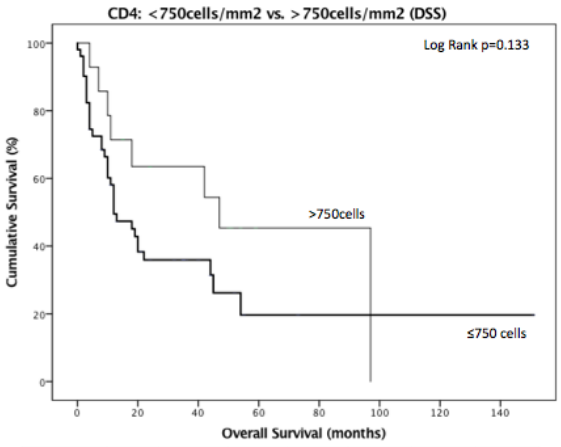


Time (months)	20	40	60	80	100	120	140
<500 cells	16	10	4	1	0		
>500 cells	16	10	4	2	1	1	0

Figure 5-17. Kaplan Meier Survival Analysis for any $CD3^+$ cell count and survival, left OS (p=0.439) and right DSS (p=0.231).

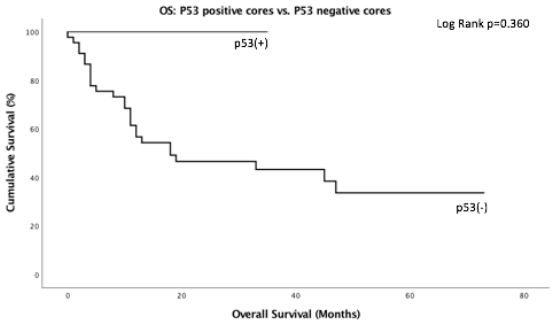


Time (months)	20	40	60	80	100	120	140
≤1000 cells	31	20	11	7	6	5	1
>1000 cells	12	9	1	0			

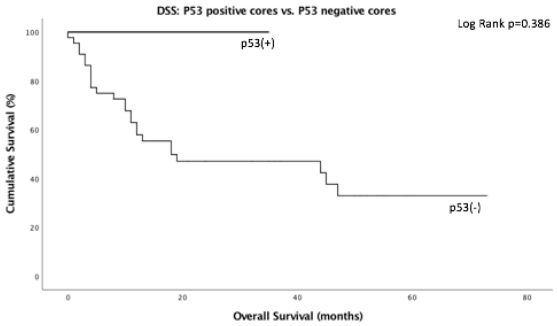


Time (months)	20	40	60	80	100	120	140
≤1000 cells	17	8	2	1	0		
>1000 cells	18	7	1	0			

Figure 5-18. Kaplan Meier Survival Analysis for any $CD4^+$ cell count and survival, left OS (p=0.321) and right DSS (p=0.133).



Time (months)	20	40	60	80
p53 (-)	18	10	1	0
p53 (+)	1	0		



Time (months)	20	40	60	80
p53 (-)	17	10	1	0
p53 (+)	1	0		

Figure 5-19. Kaplan Meier Survival Analysis for any P53+ cell count/mm2 and survival, left OS (p=0.360) and right DSS (p=0.386).

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Appendix J Figures for Hypopharyngeal Cancer Outcomes

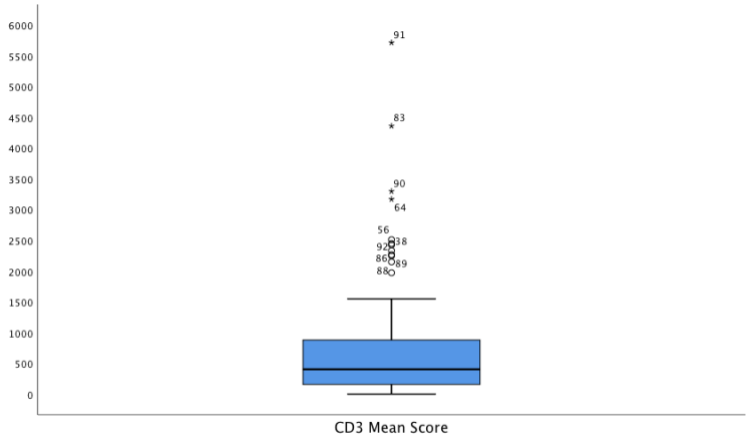


Figure 5-20. Stem and leaf plot of CD3 cell counts/mm^2 (Modal value 218.2 cells/mm^2 [IQR 725.57])

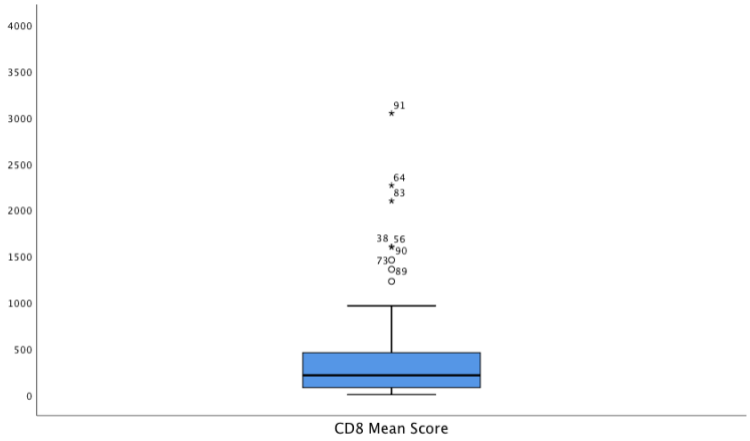


Figure 5-21. Stem and leaf plot of CD8 cell counts/mm^2. (Modal count 5.22 cells/mm^2 [IQR 380.41])

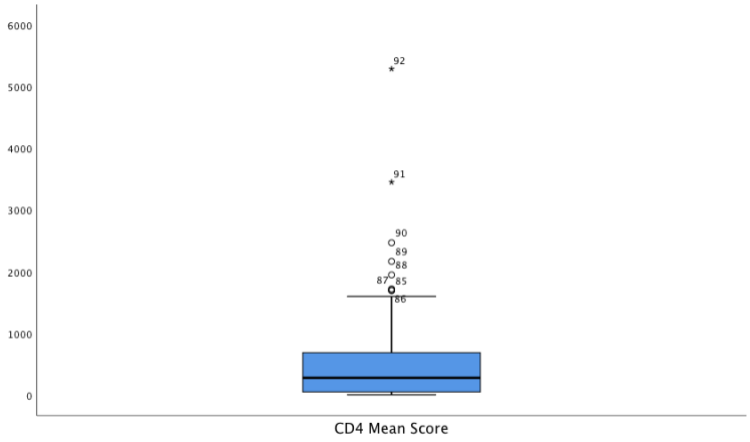


Figure 5-22. Stem and leaf plot of CD4$^+$ cell counts/mm^2

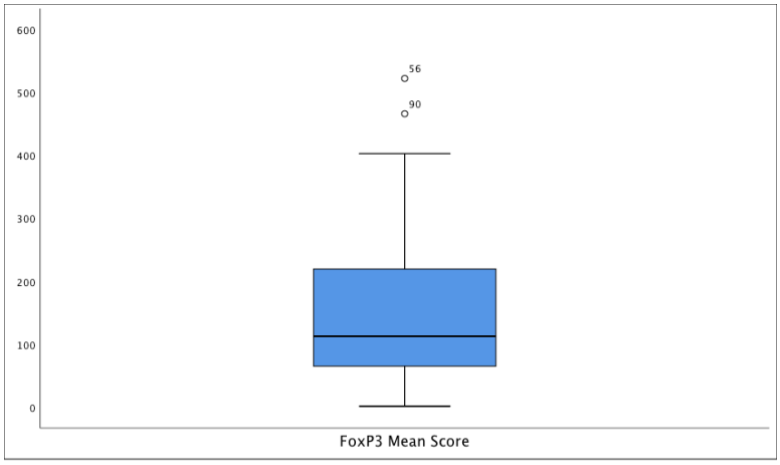


Figure 5-23. Stem and Leaf plot of $CD4^{+}CD25^{+}FOXP3+$ cell counts/mm^2.

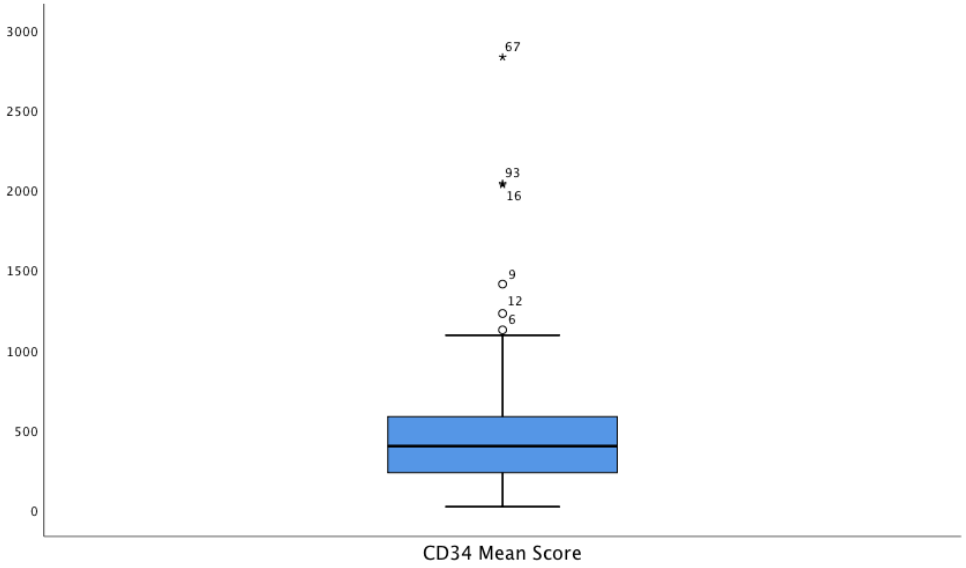


Figure 5-24. Stem and Leaf plot of $CD34^{+}$ cell counts/mm^2

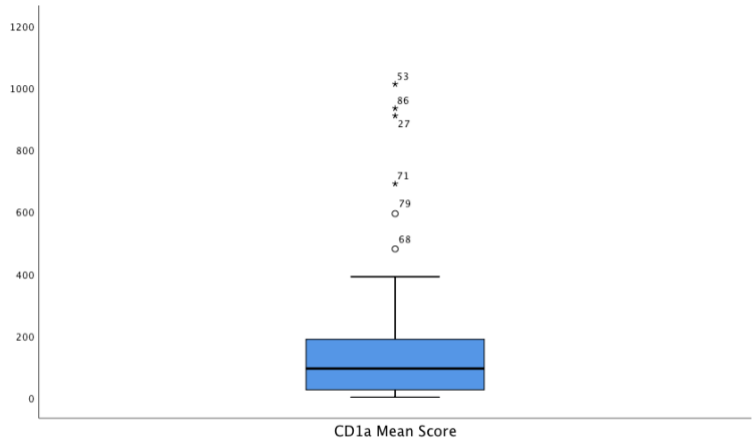
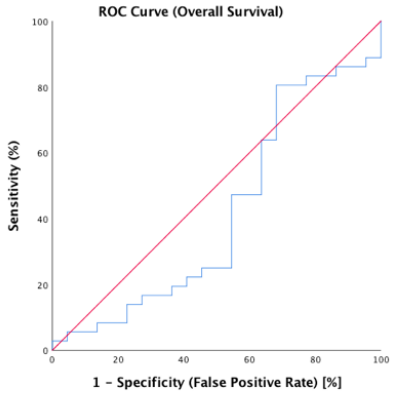


Figure 5-25. Histogram of CD1a cell counts/mm^2

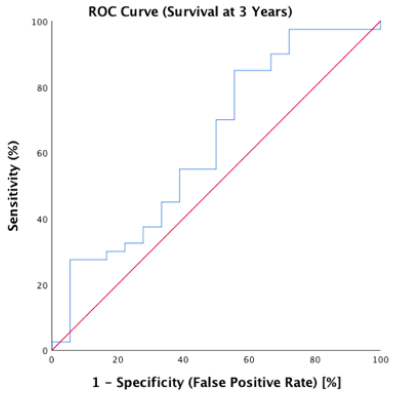
Appendix K Receiver operator characteristics of immune markers in Hypopharyngeal Cancer

Based on OS outcomes, 2 models (Fox P3+ and p53 positive ratios) were indicative of random classification models (AUROC <0.5) and were disregarded. Reversing the assumption (of a higher immune count being beneficial to survival), did not improve the predictive accuracy of the model. Only one model, $CD1a^+$ (OS = 0.42 and 3-year OS = 0.63) was indicative of a possible predicative accuracy of distinguishing abnormal (malignant) patients from controls (see Table 20).

For tests of significance, or approaching significance, likelihood ratios were calculated for $CD1a^+$ OS and 3-year mortality and these were as follows: OS (1.76, p=0.184) and 3-year OS (0.505, p=0.505). Example of the $CD1a^+$ ROC chart is shown below (see Figure 5-26).



Area under Curve	Upper Bound	Lower Bound	Sensitivity
0.417	0.257	0.576	p=0.290



Area under Curve	Upper Bound	Lower Bound	Sensitivity
0.628	0.465	0.791	p=0.083

Figure 5-26. Exemplar ROC analysis for prediction of OS and 3-year survival based on CD1a immune counts in hypopharyngeal carcinoma

Test	OS		3-year Survival	
	Area under curve	**Significance**	**Area under curve**	**Significance**
$CD3^+$	0.493	p=0.914	0.507	p=0.914
$CD4^+$	0.498	p=0.968	0.566	p=0.287
$CD8^+$	0.488	p=0.850	0.522	p=0.728
$CD34^+$	0.422	p=0.394	0.586	p=0.216
$CD1a^+$	0.417	p=0.290	0.628	p=0.083

Table 20. ROC Analyses for Immune Cell Counts

Appendix L Characteristics of salvage cases only

	HPV Positive (n=23)	**HPV Negative (n=51)**	**Significance**
Gender			
Male	15 (65.2%)	40 (78.4%)	*0.228*
Female	8 (34.8%)	11 (21.6%)	(Chi-Sq.)
Age at Diagnosis			
<50	10 (43.5%)	8 (15.7%)	***0.005***
50-69	13 (56.5%)	38 (74.5%)	(Kruskal-Wallis)
>70	0	5 (9.8%)	
Median ± SD	51 ± 8.85 years	60 ± 9.36 years	
Tumour Site			
Oral Cavity	0	4 (7.8%)	***Not performed***
Oropharynx	23 (100%)	39 (76.5%)	
Hypopharynx	0	5 (9.8%)	
Larynx	0	1 (2.0%)	
Disease Stage			
I/II	2 (8.7%)	6 (11.8%)	***0.696***
III/IV	21 (91.3%)	45 (88.2%)	*(Kruskal-Wallis)*
Type of ND			
Extended	0	0	***0.024***
Radical	4 (17.4%)	8 (15.6%)	*(Kruskal-Wallis*)
Modified Radical	17 (73.9%)	21 (41.1%)	
Selective	2 (8.7%)	22 (43.1%§)	
Number of Nodes *Mean (95% CI)*			

Total *Positive*	27 ± 22.29 2 ± 2.08	24 ± 11.82 2 ± 3.98	***0.002*** *0.160* (2 sample unpaired T Test)
Size of Positive Nodes (mm)			
Mean (95% CI)	34 ± 14.73	31 ± 14.6	*0.737* (2 sample unpaired T Test)
Extra-Capsular Spread			
Yes *No* *Unknown*	7 (30.4%) 5 (21.7%) 11 (47.8%)	10 (19.6%) 18 (35.3%) 23 (45.1%)	*0.782* (Kruskal-Wallis.)

Table 21. Clinical and pathological characteristics of Salvage/Recurrence cases

Appendix M Survival charts for Chapter 3

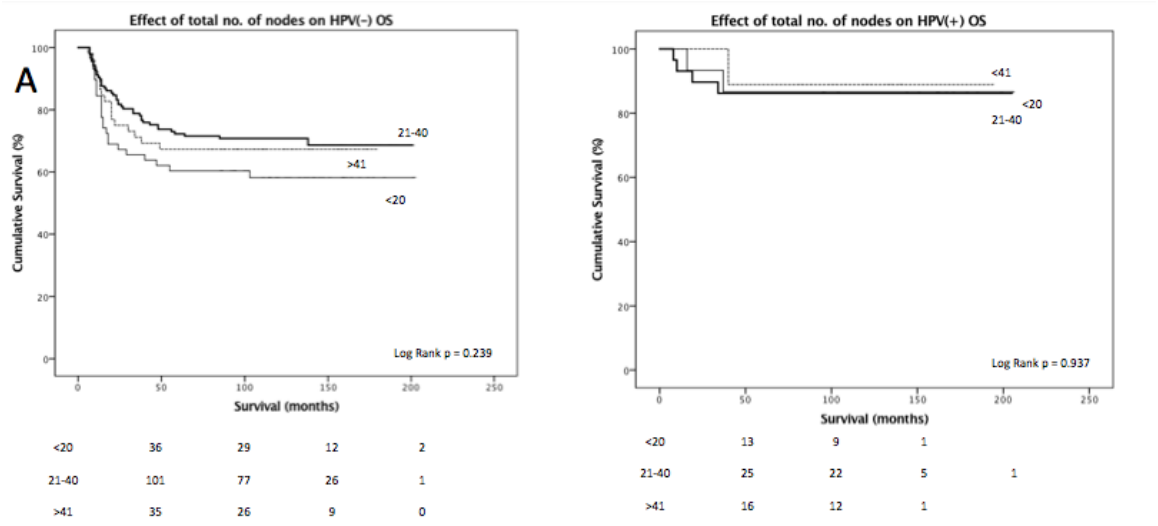


Figure 5-27. Effect of total nodal yield on OS; Left HPV(-) log-rank p=0.239, Right HPV(+) log-rank p=0.937

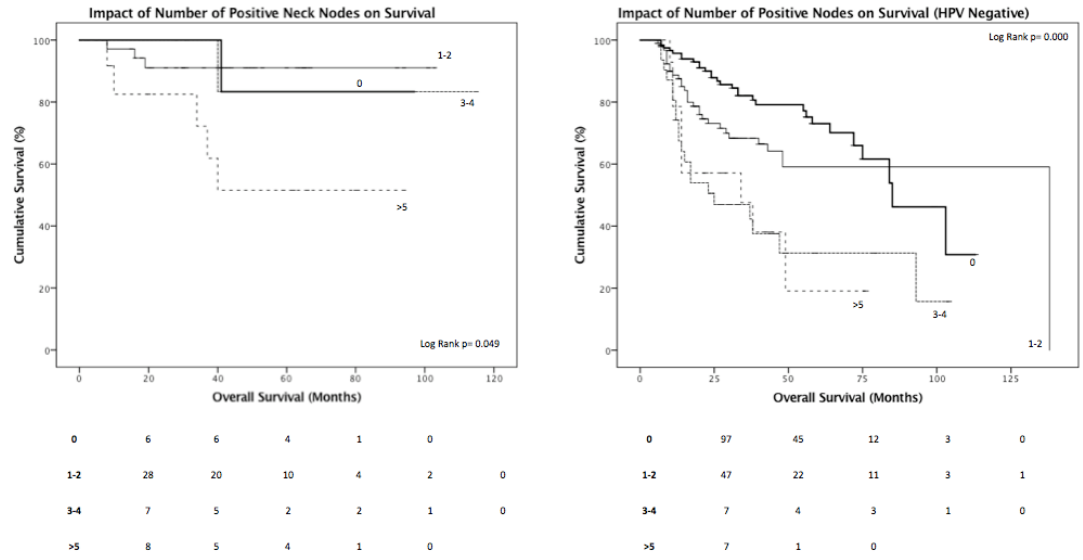


Figure 5-28. 1st Analysis. Impact of total positive nodes on OS; Left HPV(+) log-rank p=0.049, Right HPV(-) log-rank p<0.001

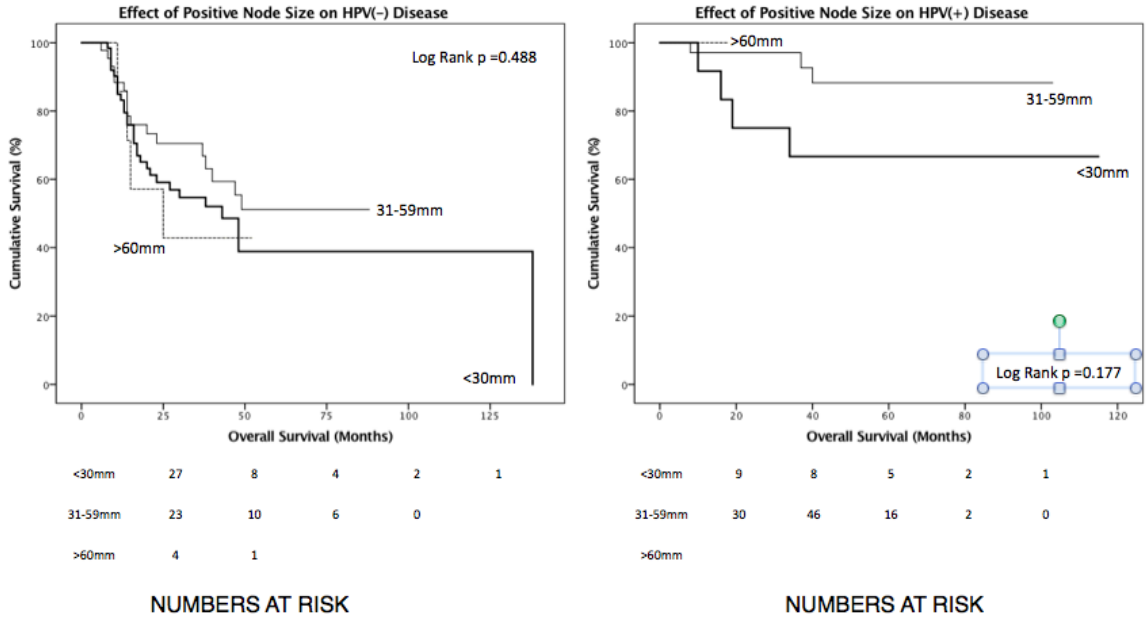


Figure 5-29. Effect of Positive Node Size on Survival. Left HPV(-), p=0.488 and Right HPV(+), p=0.177.

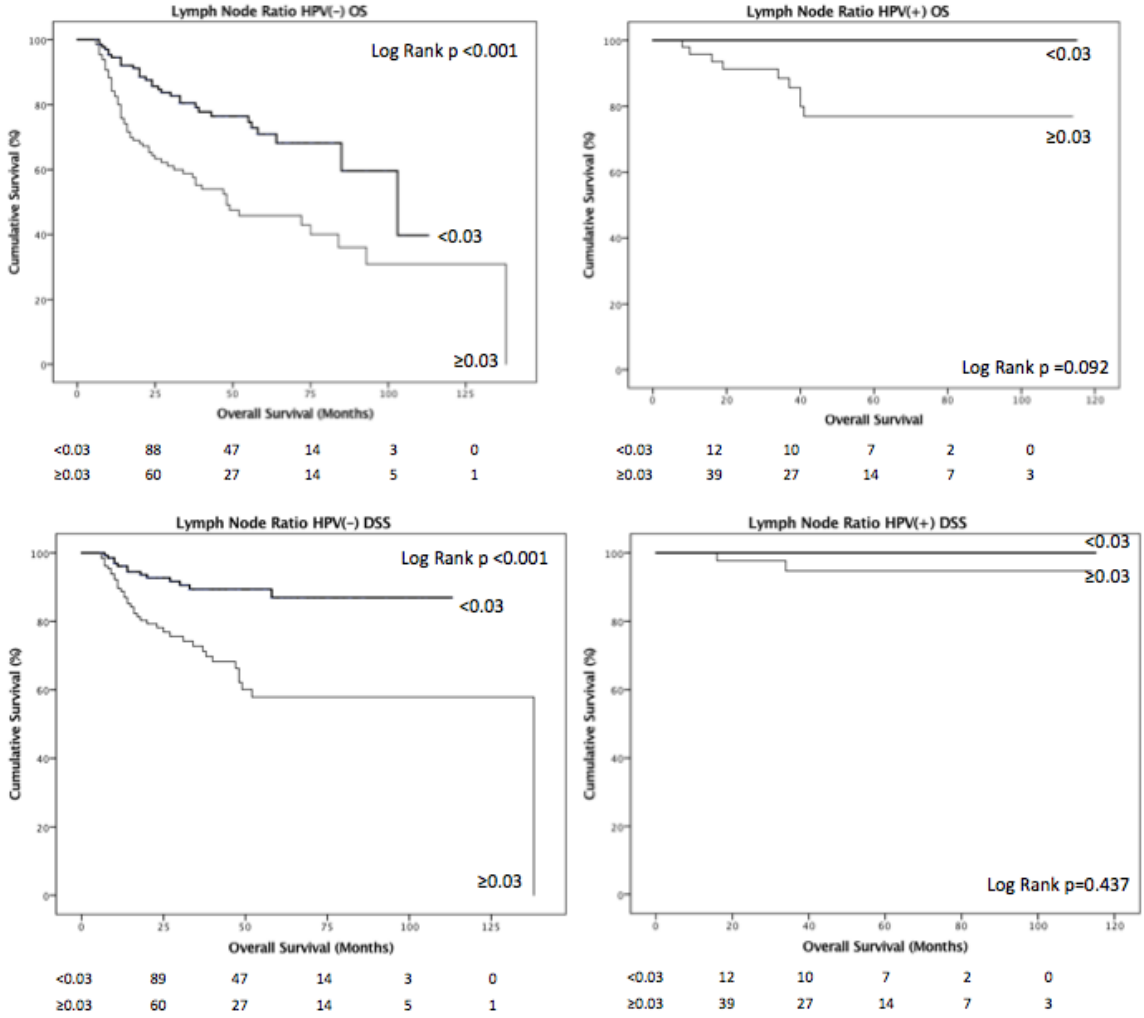


Figure 5-30. Lymph Node ratio on survival of HPV(+) and HPV(-) tumours. Top left, HPV(-) OS p<0.001. Top Right, HPV(+) OS p=0.092. Bottom Left, HPV(-) DSS p<0.001. Bottom Right, HPV(+) DSS p=0.437.

Appendix N Differences between UICC/AJCC TNM v7 and v8

7th Edition	8th Edition (specific for HPV positive OPSCC)
T Category	
Tx: Primary tumour cannot be assessed Tis: Carcinoma in situ T1: Tumour ≤2cm T2: Tumour ≥2cm, ≤4cm T3: Tumour >4cm or extension to lingual epiglottis T4: Locally advanced disease T4a: Moderate: Tumour invades larynx, tongue muscles, medial pterygoid, hard palate or mandible T4b: Advanced: Tumour invades lateral pterygoid, lateral nasopharynx, skull base, encases carotid	T0: No primary T1: Tumour ≤2cm T2: Tumour ≥2cm, ≤4cm T3: Tumour >4cm or extension to lingual epiglottis T4: Locally advanced disease, tumour invades larynx, tongue muscles, medial pterygoid, hard palate or mandible
N Category	
NX: Regional nodes cannot be assessed N0: No regional lymph node metastasis N1: Metastasis in single ipsilateral node (≤3cm), ECS negative N2a: Metastasis in single ipsilateral node (>3cm, ≤6cm), ECS negative N2b: Metastasis in multiple ipsilateral nodes (≤6cm), ECS negative N2c: Metastasis multiple bilateral/contralateral nodes (≤6cm), ECS negative N3a: Lymph nodes >6cm and ECS negative or any lymph node with ECS N3b: *Any* ECS	NX: Regional nodes cannot be assessed N0: No regional lymph node metastasis N1: ≥1 ipsilateral node (all ≤6cm) N2: Contralateral nodes (all ≤6cm) N3: Lymph nodes >6cm
M Category is unchanged	

Staging differences between HPV (+) and (-) on version 8

The below images demonstrate the staging differences between p16 positive and negative OPSCC carcinoma on version 8 (above, p16 positive, below, p16 negative).

T CATEGORY	N CATEGORY			
	N0	N1	N2	N3
T0	NA	I	II	III
T1	I	I	II	III
T2	I	I	II	III
T3	II	II	II	III
T4	III	III	III	III

T CATEGORY	N CATEGORY			
	N0	N1	N2a,b,c	N3a,b
T1	I	III	IVA	IVB
T2	II	III	IVA	IVB
T3	III	III	IVA	IVB
T4a	IVA	IVA	IVA	IVB
T4b	IVB	IVB	IVB	IVB

Appendix O The re-staging of HPV(+) disease in the 8th edition of UICC/AJCC TNM

Introduction

The new TNM Classification of Malignant diseases was introduced into clinical practice on 1st January 2018 and or the first time, makes acknowledgement of presence of an HPV positive OPSCC as a distinct disease process. The most significant changes pertain to the tumour and node classifications. In the 8th iteration, there is no differentiation for tumours that are locally advanced (i.e. no T4a or T4b) and nodal disease is down-staged by both size and number of nodes.

Overall, a movement is being made to recognise that although nodal tumour and nodal burden may be greater at first presentation, this does not necessarily relate to a more advanced stage or poor prognosis. Patients can subsequently be treated less aggressively allowing for both tumour control and an improved quality of life post management.

Our group aimed to review how a past cohort of OPSCC patients might fare under the new system and whether the new classification accurately down-staged the HPV positive patients.

Methods

333 patients were identified from a previously published study by Ward et al (22). 226 (67.9%) were male. Median age was 55.0 years ± 22.5. Median survival was 120 months ± 61.7 (Range 6-219 months). 64 (19.2%) of patients were HPV positive.

102 (30.6%) patients died in the study period.

Results

The below table reflects changes to patient groups between each iteration of TNM.

Comparisons between TNM Classification 7 and 8					
TNM Version 7			**TNM Version 8**		
HPV (-)	*HPV(+)*	*Significance*	*HPV (-)*	*HPV(+)*	*Significance*
Tx: 0 T0: 4 (1.5%) T1: 101 (37.5%) T1a: 1 (0.4%) T1b: 0 T2: 93 (34.6%) T3: 18 (6.7%)	Tx: 0 T0: 1 (1.6%) T1: 25 (39.1%) T1a: 0 T1b: 0 T2: 32 (50.0%) T3: 2 (3.1%)	*p=0.133 (Kruskal-Wallis)*	T0: 0 T1: 102 (37.9%) T2: 93 (34.6%) T3: 18 (6.7%) T4: 0 T4a: 50 (18.6%) T4b: 2 (0.7%)	T0: 1 (1.6%) T1: 25 (39.1%) T2: 32 (50.0%) T3: 2 (3.1%) T4: 4 (6.3%)	*p=0.102 (Kruskal-Wallis)*

T4: 0 T4a: 50 (18.6%) T4b: 2 (0.7%)	T4: 0 T4a: 4 (6.3%) T4b: 0				
N0: 136 (50.6%) N1: 29 (10.8%) N2a: 24 (8.9%) N2b: 54 (20.1%) N2c: 16 (5.9%) N3: 9 (3.4%)	N0: 4 (6.3%) N1: 8 (12.5%) N2a: 17 (26.6%) N2b: 29 (45.3%) N2c: 3 (4.7%) N3: 2 (3.1%)	*p<0.00001 (Kruskal-Wallis)*	N0: 136 (50.6%) N1: 29 (10.8%) N2a: 24 (8.9%) N2b: 54 (20.1%) N2c: 16 (5.9%) N3a: 4 (1.5%) N3b: 5 (1.9%)	N0: 4 (6.3%) N1: 54 (84.4%) N2: 3 (4.7%) N3: 2 (3.1%)	*p=0.135 (Kruskal-Wallis)*
M0: 267 (99.3%) M1: 2 (0.7%)	M0: 64 (100%) M1: 0	*p=0.489 (Chi-Sq.)*	As previous	As previous	
Overall tumour stage			*Overall tumour stage*		
I: 57 (21.2%) II: 47 (17.5%) III: 30 (11.2%) IVa: 123 (45.7%) IVb: 9 (3.3%) IVc: 2 (0.7%)	I: 1 (1.6%) II: 2 (3.1%) III: 9 (14.1%) IVa: 49 (76.6%) IVb: 2 (3.1%) IVc: 0	*p<0.00001 (Kruskal-Wallis)*	I: 52 (19.3%) II: 47 (17.5%) III: 24 (8.9%) IV: 0 IVa: 83 (30.9%) IVb :62 (23.0%) IVc: 1 (0.4%)	I: 55 (85.9%) II: 3 (4.7%) III: 6 (9.4%) IV: 0	*p<0.00001 (Kruskal-Wallis)*

Survival analysis was performed to assess outcomes of TNM stages in both version 7 and 8. These are demonstrated in Figure 5-31 and Figure 5-32 below. Although there appears to be consistency of outcomes in HPV negative cases in Figure 5-31 both within TNM 7 and 8 this is not the case for HPV positive cases, as seen in Figure 5-32.

Based on OS, a discrepancy of outcomes in seen in HPV(+) cases previously that is partially corrected via the revised staging system. There is some crossover of rates of mortality between Stage II and III patients which is likely, the effect of a small data set.

No statistically meaningful information is seen for DSS with either HPV(+) iteration.

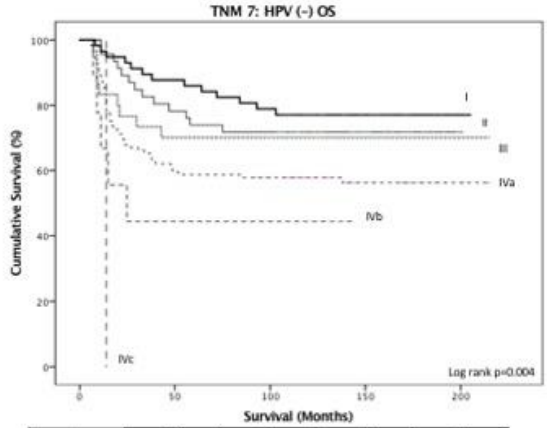
Discussion

HPV(+) disease has been down-staged in the new TNM classification system and this is seen to be effective within our historical data set. Previously, over 80% of HPV(+) patients had been diagnosed with Stage IV disease and this would likely have been associated with treatment fitting of such an advanced disease stage. Ultimately, this may have meant over-treatment and the resulting side effects associated with such invasive treatment.

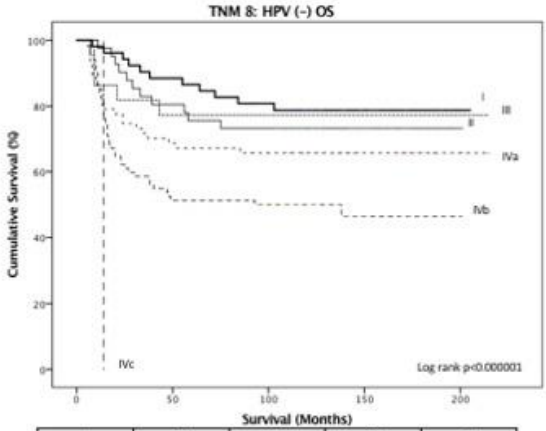
Within the revised classification, no patients are given a diagnosis of Stage IV disease and indeed, the greatest majority are found within Stage I (42.2%)and Stage II (48.4%) disease. This did not fully translate into distinctly heterogeneous outcomes based on our survival charts, but this likely reflects our study numbers as opposed to efficiency of the system.

Conclusion

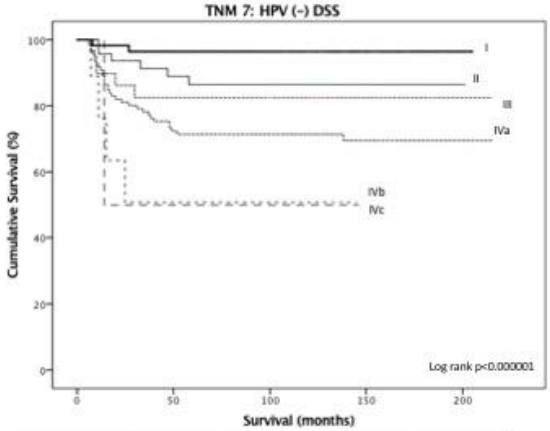
On first review, the revised TNM classification of Malignant diseases appears to satisfactorily down stage patients with HPV positive OPSCC. Our study was not able to demonstrate clear heterogeneity of outcomes based on survival charts, but this is likely the effect of an underpowered study.



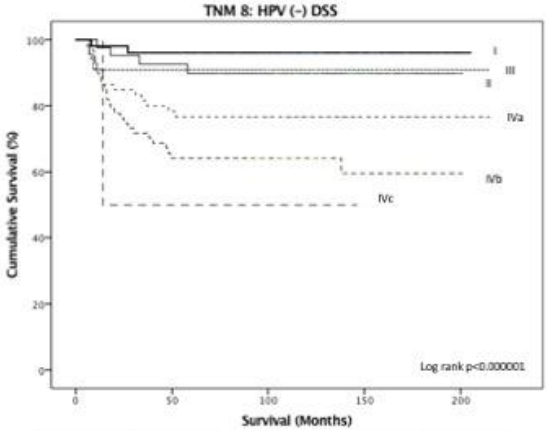
I	50	43	18	2
II	36	33	14	1
III	21	18	7	1
IVa	72	61	25	6
IVb	4	4	3	0
IVc	0			



I	46	40	16	2
II	33	30	14	1
III	17	15	6	1
IVa	46	40	19	6
IVb	42	35	9	0
IVc	0			

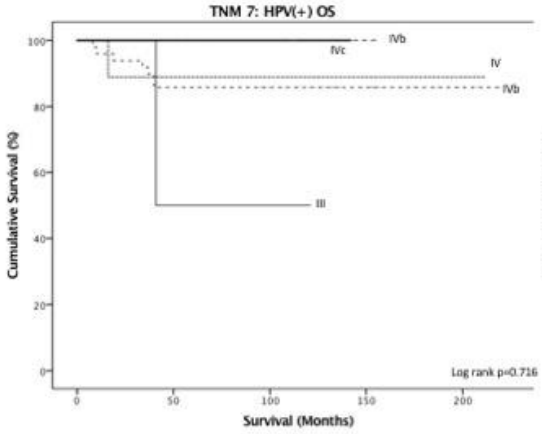


I	50	43	18	2
II	37	34	14	1
III	21	18	7	1
IVa	74	63	26	6
IVb	4	4	3	0
IVc	0			

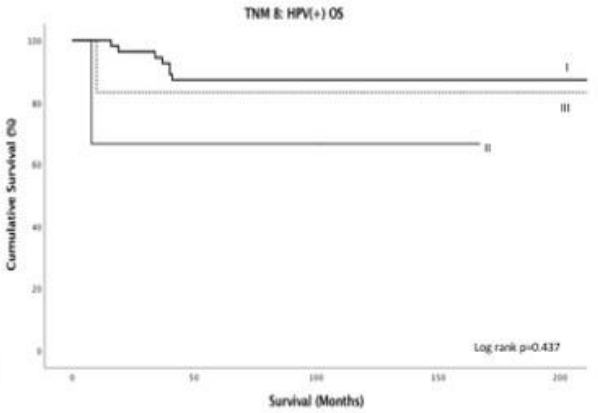


I	46	40	16	2
II	34	31	14	1
III	17	15	6	1
IVa	48	42	20	5
IVb	42	35	9	1
IVc	0			

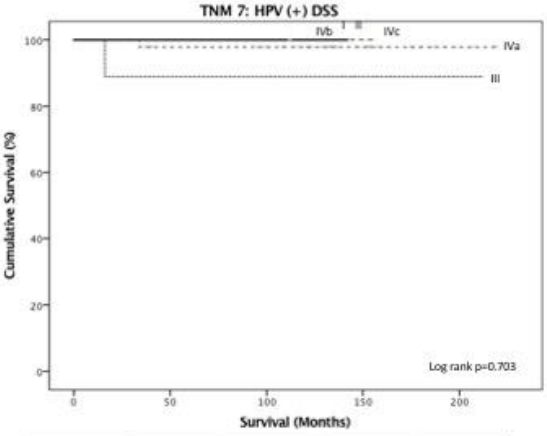
Figure 5-31. Survival analyses for HPV negative cases. Top left: TNM 7 OS p=0.004, Top right: TNM 8 OS p<0.000001, Bottom left: TNM 7 DSS p<0.000001, Bottom right: TNM 8 DSS p<0.000001



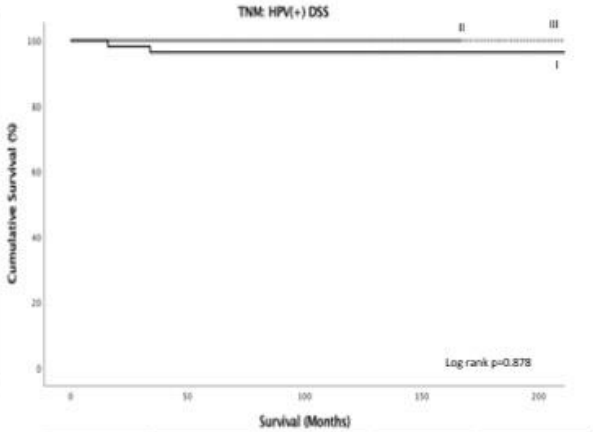
I				
II	1	0		
III	8	8	5	1
IVa	42	39	20	3
IVb	1	1	1	0
IVc	0			



I	48	24	4	0
II	2	2	1	0
III	5	5	3	1
IV	0			



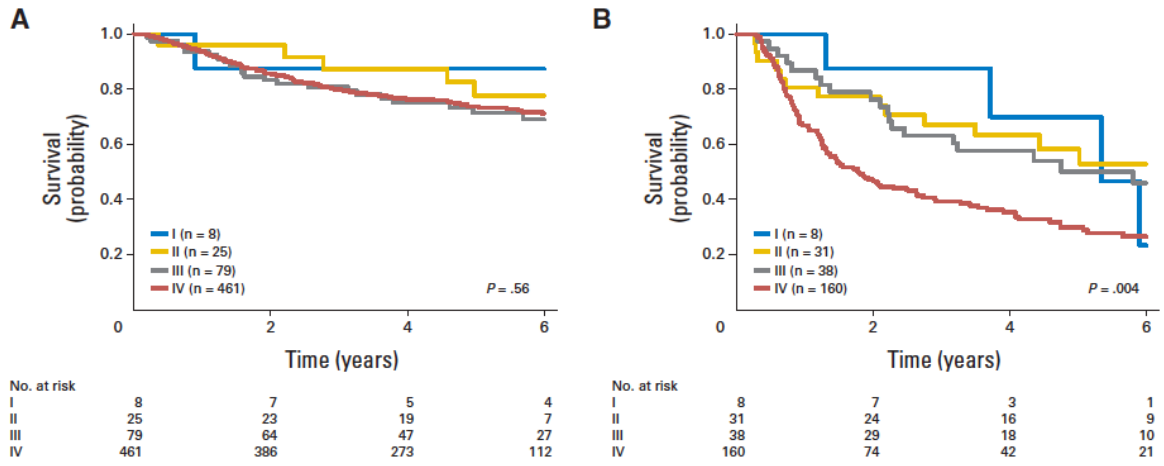
I	0			
II	1	0		
III	8	8	5	1
IVa	42	39	20	3
IVb	1	1	1	0
IVc	0			



I	48	46	24	5
II	2	2	1	0
III	5	5	3	1
IV	0			

Figure 5-32. Survival analyses for HPV positive cases. Top left: TNM 7 OS p=0.716, Top right: TNM 8 OS p=0.437, Bottom left: TNM 7 DSS p=0.703, Bottom right: TNM 8 DSS p=0.878

Appendix P Demonstration of changes to staging outcomes in HPV(+) disease



Demonstration of historical Staging outcomes for HPV(+) on the left and HPV(-) on the right. From (239)

Appendix Q ICD 10 and OPCS Codes for Chapter 4

Head and Neck Cancer Diagnosis
ORAL TONGUE (C020, C021, C022, C023). **MANDIBLE/FOM** (C040, C041, CO48, CO49, C411). **GUM/CHEEK/LIPS/ORAL VESTIBULE** (CO30, CO31, CO39, CO60, CO61, C001, C002, C003, C004, C005, C006, COO8, C009). **TONGUE (UNCLEAR)** CO68, CO69, CO29. **OROPHARYNX** (CO50, CO51, CO52, CO58, CO59. **TONGUE BASE/OROPHARYNX, TONSILS, RETROMANDIBULAR TRIGONE** (C100, C101, C102, C103, C104, C108). **LARYNX/HYPOPHARYNX** (C12X, C130, C131, C132, C138, C139, C150, C153, C159, C320, C321, C322, C323, C328, C329). **CERVICAL METASTASIS** (C770), **DISTANT METASTASES** (C771, C772, C773, C774, C775, C778, C779, C780, C781, C782, C783, C784, C785, C786, C787, C788, C790, C791, C792, C793, C794, C795, C796, C797, C798)
Morbidities
HYPERTENSION (I10X, I150, I151, I152, I158, I159). **HYPERLIPIDAEMIA** (E780, E781, E782, E783, E784, E785). **DIABETES** (E100, E101, E102, E103, E104, E105, E107, E108, E109, E110, E111, E112, E113, E114, E115, E116, E117, E118, E119, E120, E121, E122, E123, E124, E125, E126, E127, E128, E129, E130, E131, E132, E133, E134, E135, E136, E137, E138, E139, E140, E141, E142, E143, E144, E145, E146, E147, E148, E149. **SMOKING** (F170, F171, F172, F173, F174, F175, F176, F177, F178, F179, T652, Z720). ALCOHOL (E244, F100, F101, F102, F103, F104, F105, F106, F107, F108, F109, G312, G621, G721, I426, K292, K700, K701, K702, K703, K704, K709, K860, T519, Z502, Z721). **ISCHAEMIC HEART DISEASE** (I250, I251, I252, I255, I256, I258, I259, Z951, Z955, Z958, Z959). HEART FAILURE (I500, I501, I509). **CEREBROVASCULAR DISEASE** (I658, I659, I660, I661, I662, I663, I664, I668, I669, I670, I671, I672, I673, I675, I676, I677, I678, I679, I690, I691, I692, I693, I694, I698, F010, F011, F012, F013, F018, F019, G214, G450, G451, G452, G453, G454, G458, G459, G460, G461, G462, G463, G464, G465, G466, G467, G468). **PERIPHERAL VASCULAR DISEASE** (I711, I712, I713, I714, I715, I716, I718, I719, I720, I721, I722, I723, I724, I728, I729, I730, I731, I738, I739, Z867). **RENAL DISEASE** (C64X, C65X, C790, D300, D301, D410, D411, E102, E112, E122, E132, E142, I120, I131, I132, I139, I150, I151, I701, I722, I823, K767, M103, M1030, M1031, M1032, M1033, M1034, M1035, M1036, M1037, M1038, M1039, N030, N031,N032, N033, N034, N035, N036, N037, N038, N039, N040, N041, N042, N043, N044, N045, N046, N047, N048, N049, N050, N051, N052, N053, N054, N055, N056, N057, N058, N070, N071, N072, N073, N074, N075, N076, NO77, N078, N079, N080, N081, N082, N083, N084, N085, N088, N10X, N181, N182, N183, N184, N185, N189, N19X, N250, N251, N258, N259, O102, O103, N010, N011, N012, N013, N014, N015, N016, N017, N018, N019). **GORD** (K210, K219). **ASTHMA** (J450, J451, J458, J459). COPD (J430, J431, J432, J438, J439, J448, J449). **LIVER DISEASE** (K700, K701, K702, K703, K704, K709, K710, K711, K712, K713, K714, K715, K716, K717, K718, K719, K721, K729, K730, K731, K732, K738, K739, K740, K741, K742, K743, K744, K745, K752, K753, K754, K758, K759, K760, K761, K762, K769, K770, K778, B150, B159, B160, B161, B162, B169, B170, B171, B172, B178, B180, B181, B182, B188, B189, B190, B199, B251.
Resection Codes
TOTAL GLOSSECTOMY (F221). **PARTIAL GLOSSECTOMY** (F222, F228, F229, F231). **MANDIBULECTOMY** (V141, V142, V143, V148, V149). MAXILLECTOMY (V061, V068, V069**). FOM RESECTION** (F381). **PALATE EXCISION** (F281). **ORAL VAULT EXCISION** (F201, F202, F382, F428). **PHARYNGECTOMY** (E191, E192, E198, E199). **LARYNGECTOMY** (E291, E292, E293, E294, E296, E356, E295, E298, E299, E302, E341). **SALIVARY RESECTION** (F441, F442, F443, F448, F449, F444, F445).

Other Procedures
NECK DISSECTION (T851, T861, T872). **GASTROSTOMY** (G341, G342, G343, G344, G345, G348, G349). **OESOPHAGEAL RESECTION** (G011, G012, G013, G018, G019, G020, G021, G022, G023, G024, G025, G028, GO29, G030, G031, G032, G033, G034, G035, G036, G038, G039, G040, G041, G042, G043, G048, G049, G050, G051, G052, G053, G054, G055, G056, G058, G059 **THYROIDECTOMY/PARATHYROIDECTOMY** (B081, B082, B083, B084, B085, B086, B088, B121, B124, B128, B129, B141, B143, B144, B145, B148, B149)
Complications
ACUTE CARDIOVASCULAR EVENT (I200, I201, I202, I208, I209, I210, I211, I212, I213, I214, I219, I220, I221, I228, I229. STROKE (I600, I601, I602, I603, I604, I605, I606, I607, I608, I609, I610, I611, I612, I613, I614, I615, I616, I618, I619, I620, I621, I629, I630, I631, I632, I633, I634, I635, I636, I638, I639, I64X. **PERIPHERAL VASCULAR EVENT** (I740, I741, I742, I743, I744, I745, I748, I749, I978, I979, K550, K763). **LOWER RESPIRATORY TRACT** INFECTIONS (B392, B400, B402, B420, B440, B441, B450, B460, J100, J121, J122 J123, J128, J129, J13X, J14X, J150, J151, J152, J153, J154 J155, J156, J157, J158, J159, J160, J168, J170, J171, J172, J173, J180, J181, J182, J188, J22X, J440, I691, I698, J851. **VENOUS THROMBOEMBOLSIM** (I260, I269, I801, I802, I803, I808, I809, I821, I823, I828, I829, L791, L792, L793, L794, L795. SEPSIS (A021, A227, A267, A327, A400, A401, A402, A403, A408, A409, A410, A411, A412, A413, A414, A415, A418, A419, B377, A483, R572). **CARDIO-RESPIRATORY ARREST** (I460, I469, R090, R092, G931). ACUTE RENAL FAILURE (N170, N171, N172, N178, N179, N19X, X401, X402, X403, X404, X405, X406, X407, X408, X409). **GASTROINTESTINAL COMPLICATIONS** (A040, A041, A042, A043, A044, A045, A046, A047, A048, A049, K250, K251, K252, K253, K254, K255, K256, K260, K261, K262, K263, K264, K265, K266, K270, K271, K272, K273, K274, K275, K276, K280, K281, K282, K283, K284, K285, K286, K287, K289, K290, K521, K522, K528, K529, K560, K562, K625, K650, K658, K659, K913, K920, K921, K922, R100, K226, K570, K572, K574, K578. **TRACHEOSTOMY MALFUNCTION** (J950). **UTI** (N930). **SKIN COMPLICATION** (A480, L890, L891, L892, L893, L899, L89X, R02X. **HAEMORRHAGE** (D500, T810, T811, R571, R58X, Y221, Y321). **SURGICAL SITE COMPLICATIONS** (J390, J391, J853, J860, J869, K122, L020, L021, L028, L029, L032, T793, T813, T814, T842, T843, T844, T845, T846, T847, T848, T849, E272, S471, S561, S562, S567, S568, S569, S571, S572, S577, S578, S579, S581, S582, S588, S589, Y228, Y229, S561, S562). **IATROGENIC COMPLICATIONS** I772, S047, S048, S049, S150, S153, S840, S841, S847, S848, T812, T815, T816, T817, T818, T819, T301, T302, T303, T551, T552, T553, T554, T555, T556, T558, T559, X091, X092, X093, X094, X095, X099, Y322, Y323, Y328, Y329). **GASTROSTOMY MALFUNCTION** (K914, T855, T856, T857, T858, G447).

Appendix R Patient Demographics

	LSD Quintile 1-2 (n=5,051)	MSD Quintile 4-5 (n=7,282)	Significance
Sex			
	63 ± 12.4	61 ± 11.3	**p<0.000001** (Independent T-test)
Age Range			
18-34 *35-44* *45-54* *55-64* *65-74* *75-84* *>85*	89 (1.8%) 262 (5.2%) 665 (13.2%) 1686 (33.4%) 1366 (27.0%) 817 (16.2%) 166 (3.3%)	94 (12.9%) 407 (5.6%) 1249 (17.2%) 2750 (37.8%) 1893 (25.9%) 763 (10.5%) 126 (1.73%)	**p<0.000001** (Kruskal Wallis)
Sex			
Male *Female*	3386 (67.0%) 1665 (33.0%)	5387 (74.0%) 1895 (26.0%)	**p<0.000001** (Chi-Sq.)
Cancer Site			
Oral Cavity *Oropharynx* *Hypopharynx-Larynx*	2626 (52.3%) 902 (17.9%) 1490 (29.7%)	3136 (43.3%) 1143 (15.8%) 2965 (40.9%)	**p<0.000001** (Kruskal Wallis)
Individual Co-morbidities			
Hypertension Smoking Alcohol excess Obesity Diabetes Ischaemic Heart Disease COPD Hyperlipidaemia Peripheral vascular disease Asthma Liver disease Renal disease Heart failure GORD Cerebrovascular disease	1233 (24.4%) 688 (13.6%) 355 (7.0%) 38 (0.8%) 406 (8.0%) 276 (5.5%) 195 (3.9%) 233 (4.6%) 216 (4.3%) 215 (4.3%) 60 (1.2%) 66 (1.3%) 60 (1.2%) 69 (1.4%) 31 (0.6%)	1817 (24.9%) 1881 (25.8%) 962 (13.2%) 74 (1.0%) 609 (8.4%) 568 (7.8%) 739 (10.1%) 397 (5.4%) 403 (5.5%) 381 (5.2%) 175 (2.4%) 116 (1.6%) 107 (1.5%) 114 (1.6%) 80 (1.1%)	p=0.494 **p<0.000001** **p<0.000001** p=0.129 p=0.518 **p<0.000001** **p<0.000001** **p=0.037** **p=0.002** **p=0.013** **p<0.000001** p=0.195 p=0.183 p=0.368 **p=0.005**
Morbidity Score			

Strata 1 (<5) Strata 2 (5-10) Strata 3 (>10-20) Strata 4 (>20-30) Strata 5 (>30)	2864 (56.7%) 578 (11.4%) 1157 (22.9%) 377 (7.5%) 75 (1.5%)	3582 (49.2%) 866 (11.9%) 1926 (26.4%) 702 (9.6%) 206 (2.8%)	**p<0.000001** (Kruskal Wallis)
Primary Surgery			
	3130 (62.0%)	4557 (62.6%)	p=0.491 (Chi-Sq.)
Surgery during an emergency admission			
	317 (6.3%)	761 (10.5%)	**p<0.000001**
Resection Strata			
RS1 RS2 RS3 RS4 RS5 RS6	1320 (26.1%) 510 (10.1%) 992 (19.6%) 673 (13.3%) 1104 (21.9%) 419 (8.3%)	1270 (17.4%) 579 (8%) 1414 (19.4%) 956 (13.1%) 2146 (29.5%) 879 (12.1%)	**p<0.000001** (Kruskal-Wallis)
Adjunctive Surgeries			
Neck Dissection Tracheostomy Any Surgical Reconstruction	3862 (76.5%) 2534 (50.2%) 3634 (71.9%)	5579 (76.6%) 3712 (51.0%) 4720 (64.8%)	p=0.843 p=0.378 **p<0.000001**
Gastrostomy tube			
	679 (13.4%)	925 (12.7%)	p=0.229 (Chi-Sq.)

Table 22. Table of Patient Demographics from Chapter 4

Glossary of Terms

Human Papilloma Virus (Types 16 and 18)

An oncogenic virus, transmitted sexually that has been demonstrated as a significant causative agent of oropharyngeal carcinoma. It is thought to be the cause of up to 70% of oropharyngeal cancers.

Hypopharynx

A term referring to the area of the throat, lying below the oropharynx, but above the glottis.

Neck Dissection

The systematic removal of all lympho-fibrofatty tissue of the neck in order to remove cancer or stage disease. Several types of neck dissections exist, extending from Radical neck dissections (removal of all ipsilateral cervical lymph nodes alongside the internal jugular vein, the sternomastoid muscle and the spinal accessory nerve), to selective neck dissections (which specifically and exclusively remove a particular set of nodes and no other anatomy).

Oropharynx

A term referring to the area of the throat containing the base of tongue and pharyngeal tonsils, arising just distal to the oral cavity.

Social Deprivation

Referring to socio-economic deprivation. A complex term, encompassing a host of parameters that lead to patient inequalities. In the context of this work, it refers to the the relative affect of patients who are more or less socio-economically deprived and their resultant effect on healthcare outcomes.

Squamous Cell Carcinoma

An epidermoid based cancer arising from squamous cells, in the context of this work, referring to the lining of the hollow head and neck viscera.

Survival Analyses

Often used to assess end points in medical studies and used extensively in the context of this thesis. This analysis addresses the expected duration of time until an event occurs and also its associated rate. In oncology, this is often related to living status (overall survival), death directly

attributable to disease (disease specific survival), treatment failure (the persistence of tumour following definitive oncological treatment) and tumour recurrence (the return of oncological disease following a previous cured state).

Tissue Micro-array

A compilation paraffin block that is comprised of multiple small donor cores that allow the efficient analysis of an ideally representative sample of tumours representing a particular immunostain.

TNM Staging System

A system used to stratify patients diagnosed with solid malignant tumours, both in terms of diagnosis and treatment, but prediction of outcome. Criteria based of UICC/AJCC standards and recently updated in version 8.

Tumour Infiltrating Lymphocytes

A term used to describe lymphocytes found in tumour tissue that represent an underlying protective or inhibiting effect on the body to the cancer. Their location, number and type can be crucial in understanding the natural history of tumours and how said tumours may respond to treatment.

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