THE UNIVERSITY OF HULL

Immunomodulation within the Head and Neck Tumour Microenvironment

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By

Patrick Francis Murray
MB ChB, MRCS, MRCP

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Abstract

Changes in the immune response have been implicated in the progression of squamous cell carcinoma of the head and neck (HNSCC). Evidence is emerging that processes within the tumour microenvironment can lead to immune modulation and subsequent tumour growth or metastasis.

The hypothesis of this thesis is that the HNSCC tumour microenvironment will have increased levels of cytokines that produce an overall negative effect on the cellular cytotoxic immune response against the malignant cells. Specifically, it is hypothesised that a Th-2-like anti-inflammatory response will favour tumour cell progression and be associated with advanced stage HNSCC.

This thesis examines the levels of a panel of immune cytokines to investigate whether difference in these levels have an association with the progression of the disease and other standard clinico-pathological factors. A method of protein extraction from tumour tissue and detection of quantitative cytokine levels was developed and optimised. A cohort of 69 patients newly-presenting with HNSCC was recruited prospectively to the study, with a total of 83 samples of primary HNSCC tumour tissue and metastatic nodal tissue being investigated.

Increased levels of TGF-β, described as concentration of cytokine/mg total protein extracted, (median 1051 pg/mg *vs*. 659 pg/mg, $p = 0.004$) and reduced levels of IL-17 (median 4.2pg/mg *vs.* median 18.6 pg/mg, $p = 0.009$), compared with normal tissue from control patients were reported. The HNSCC samples were also found to have higher levels of VEGF in tumour samples (83 pg/mg *vs*. 27.6 pg/mg, $p=0.026$) compared with control tissue. No difference was found in the levels of IL-2, IL-10, IL-12, IL-15, or IL-17. When comparing early stage (I-III) to late stage IV HNSCC patients it was found that there were significantly lower levels of the Th1-like IL-12 in the higher stage IV patients (median 50pg/mg *vs*. 21 pg/mg, $p = 0.01$), and significantly higher levels of IL-15 in stage IV patients (median 52 pg/mg, *vs*. 20 pg/mg $p = 0.03$).

In summary, analysis of cytokine levels within the tumour microenvironment of HNSCC may be of prognostic value, and further study of the immune suppressive nature of HNSCC could open potential therapeutic approaches, especially if such data are correlated with other cellular parameters, e.g. T regulatory or CD8+ve T cell levels.

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Presentations resulting from Research

1. Murray P, Green V, Stafford N, Greenman J (2010). Investigating the Treg/TH17 Cytokine axis within HNSCC. *Otolaryngol Head Neck Surg* **142**(2):64.

   Oral Presentation at American Academy of Otolaryngology- Head and Neck Surgery annual conference 2009.

2. Oral Presentation Society of Academic and Research Surgeons, Bristol 2008.

3. Oral Presentation North of England ENT society, York 2007.

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Chapter 1: Immunoregulation within the Head and Neck Tumour Microenvironment

1.1 General Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is an aggressive epithelial solid malignancy. HNSCC is the commonest cancer arising within the head and neck region, excluding skin cancer, with the SCC histology making up 95% of the total cancers. The total incidence has been stable overall in the western world although there are reports of increasing frequency in certain groups; in contrast it continues to rise in eastern European countries and this has been attributed to increasing tobacco use (Garavello *et al* 2010, Jemal *et al* 2011).

The treatment of HNSCC involves surgery, chemotherapy and radiotherapy, or a combination of these modalities. A definitive treatment has been elusive in the advanced stages, with 2 year survival of approximately 40% overall for pharyngeal squamous cell carcinoma (SCC) rising to 60% overall for oral SCC. In the UK projections have been made that suggest a decline in the mortality rate of HNSCC over the next 25 years (Olsen, 2008). Life-style choices have been implicated as risk factors for developing HNSCC and therefore a proportion of malignancies are preventable: smoking, excessive alcohol consumption, and exposure to oncogenic viruses such as certain types of Human Papilloma Virus (HPV) possibly related to sexual preferences (Chin *et al* 2006, van Monsjou *et al* 2012).

There has been wide ranging research into the suppression of the host immune responses in cancer patients, and this has been investigated extensively in HNSCC (Young 2006, Whiteside 2012). Study of the inflammatory infiltrates of Head and Neck tumours has led to hypotheses relating to the tumour micro-environment causing a reduction of the host immune surveillance capability allowing disease progression in HNSCC (Bergmann 1998, Marincola *et al* 2000, Shiozaki *et al* 2003, Zitvogel *et al*

2006, Whiteside 2008). The concept of chronic inflammation leading to oncogenesis has been linked since Virchow, in 1863, attributed the suggestion of a functional relationship between the inflammatory infiltrating leucocytes with the developing tumour cells (Balkwill and Mantovani 2001). Dvorak made the analogy between the generation of stroma in the tumour microenvironment and the process of wound healing, stating 'Cancer is a wound that doesn't heal' (Dvorak 1986).

Some elements of the human immune system have been shown to be active in patients with HNSCC, and been described as 'immune surveillance' (Young 2006, Whiteside 2009). Antibodies specific for tumour-associated antigens (TAAs) have been found circulating in patients with cancer, such as MAGE-1 first identified in melanoma patients (Sahin *et al* 1997). T lymphocytes specifically able to recognise TAAs have also been demonstrated (Whiteside 2010). TAAs such as the hyaluronan RHAMM, and the membrane associated carbonic anhydrase G250/CAIX have been shown to have simultaneous expression in HNSCC (Schmitt *et al* 2009).

Immune effector cells such as natural killer (NK) cells, cytotoxic $CD8^+$ T cells, NKT cells and macrophages have been shown to have activity in cancer patients, and patients with cancer can produce measurable systemic responses to infection by both bacteria and viruses. This suggests that products of the tumour micro-environment are active in tumour immune escape, and may affect subsequent progression.

1.2 Basic Anatomy of the Head and Neck

1.2.1 Embryology

The anatomy of the adult head and neck region is complex, and an understanding of embryology helps the understanding the potential paths of local invasion, and lymph node spread of HNSCC. The importance of cytokines in embryological development, such as the absolute requirement of Transforming Growth Factor-β (TGFβ) in murine embryological development to proceed also hints at the requirement of functional cytokine networks to avoid cancer development (Gorelik and Flavell 2002).

The most typical features in the embryology of the head and neck are formed from the pharyngeal arches (Langman's Medical Embryology, 2006). These form within the fourth to fifth week of development. They initially consist of bars of mesenchymal tissue separated by deep clefts known as the pharyngeal or branchial clefts (relating to embryological gill development in fishes and amphibian). As they develop a number of out-pockets of tissue form, known as the pharyngeal pouches, along the border of the pharyngeal gut tract. These contribute significantly to the development of the structures of the head and neck. The epithelium arises from the embryological pharyngeal portion of the foregut. The epithelial lining of the larynx arises from the respiratory diverticulum, or lung bud, which appears as an outgrowth from the ventral wall of the foregut. It arises from the endoderm which also creates the endothelial lining of the trachea, bronchi and lungs. Each pharyngeal arch has a supplying artery, nerve and associated lymphatic drainage.

1.2.2 General Adult Anatomy of the Head and Neck

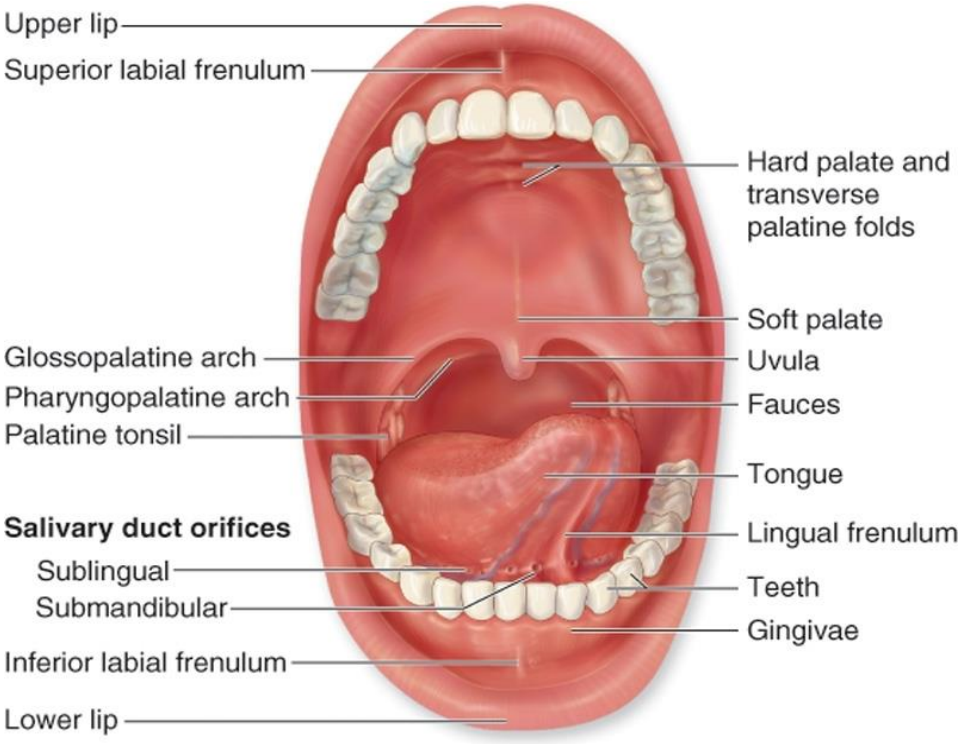
1.2.3 Oral Cavity

The oral cavity extends from the lips anteriorly, to the palatoglossal folds (anterior pillars of the fauces) posteriorly. The lateral borders are made up by the buccal mucosa of the cheeks, overlying the buccinator muscles, and enclosing the vestibule of the mouth which is a slit-like space between the cheeks and the gingivae, alveolar processes and teeth (if present). The floor of the mouth is a musosal lining of the mylohyoid muscle, and the extrinsic muscles of the tongue, the genioglossus, hyoglossus, and styloglossus. The roof is formed by the hard palate of the maxilla. Posteriorly the limit of the oral cavity is classified as the sulcus terminalis, dividing the anterior two thirds and posterior third of the tongue, containing the foramen caecum, the obliterated remains of the thyroglossal duct, amongst the line of circumvallate papillae.

Latero-posteriorly lies the retromolar trigone region. It is the point of transition between the oral cavity and the oropharynx. The mucosa overlies the pterygomandibular raphe, the boundary between the palatoglossal muscle of the anterior pillar of the fauces and the vestibule of the oral cavity. Both the buccinators muscle of the oral cavity and the superior constrictor of the pharnx attach here. At the point of the retromolar trigone the floor is deficient in musculature as the mylohyoid stops and allows easier potential spread of infection or neoplastic cells into the neck. The retromolar trigone is also associated with late stage presentation of oral SCC (Seaone-Romero *et al* 2012).

The oral cavity is lined with a stratified squamous epithelium. This is keratinised in certain areas of the gum, tongue and hard palate to a variable degree, but is non-keratinised elsewhere.

Fig 1.1 Oral Cavity (From http://www.familymedschool.com/2011/09/13/anatomy-of-the-mouth)



1.2.4 The Pharynx

The pharynx is a fibromuscular tube that runs from its attachment at the base of the skull to the oesophagus inferiorly. It can be divided into three parts the nasopharynx, the oropharynx and the laryngopharynx. Below are the descriptions of the oropharynx, larynx and hypopharynx on which head and neck squamous cell carcinoma sites of origin are commonly classified.

Oropharynx

The oropharynx extends from the lower surface of the soft palate to the upper border of the epiglottis. It is posteriorly bounded by the constrictor muscles of the pharynx; the superior, middle and inferior constrictors which act to push a bolus of food into the oesophagus in a peristaltic mechanism. Anteriorly, the oropharynx commences with the posterior third of the tongue, the lingual tonsil, and the oral cavity. Laterally are the

palatopharyngeal and palatoglossal arches (pillars of the fauces) which contain a fossa containing the palatine tonsils. The floor is made of the valecula which is a shallow fossa between the tongue base and the epiglottis, limited by the glossoepiglottic folds of mucosa. Its nerve supply is the internal laryngeal nerve.

Larynx

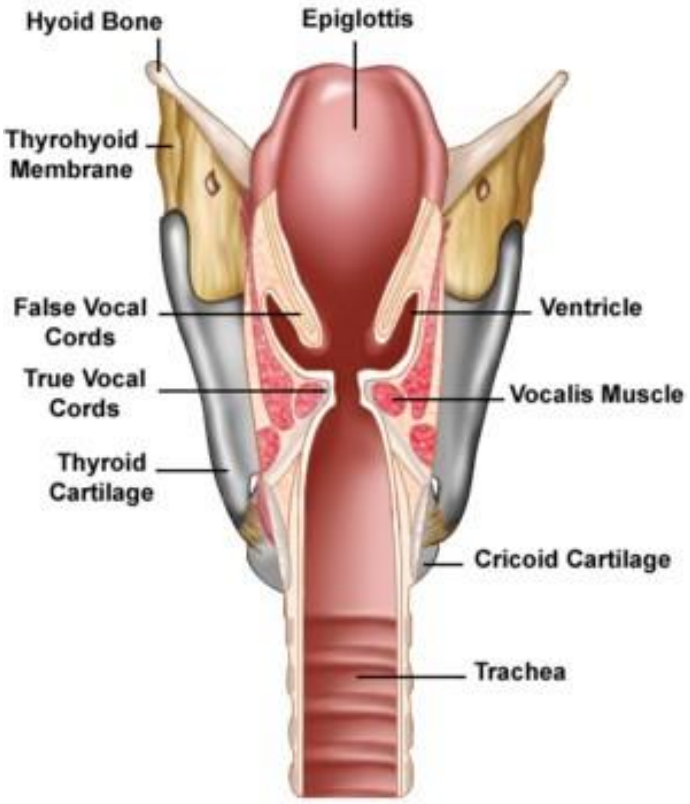
The larynx is technically a respiratory organ, derived from the respiratory diverticulum of the distal pharyngeal embryonic gut. Its main function is to protect the airways below from foreign bodies and potential infection. It also is used for phonation and is important in communication.

The skeleton of the larynx is made up of three individual cartilages (thyroid, cricoids, and epiglottic), and three pairs of cartilages (arytenoids, corniculate and cuneiform). A number of extrinsic and intrinsic ligaments and membranes make up the familiar shape and structure of the larynx (See Fig. 1.2).

The larynx is suspended from the hyoid bone by the thyrohyoid membrane, and attached to the epiglottis via the hyoepiglottic and thyroepiglottic ligaments. The intrinsic membranes of the quadrangular membrane extending between the epiglottis and the arytenoids cartilages has a free lower border which constitutes the vestibular ligament, and covered in respiratory epithelium makes up the false cords, and aryepiglottic folds. The cricothyroid membrane is made up of two parts; the anterior (or median) cricothyroid ligament, and the paired lateral or cricovocal ligaments whose free edge lined with mucosa make up the 'true' vocal ligaments used in phonation. The muscles of the larynx are able to open (posterior cricoaretenoid) and close the glottis (transverse and oblique arytenoids, and aryepiglottic muscle), the triangular space between the true cords, and can alter the tension of the cords resulting in pitch change (thyroarytenoid).

The mucus membrane of the larynx is mainly pseudostratified columnar ciliated epithelium as part of its respiratory function, however the vocal cords are strictly covered with non-keratinised stratified squamous epithelium. When this becomes abnormally keratinised it can indicate an abnormality.

Fig 1.2 The Larynx (Posterior view, from http://myweb.csuchico.edu/)

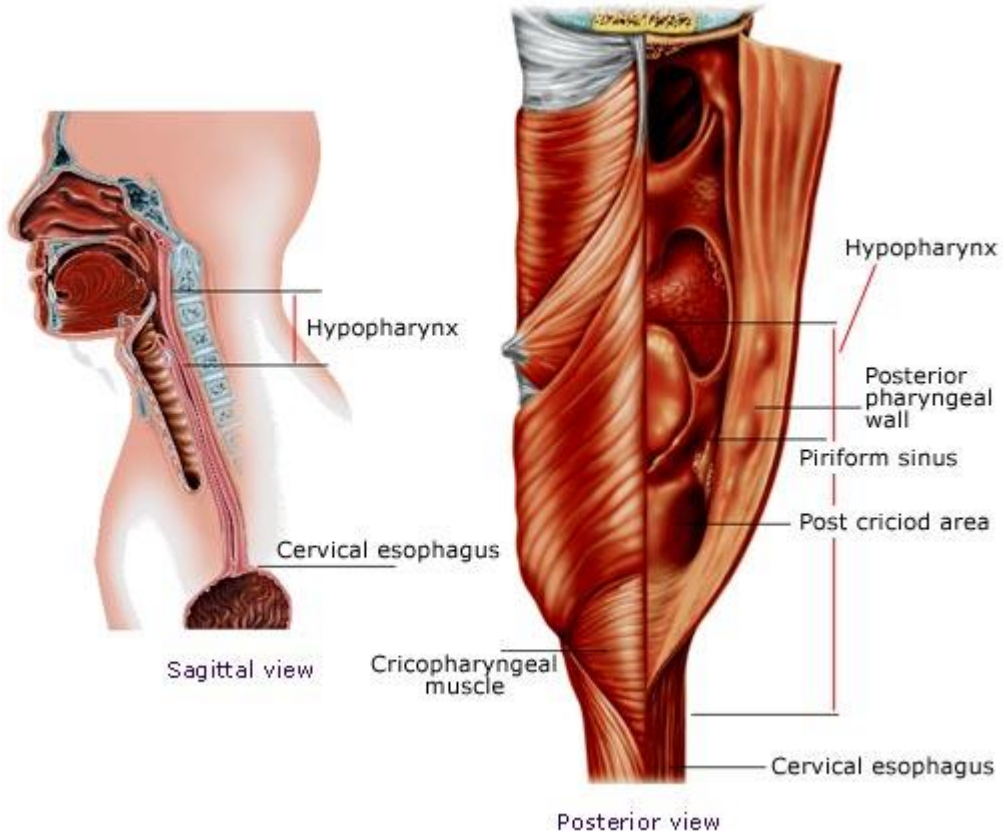


Hypopharynx

The hypopharynx is the term describing the region of the pharynx below the larynx. The Piriform fossae run laterally (see Fig. 1.3), which are inverted pyramidal folds of mucosa commencing either side of the opening of the larynx, and are limited by the lateral glossoepiglottic fold anteriorly. The inferior constrictor composes the posterior wall below the level of the vocal cords. The superior and middle constrictors end at this point, leaving a potential weakness in the posterior pharyngeal wall known as the dehiscence of Killian (through which a pharyngeal pouch may extend). The hypopharynx ends at the cricopharyngeal sphincter. The piriform fossae are important

clinically, as a neoplasm may grow in this region for some time before symptoms become apparent to the patient due to the lack of local symptoms.

Figure 1.3 The Hypopharynx (From http://www.kuleuven.ac.be/cltr/nl/hypopharynx)



1.2.5 Lymphatic Drainage

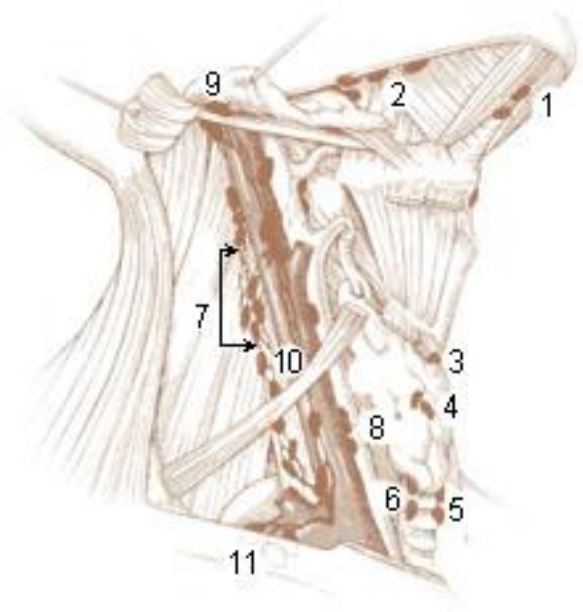
The function of the lymphatic system is to continue the circulation of tissue fluid. During the systemic blood circulation, blood pressure at the capillary level forces fluid, and depending on fenestration proteins, into the extracellular matrix to bath the cellular level. Water can be reabsorbed by oncotic pressure in the distal capillary and venules. The extracellular fluid is circulated and returned to the venous system via the lymphatics. These are simple endothelial structures, similar to veins, but with more valves to prevent retrograde flow. In general the superficial lymphatic system follows veins, whereas deep lymphatic tissue follows arteries. The lymphoid system is the macroscopic anatomical structure of the immune system. The primary lymphoid organs

are the bone marrow and the thymus. Secondary organs include the palatine tonsils, adenoids, lymph nodes and the spleen.

The lymphatic drainage of the head and neck has been described in three stages (Stell and Maran 2004). The two Waldeyer's rings and the deep cervical lymph node system. The internal Waldeyer's ring surrounds the oropharynx and is a ring of lymphoid tissue consisting of the adenoids in the post-nasal space, the palatine and the lingual tonsils. The external Waldeyer's ring consist of the superficial lymph nodes draining the head and neck consisting of the occipital, postauricular, parotid and facial nodes, in continuity with the submental, submandibular and superficial cervical nodes.

Figure 1.4. The Cervical lymph nodes of the neck

(From http:// wikidoc.org/index.php/Supraclavicular_lymph_nodes)



Cervical Lymph Nodes

1. Submental
2. Submandibular
3. Prelaryngeal
4. Thyroid
5. Pretracheal
6. Paratracheal
7. Mid Cervical
8. Lower cervical
9. Jugulo-digastric
10. Jugulo-omohyoid
11. Supraclavicular

The deep cervical lymph nodes drain the lymph from both the superficial lymph system and the surrounding deep fascial layers of the neck. The drainage of lymph has been studied using lymphangiography to identify the most likely direction of spread of tumour metastatic disease. Linberg first described the method of likely primary site identification based on cervical metastatic node involvement (Lindberg 1972, see Fig

1.5). The neck has been divided into levels in order to classify the nodal regions to which metastases may develop away from the primary site of disease (see Fig 1.6).

Figure 1.5. Common sites of metastatic disease to the Cervical neck nodes (From http://dentallecnotes.blogspot.co.uk/2011/10/differential-diagnosis-of-head-and-neck.html)

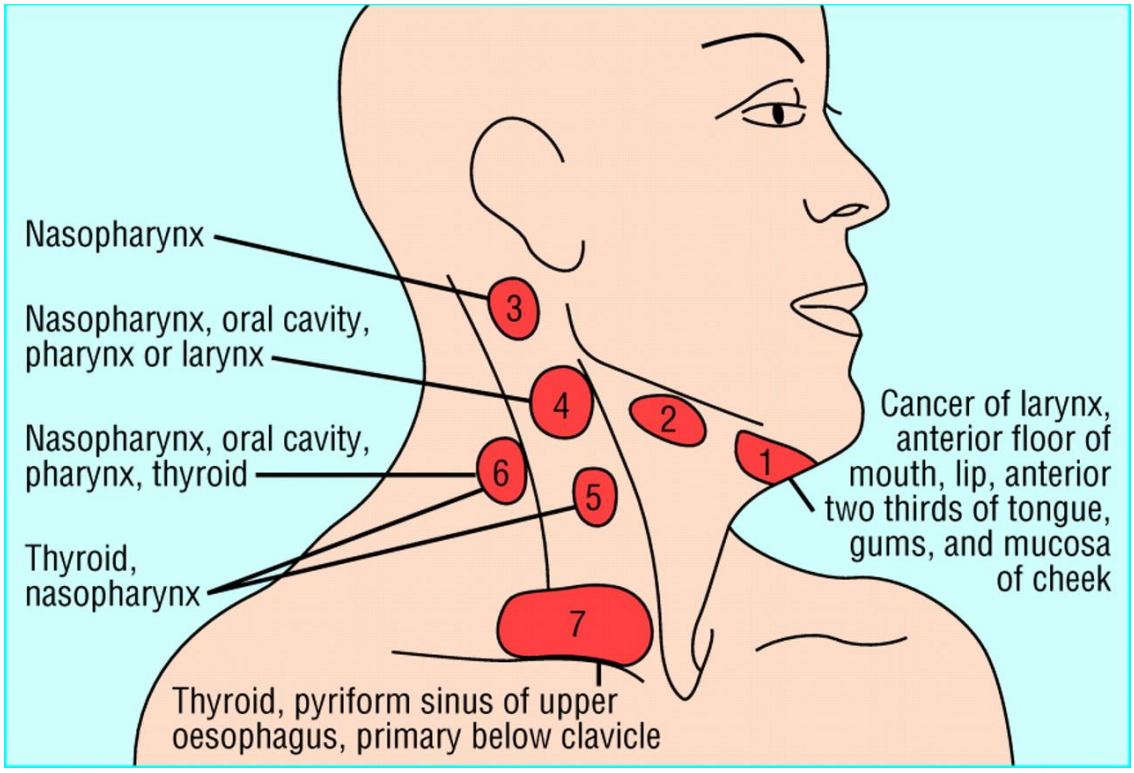
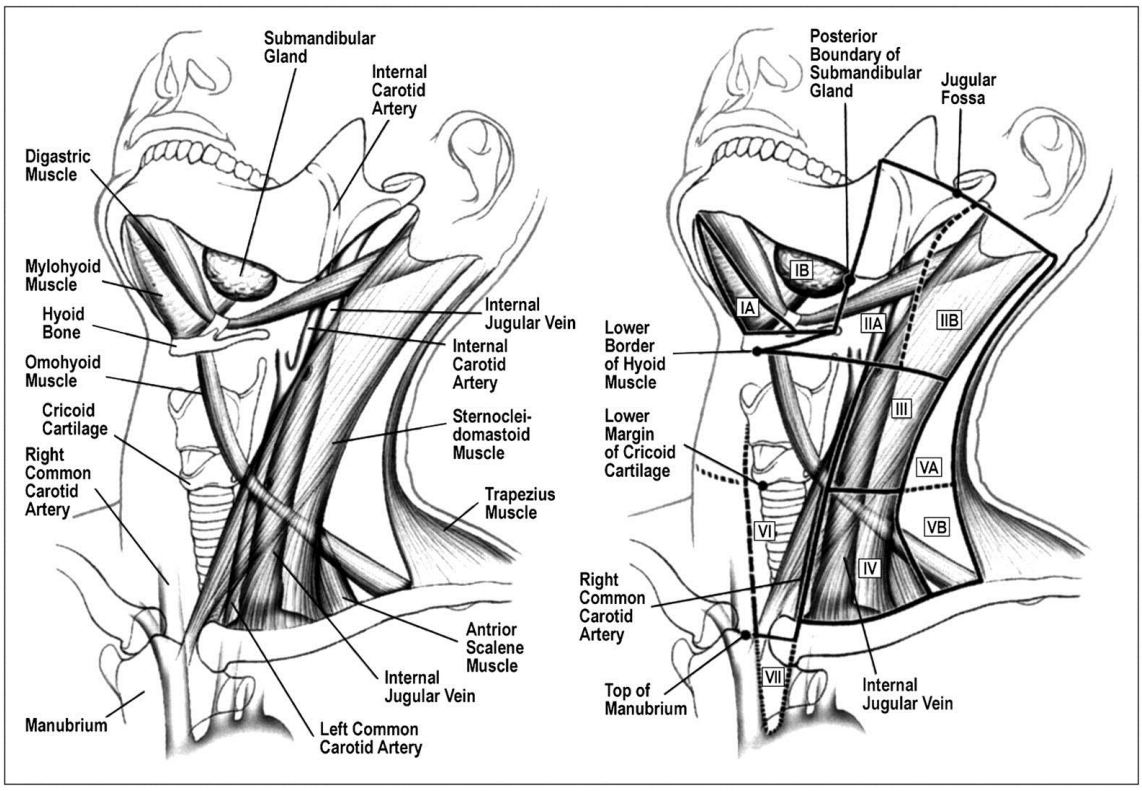


Fig 1.6. Levels of the Neck (From Mar *et al* 2007).

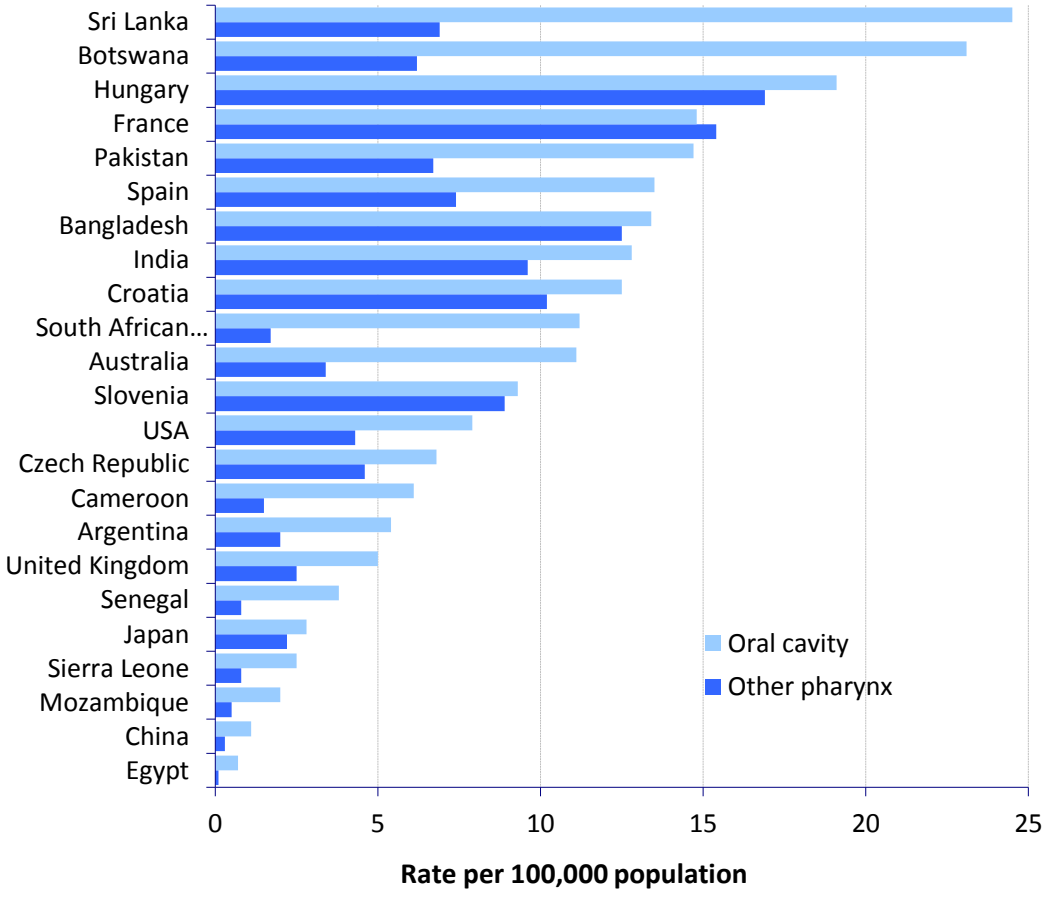


1.3 Epidemiology of HNSCC: Incidence

1.3.1 Worldwide Incidence of HNSCC

HNSCC is the sixth most common malignancy worldwide, and accounts for 7000 new cases of cancer in the UK in 2005/06 (National Office of Statistics 2009). In the US the estimate of new cases for 2008 was 47,300, with 11,100 estimated deaths (Jemal *et al* 2008). Across the world there is an increased risk of developing oral SCC in South-central and South-eastern Asia, western and southern Europe, and South Africa. Laryngeal SCC has a higher prevalence in Southern and Eastern Europe, South America and Western Asia (Jemal *et* al 2011, WHO 2002, Fig. 1.7).

Fig. 1.7. World Age standardised male incidence rates for oral cancers, selected countries, 2002 estimates. (WHO 2002)



1.3.2 UK Incidence and Mortality Statistics for HNSCC

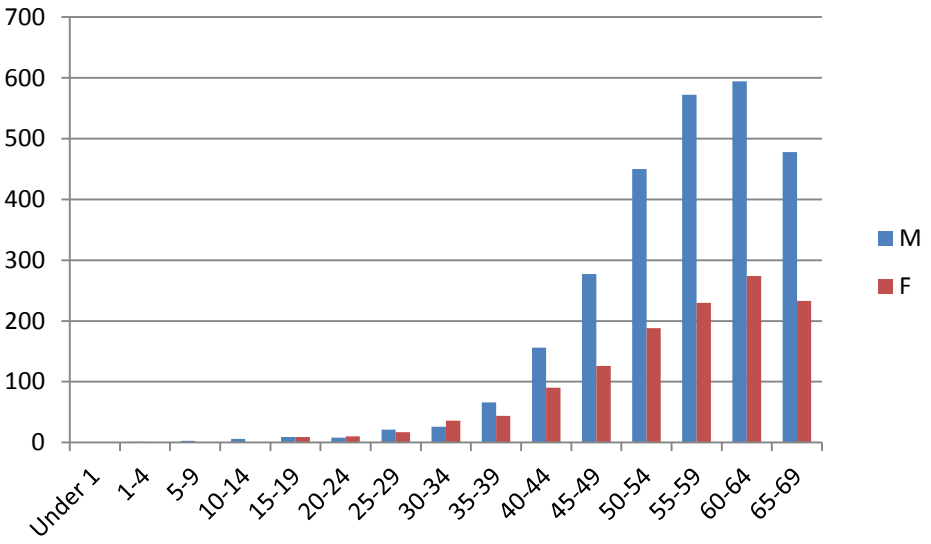
The Cancer research UK HNSCC incidence and mortality statistics divide the cohort into two groups, those diagnosed with laryngeal SCC and those with lip, oral, and pharyngeal SCC. Between 2006 and 2008 approximately 306,000 patients in the UK were diagnosed with cancer (excluding non-melanoma skin cancer), 50.2% were male, 49.8% female.

DAHNO (Data for Head and Neck Oncology) released the findings of its continuing Audit of head and neck cancer across the UK (DAHNO 2011). They recorded 6458 registrations of patients with new HNSCC across England and Wales between the 1st of November 2009 and 31st of October 2010. This was compared to an estimate of 6740 patients based on the cancer registry data from 2006-08; a 95.8% estimated data capture of registered patients. Within this 79% of patients were recorded as having a stage where T and N of the TNM staging system are used, however more patients were classified as T_x or N_x (see section 1.5.1)

The ONS cancer registration in 2009 recorded approximately 7550 patients who were diagnosed with HNSCC, of these approximately 3700 males and 2000 females were diagnosed with lip, oral cavity or pharyngeal SCC, which is the commonest subsite of HNSCC. Approximately 1500 males and 300 females were diagnosed with laryngeal cancer.

Overall mortality from 2006-08 (ONS, 2011) revealed 1367 males died with lip, mouth and pharyngeal cancer, compared with 721 females; 639 males dies with laryngeal cancer, compared with 159 females.

Fig 1.8 Total number of Lip, oral and pharyngeal Cancers by Age (UK,



Data for survival from CR-UK and the ONS has shown a slight increase in overall survival of laryngeal cancer of around 62% five-year survival.

The CR-UK data on oral cavity and pharyngeal cancer from 1996-2000 showed an overall 2 year crude survival of 62%. The prognostic value of stratifying the cohort by extent of tumour spread is discussed below (Section 1.5).

La Vecchia *et al* analysed data from the WHO and examined cancer mortality in 34 European countries between 2000-04, and compared trends from 1975-2004. They found that overall cancer rates peaked in 1988. On examining oral and pharyngeal SCC they found mortality had declined overall by 10% from 6.6 to 6.0 per 100,000 population. They did find an increase in female mortality overall from oral/pharyngeal SCC to 1.2 per 100,000 population.

So, in summary, better cancer registration is leading to better data for incidence and outcome in HNSCC.

1.4 Diagnosis, Staging and Prognostic factors in HNSCC

The diagnosis of HNSCC depends mainly on clinical history and the site of origin of the tumours. Accurate staging of the disease at presentation is essential due to prognostic consequences, and significant correlation with poor survival rates (Layland *et al* 2005).

The disease is more likely to present in males, with a median age of 60 years, and around 70% of patients present with local metastases to the cervical lymph nodes (Parkin *et al* 2002).

Progression of the disease causes increased morbidity due to its location through loss of function and aesthetics. This can have a dramatic effect on the patient's quality of life. Despite advances in surgical, radio- and chemo-therapy there has not been the expected improvement in five-year survival of the disease over the last thirty years, which remains around 60% (Rachet *et al* 2008). Current treatment regimens also lead to significant morbidity even when local cure is achieved (van den Broek *et al* 2009, Duvvuri *et al* 2004, and Weber *et al* 2003).

1.4.1 Staging

The Tumour-node-metastasis (TNM) staging system is widely used, it was first reported by PF Denoix in 1944. The 7th edition has been developed with a consensus in 2009 between the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC). It is an anatomical staging system, based on size and spread of the primary tumour, local lymph node metastatic disease, and distant metastatic disease. The tables which list the T, N, and M categories for the subsites of HNSCC used in this thesis are in Appendix 2.

Further categorisation into staging of the disease is made depending on the TNM score, and therefore spread of the disease. Staging ranges from Stage I, being most localised, to stage IVC locally advanced disease with distant metastases.

The American SEER database provides groups tumours according to metastatic data, in situ, local, regional nodal disease, and distant metastatic disease.

The TANIS scoring systems are adjuncts to the TNM score, which gives an extra level of stratification (see table below), which may improve prediction of prognosis, but has not been widely assumed. (Snyderman and Wagner 1995, van der Schroeff and Jung 2009).

The TNM system has provided an international standard of reporting tumour size, invasion and lymph node metastasis, allowing standardised comparison between populations (Patel 2005). The scoring systems have allowed stratification of patients to compare tumour progression and survival. As further molecular evidence of heterogeneity of HNSCC arises, further stratification will be necessary, especially with the evidence produced with the HPV-positive tumours (Section 1.5.8).

1.4.2 Prognostic Factors in HNSCC

The most important prognostic indicators are those of TNM, the T-stage, N-nodal involvement and M-distant metastases (Mehanna *et al* 2010).

The T stage is generally related to tumour size r, due to the inverse proportion of tumour volume and prognosis. Comparing T-stage T1/T2 patients with T3/T4 staged patients showed an increased relative risk of 1.4 (Cerezo *et al* 1992). However Knegjens and colleagues (2011) compared imaging voluming of disease with TNM, and found an increase in the hazard ratio of 14% for each $10cm^3$ increase in tumour volume. Whereas there was no statistical significant difference in T-stage. They confirmed the importance of N stage as a statistically significant independent factor for overall survival.

Nodal involvement dictates that the tumour is already advanced enough to have metastasised locally, and this is also associated with a poor prognosis, hence the N-stage of TNM. However, the N-stage is based on side and size and number of metastatic lymph nodes in HNSCC staging. There is no scoring of subsite of nodal involvement, or extra-capsular spread which both have an effect on worsening prognosis (Woolgar 1999, Myers *et al* 2001). In Cerezo *et al*'s study patients with N2 disease had a 1.3 times higher risk of death than those with N1, and those with N3 had twice the risk of death (all patients in the study had nodal disease). They confirmed Hahn *et al*'s work that nodal involvement had more prognostic significance than T stage. (Cerezo *et al* 1992, Hahn *et al* 1987).

Distant Metastases

All patients with distant metastatic disease with an HNSCC primary are classified as Stage IVC by the TNM staging classification. There is a highly significant association of survival (Datema *et al* 2010) in 1662 patients in Leiden, Netherlands between 1981-1998.

Table 1.1 Metastatic HNSCC and survival (From Datema *et al* 2010)

M Classification	1-year survival	3-year survival	5-year survival
M0	0.85	0.71	0.54
M1	0.15	0.08	0.00

Local Recurrence

Cerezo *et al* retrospectively reviewed 492 patients with HNSCC (1992) and clinically positive neck nodes following radical radiotherapy treatment alone. They found that local recurrence at the primary site of treatment was the most important cause of treatment failure in their patients with 20%, followed by distant metastatic disease in 10%.

Co-morbidity

Co-morbidity can be defined as the concurrent presence of two or more diseases diagnosed in the same patient in relation to another disease being studied (Gimeno-Hernandez *et al* 2011). In HNSCC patients these diseases directly unrelated to the cancer may be related to epidemiological factors such as tobacco smoking or alcohol. Investigating patients with laryngeal cancer and co-morbidity using the Charlson co-morbidity index (CCI), Gimeno-Hernandez and colleagues found that severe co-morbidity was associated with a significantly increased risk of dying from all causes than those with null, mild or moderate scores.

Datema *et al* (2010) had previously examined the effects of co-morbidity as defined by the ACE27 co-morbidity evaluation, also known as the modified Kaplan Feinstein (KFI) co-morbidity index, grading 1- mild, 2- moderate, 3 - severe decompensation (2 or more grade 2 ailments were combined to give a grade 3score). A comparative study had previously compared the KFI with the CCI, the cumulative illness rating score, and the index of coexistent disease, and found the KFI most successful in stratifying patients with head and neck cancer (Hall *et al* 2002). They found a significant association between increasing co-morbidity and risk of death of any cause. A grade 0 patient had a 5 year survival probability of 0.58 compared with a Grade 3 5 year survival of 0.25. They also found similar associations with T and N score (T1 5 year survival probability of 0.74, T4 - 0.27; N0 - 0.65, N3 - 0.12).

1.5 HNSCC and the Immune response

1.5.1 Background

The tumour microenvironment of HNSCC involves tumour cells with the epithelial layer, surrounding stromal cells, and tumour-infiltrating cells, which include

lymphocytes, antigen presenting cells, and inflammatory cells (Whiteside 2009). The ideal environment to induce complete tumour regression is not known, and probably varies from person to person and by type of tumour. It is also by definition difficult to sample in human HNSCC, as the ideal infiltrate would be expected to result in tumour eradication.

It is important to understand the normal function of the human immune system in order to discover its function in HNSCC. A number of elements affecting the initiation and progression of cancer will be summarised.

Percival Pott, an Eighteenth Century London Physician, first noted the increase incidence of scrotal cancer amongst chimneysweeps, and first described the association between an environmental agent and cancer. His hypothesis was the coal dust and tar exposure led to the development of scrotal cancer.

The concept of a multi-step process of initiation, promotion and progression begins with the irreversible mutation within a cell to promote proliferation, via activation of a proto-oncogene, or loss of tumour-suppressor genes (Barrett 1993). However cellular proliferation on its own is not associated with cancer formation. Therefore further genetic or epigenetic mutations or insults are necessary for cancer formation.

1.5.2 Field Change and Cancerisation

The theory of "Field Cancerization" within HNSCC was first proposed in 1953 (Slaughter *at al* 1953). A case series of 783 HNSCC patients histology was analysed, and found that the lateral extension of the tumour was always greater than deep extension; microscopic analysis of the 'normal' margins of the tumour revealed marked hyperplasia, hyperkeratinisation, with 'round-cell' infiltrates and capillary telangiectasia. This may be an early description of tumour infiltrating lymphocytes and

tumour associated neovascularisation. Slaughter and colleagues also found that 88 patients had synchronous primaries, and concluded that cancerous changes were occurring over regional areas.

The concept of field cancerisation in head and neck cancer is that a region or subsite is genetically damaged, in a way which predisposes to malignancy, but avoids apoptosis or immune detection. This patch can then extend through proliferation and involve a wider area known as field change (Braakhuis *et al* 2003). Further exposure to carcinogenic factors then leads to further genetic alteration, which can lead to clonal divergence and the development of cancer within a pre-malignant field.

The concept of field cancerisation has clinical implications due to the risk of local recurrence or second primary cancers. The incidence of second primary tumours at diagnosis is as high as 20% of patients (de Vries *et al* 1986). This can also affect treatment, as field size, or multifocal disease may make the morbidity of complete surgical excision too risky for the patient.

Microsatellite typing uses polymerase chain reaction (PCR) to amplify DNA sequences which include sequence repeats. Alleles are copies of genes within the genetic code, and one is inherited from each parent. The copies have varied lengths of repeats, and therefore can be distinguished by size (Viega *et al* 2003). In a tumour cell which has had a deletion of one copy of a tumour suppressor gene or allele, loss of one allele, or loss of heterozygosity, can be detected in nearby microsatellite repeats within the tumour. Grati *et al* demonstrated 32 of 45 chromosomal regions within oral and oropharyngeal carcinoma had losses of LOH of >20%. (Grati *et al* 2000)

Tabor *et al* (2001) analysed LOH by microsatellite analysis and p53 mutation analysis comparing tumour tissue with macroscopically surrounding normal tissue, found that 36% (10 of 28) specimens showed molecular evidence of field cancerisation. They also found evidence the field extended beyond the surgical margins of the specimens.

1.5.3 Genetic alteration and HNSCC

In 1990 Fearon and Vogelstein presented a model for the genetic basis of colorectal neoplasia, focussing on the mutative activation of oncogenes with the mutative inactivation of tumour suppressor genes. They stated that at least four genes were needed to be affected by mutation in order to cause malignancy, whereas fewer changes resulted in benign tumour development. These changes, although displayed in the usual linear chronological fashion, did not require the mutations to occur in that order, but cumulatively would result in the same result of malignancy. The activation of oncogenes, and inactivation of tumour suppressor genes can lead to growth promotion within the affected cell, and confer a survival benefit (Suarez *et al* 2006).

The step-wise model has been demonstrated only in Oral SCC, probably due to the ease of access to regular biopsy analysis. It has also the greatest research into pre-malignant lesions and the potential development of cancer (Califano *et al* 1996). Further work analysed progression of HNSCC, using microsatellite analysis for allelic loss from 10 major chromosomal loci. Califano and colleagues analysed 87 biopsies from benign lesions associated with carcinogen exposure, then pre-malignant lesions, to invasive tumours. The authors found a pattern of increasing chromosomal loss across the loci with each histopathological progression. These were 9p21 which codes for p16 or INK4A a cell cycle inhibitor (Hunter *et al* 2005), 11q13 bcl-1/int-2 coding for the proto-oncogene cyclin D1, 17p13 for p53, and Chromosome 3p which contains tumour suppressor gene loci (Califano *et al* 1996). They found loss of heterozygosity at chromosomes 3p. 9p and 17p in apparently early stages associated with dysplasia and early carcinogenesis, and that further alterations at 11q, 4q, and chromosome 8 were found in carcinoma tissue suggesting a later stage in carcinogenesis (Leemans *et al* 2011).

Sidransky *et al* presented evidence that synchronous primaries may arise from a common clonal origin of tumour cell within HNSCC. Using X-chromosome inactivation they sampled 18 synchronous lesions from 8 female HNSCC patients (Bedi *et al* 1996). The principle being that one X-chromosome is inactivated in a random fashion early in embryogenesis, and this pattern is then stably passed on to further daughter cells. Only four patients had interpretable results of X-chromosome inactivation, and of these all four demonstrated the same pattern of X-chromosome inactivation.

Further evidence of clonal expansion has been demonstrated by using comparative genomic hybridisation in order to detect regions of DNA sequence copy number alterations (Bockmuhl *et al* 2002, Kujawski *et al* 1999, and Patmore *et al* 2004). Computer analyses of gains or deletions at locations of the genome were compared between primary HNSCC tumour sites and metastatic lymph node deposits. Patmore *et al* found >95% concordance in 22 from 23 specimens suggesting a strong clonal relationship.

Jin *et al* (2002) examined a total of 42 samples taken from 19 HNSCC specimens for karyotypic heterogeneity. They demonstrated a mixture of cytogenetically related and unrelated clones indicating cells at different stages of abnormal chromosomal rearrangement. They reported one specimen which showed two complex clones which weren't cytogenetically related indicating a tumour of multicellular origin.

Patmore and colleagues also examined 68 HNSCC specimens using comparative genomic hybridisation to analyse HNSCC at a subsite level (Patmore *et al* 2007). They found multiple genetic aberrations affecting the tumour genomes, and also laryngeal carcinomas had a statistically significant increase in aberrations in comparison to oral SCC.

1.5.4 Tumour progression in HNSCC

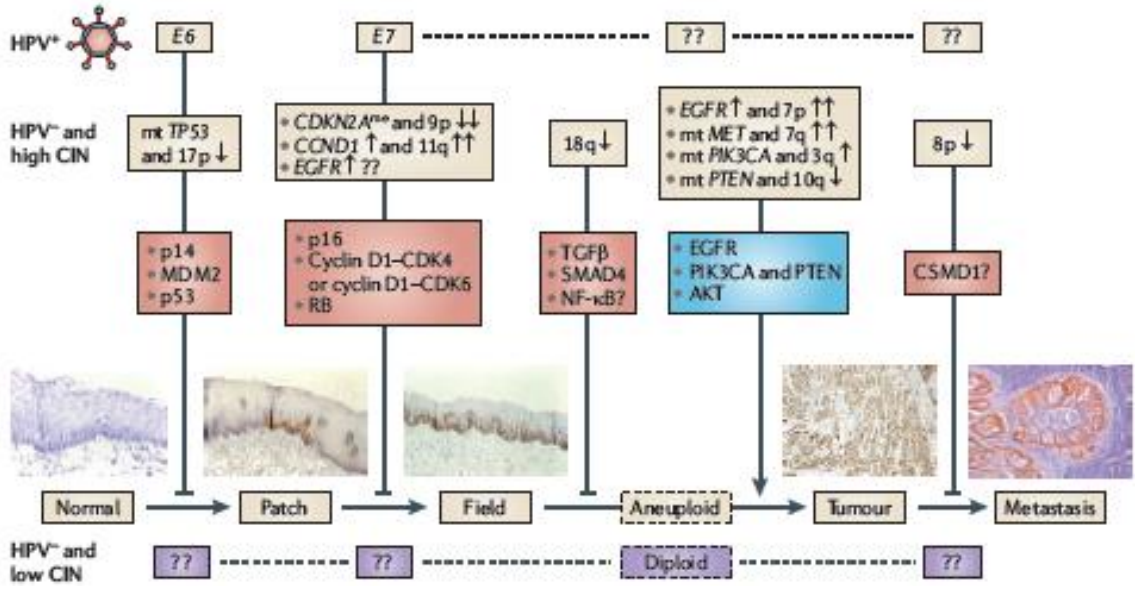
The natural progression of HNSCC historically has been assessed on the basis of histological features. These include dysplasia in which histological cell atypia such as pleomorphism and increased mitotic division, can be graded as 'mild', 'moderate' and 'severe' depending on the involvement of the epithelial layer. Carcinoma-in-situ is defined as malignant cells at the most severe end of the dysplastic spectrum, but these have not yet penetrated the epithelial basement membrane and therefore demonstrated invasion. Invasive carcinoma can be categorised as 'well', 'moderate', or 'poorly' differentiated cells depending on the tumours retention of normal tissue architecture. Anaplastic carcinoma has no cytological or architectural resemblance to the original tissue.

The genetic and epigenetic changes lead to proteomic changes within the cell, and alterations to signalling pathways. These signalling pathways in turn alter the cellular response, and lead to malignant phenotypes such as limitless cell proliferation, self-sufficient in autocrine and paracrine growth signals, avoidance of apoptosis, stimulate neo-angiogenesis, and the ability to invade surrounding tissue and metastasise (Hanahan and Weinberg 2011).

The key in avoiding genomic instability and development of cancer is effective DNA repair. The detection of DNA damage has been associated with over 130 genes identified by the Human Genome project. If DNA damage or nuclear stress is detected, protein kinase cascades are activated and the cell cycle is halted at this point. If the DNA damage is irreparable, then apoptosis is activated via the *p53* gene. Cell cycle 'check points' are mechanisms by which the cell actively halts progression of the cycle in order to confirm the previous processes, such as DNA replication or mitosis, are complete (Kastan and Bartek 2004).

Leemans *et al* propose a multistep model of carcinogenesis of HNSCC (see fig 1.9) based on the acquired cancer-associated phenotypes described by Hanahan and Weinberg.

Fig. 1.9. Potential factors in a step-wise development of HNSCC (from Leemans *et al* 2011)



Replication

The step that allows unlimited replicative potential in HSNCC is controlled by the p53 and the RB pathways. The retinoblastoma pocket proteins control the first restriction point of the cell cycle or G1. At induction of mitosis, complexes of cyclins and cyclin-dependent kinases are activated. These phosphorylate the RB pocket proteins leading to activation of E2F, which in turn activate the expression of S phase genes.

DNA damage and stress can be detected by ataxia-telangiectasia (ATM) and ataxia-telangiectasia and Rad3-related (ATR) DNA damage sensors to phosphorylate and active p53 to induce expression of p21 which halts the cell cycle. P53 is also a master regulator of apoptosis. TP53 which codes for p53 has been found in 60-80% of HNSCC

cases (Poeta *et al*, 2007). It is also inactivated by HPV 16 E6 protein in HPV-induced HNSCC.

Growth Factors in HNSCC

The epidermal growth factor receptor (EGFR) is a cell surface protein of the ErbB family. Activation leads to a downstream phosphorylation cascade via MAPK, ERK, and Jak/STAT which have been related to subsequent proliferation, apoptosis, invasion, angiogenesis, and metastasis (Chang and Califano, 2008). EGFR signals via the Ras-MAPK, P13K-PTEN-AKT and phospholipase C pathways (Hynes and Lane 2005). It is also able to transfer to the cell nucleus and assist in activating other transcription factors such as signal transducer and activator of transcription proteins (STAT) 3 and 5 (Lo 2010). It has been shown to be over-expressed in HNSCC, and is an important therapeutic target, for example the use of the monoclonal antibody against EGFR, Cetuximab (Cooper and Cohen 2009).

The transforming growth factor-β (TGF-β) receptor signalling pathway acts in an inhibitory fashion, by signalling via SMAD phosphorylation it promotes gene expression of cell cycle suppressors such as CDKN2B. Reduced expression of TGF-β receptors have been found in HNSCC (Ikushima and Myozono 2010), and this has been associated with an increase in nuclear factor -κB (NF-κB) (Leemans *et al* 2011).

Cell Death – Apoptosis to Necrosis

The distinct pathway of programmed cell death, or apoptosis, is essential in normal embryonal development and also the ongoing maintenance of normal tissue homeostasis. The key genes within the cell death pathway are closely related to inflammatory cytokines. Caspase 1, a protease, is closely related to the interleukin(IL)-1β-converting enzyme, and induces cell death (Danial and Korsmeyer 2004). The

caspase pathway was initially found to require three apoptotic protease activating factors, Apaf-1, -2, and -3, which were needed to activate caspase protease cascade in an energy (ATP) dependent activity in vitro, with Apat-2 and -3 subsequently identified as cytochrome c and caspase 9 (Li *et al* 1997).

The proto-oncogene BCL-2 was initially identified at the breakpoint of t(14;18) in human follicular B cell lymphoma, and was found to block the cell death pathway, specifically at the mitochondrion. Murine studies using transgenic bearing BCL-2 immunoglobulin mini-gene develop polyclonal follicular hyperplasia due to accumulation of resting B cells, as opposed to increased proliferation (McDonnell *et al* 1989). These mice were found to develop high grade monoclonal lymphoma, associated with a complementary activation of c-myc. *Bcl2/myc* transgenic mice demonstrated the danger of a pro-proliferative in c-myc activation and loss-of apoptosis in Bcl2 as the mice developed undifferentiated leukaemia.

The Bcl2 family also contains the pro-apoptotic molecule BAX, and the ratio of Bcl2/BAX can act as a balanced threshold to apoptosis.

Apoptosis is characterised by non-inflammatory cellular phagocytosis with the dying cell presenting cell membrane signals such as phosphatidylserine by macrophages, which release TGF-β and IL-10 to reduce the inflammatory response (Danial and Korsmeyer 2004).

Cell death can be mediated by other means. Cytotoxic T cells and NK cells exocytose perforin and granzymes. Perforin induces membrane pores to cause cell death, whereas Granzyme-B can activate both caspase-3 as well as DNases resulting in DNA breakdown, whereas Granzyme-A can activate DNases via a caspase independent route.

Other death receptors have been shown to be important in immune cell function, including the Fas death receptor and the Trail/Tnf pathway (Strauss *et al* 2009).

Necrosis can result from a range of cellular insults such as ischaemia and acidosis, and is characterised by organelle distortion and degradation as opposed to the organised condensation and fragmentation of nuclei in apoptosis. The pathway beyond the initial insult usually leads to uncontrolled release of Ca^{2+} from the endoplasmic reticulum.

1.5.5 Immuno-surveillance and HNSCC

The theory that the human immune system is able to monitor, recognise and remove neoplastic cells is known as tumour immune surveillance (Dunn *et al* 2004). The classical elements of the immune response can be divided into the innate and adaptive immune systems. The innate immune system is made up generally of cells which need no previous priming in order to be activated, such as macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells and natural killer cells. The adaptive immune system (T and B cells) depend upon activation by professional antigen-presenting cells processing abnormal molecules through the major-histocompatability complex (MHC) and coupling with the T-cell receptor (TCR) and associated co-stimulatory molecules.

However, there are cells which bridge the classic divide between the adaptive and the innate immune system. For example δγT cells and NK-T cells, both have the ability to directly interact with tumour cells without activation by the TCR (Gajewski *et al* 2013). Activated NK cells have been shown to eradicate murine large solid cancer models using IL-15 (Liu *et al* 2012).

Tumour-associated antigens are present on the cells of HNSCC, however they may have shared expression with other normal cells within the periphery or in the thymus. Immune tolerance is generated within the thymus, were the highest avidity interactions between MHC-peptide-TCR can lead to selective deletion of those T cells. However,

point mutations within normal genes are less likely to be replicated in normal tissue and may result in a more effective immune response (Sakaguchi *et al* 2008).

The theory of immunosurveillance, in which the immune system monitors normal cells for the development of malignant change, and removes them; and the theory of immunostimulation in which a chronic condition or inflammatory response leads to an environment which encourages tumour transformation, have both been put forward and evidence for both have been demonstrated (Ichim 2005, Zitvogel *et al* 2006, Ridolfi *et al* 2009).

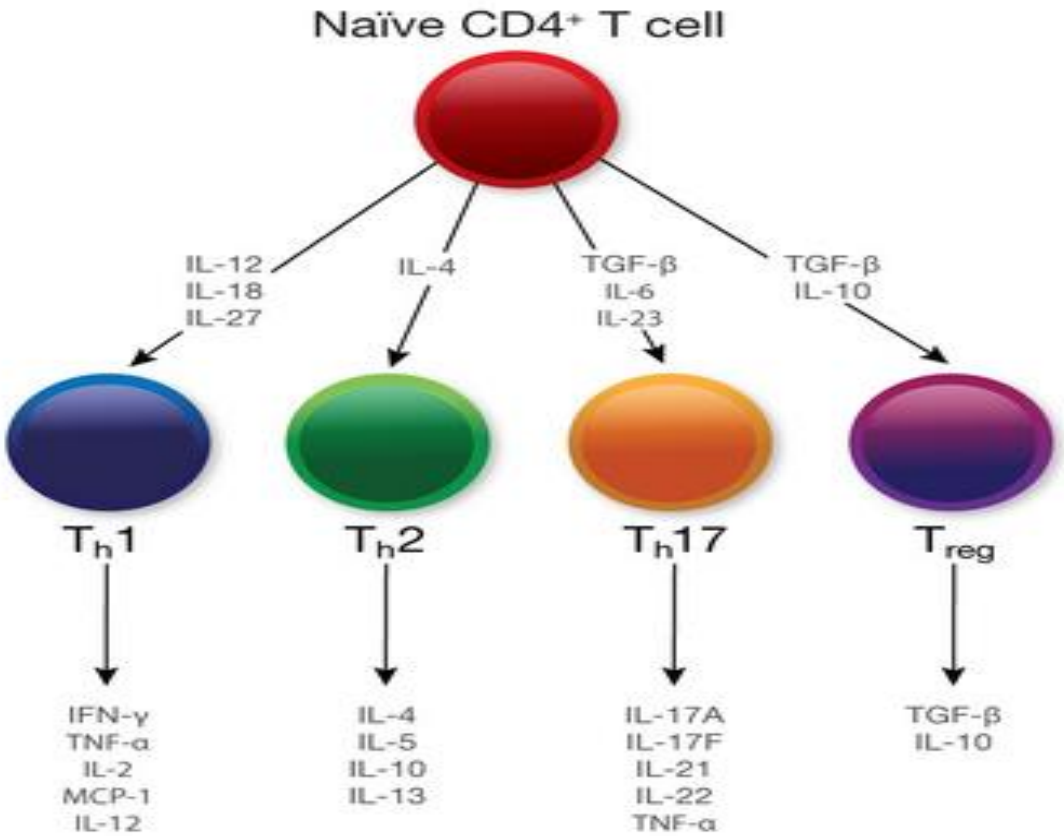
The principles behind this can be proposed in two immune models of the tumour microenvironment. The first is in which an active T cell-infiltrated phenotype is apparent. Tumour-infiltrating lymphocytes have been shown to be associated with a significantly positive outcome in colorectal cancer (Tosolini *et al* 2011). Patients with stage 1 and 2 disease without activated $CD8^+$ T cells developed recurrent disease within 5 years. Whereas more advanced stage 3 patients with a T cell infiltrate had prolonged disease-free survival. They also found that Th1 associated genetic clusters (Tbet, Stat4, IRF1) was associated with a prolonged disease-free survival, whereas Th17 associated genes (RORC, IL17A) had a worse prognosis.

Uppalari reviewed the literature examining TILs in prognosis in HNSCC (Uppalari *et al* 2008). Five studies found a positive prognostic association with TILs, whereas two studies showed no difference by immunohistochemistry. These studies were small however, and utilised mixed analytical techniques, the largest with 208 oral SCC patient's samples examined by H&E standard staining and pathologic grading of the infiltrate.

1.5.6 T helper Cell subsets and their Cytokines in HNSCC

The T helper subsets: Th1, Th2, Th17, fTh and Treg cells are all derived from CD4+ T helper cell progenitors. Their development is controlled by signal transducer and activator of transcription (STAT) transcription factors and master transcription factor STAT1 and transcription of T-Bet. IFN-γ stimulates TH-1 cells, which can maintain self-propagation of the subgroup due to production of IL-12. IL-12 activates STAT4 which in turn produces IL-12 and IL-12Rβ2 and IFN-γ (Usui 2006, Robinson 1997, Weaver *et al* 2006).

Fig 1.10. T-Helper cells lineage
(https://www.caymanchem.com/app/template/Article.vm/article/2177)



Further positive feedback loops also exist for the other Th cells. The Th2 cells have GATA3 and STAT6, Th17 cells have RORγt and STAT3, and the Tregs have FoxP3 and STAT5 (Zhu 2010).

Follicular helper T cells (fTh) are a recent addition to the Th cell subsets. These cells are important in the T cell-B cell interaction and development within germinal centres of secondary lymphoid structures such as the tonsil and regional lymph-nodes (Crotty 2011). fTh cells are controlled by Bcl6, and are positive for the chemokine receptor CXCR5, and is required for B cell migration in response to CXCL13. This allows migration of fTh cells into germinal centres. They also are positive for IL-21 and PD-1. Bcl6 inhibits Blimp-1, and can inhibit the differentiation of the other Th cell subsets, in addition Bcl6 also inhibits T-bet, GATA3-protein and RORγt function thus inhibiting Th1, Th2 and Th17 cell differentiation directly (Fazilleau *et* al 2009, Crotty 2011).

Table 1.2 Summary of T helper cell subsets, transcription factors and cytokine secretion

T Helper Cell	Inducers	Transcription Factors	Secreted Cytokines
Th-1	IL-12, IFN-γ	T-bet, STAT-1	INF-γ, IL-2, TNF-α
Th-2	IL-2, IL-4	GATA3, STAT6	IL-4, IL-5, IL-10, IL-13
Th-17	IL-6, IL-21, TGF-β	RORγt, STAT3	IL-17A, IL-17F, IL-21, IL-22
T-reg	TGF-β, IL-2	FoxP3, STAT5, SMAD	TGF-β1, IL-10
fTh	IL-6, IL-21	STAT3, Bcl6	IL-21, IL-4, CXCR5, PD-1

As a proportion of all white cells, 5-10% of circulating $CD4^+$ cells in both mice and humans are CD4+CD25+ T regulatory cells, which have a role in controlling auto-immunity and are also important in maintaining transplantation tolerance (Baecher-

Allan *et al* 2004). TGF-β has been shown to be an important regulator of T cell growth and development by inhibiting IL-2 production, up-regulating cell-cycle inhibitors and anti-proliferative actions on $CD4^+$ T cells (Gorelik 2002). The authors hypothesise that increased numbers of Treg cells would be beneficial in maintaining transplant organ function. This may be a reciprocal arrangement in the tumour microenvironment, in which decreased Tregs may lead to improved anti-tumour activity. It is necessary for TCR, IL-2 and TGF-βR (receptor) stimulation being required for induction of CD4+CD25+Foxp3+ Treg cell induction (Fu *et al* 2004).

A mouse model of premalignancy and invasive HNSCC was devised to investigate the proportions and total numbers of specific immune cells within cervical lymph nodes of mice compared with controls (De Costa *et al* 2012). This was created by inducing dysplastic and malignant lesions by oral ingestion of 4-nitroquinoline 1-oxide (4NQO), a substance known to induce murine oral carcinogenesis. This model allowed study of the immune response in a step-wise development model of oral HNSCC as opposed to other studies which involve direct injection of malignant cells. This is potentially more comparable to environmental factors in humans. They found an increase in both conventional cytotoxic T cells and Tregs in the cervical lymph nodes of HNSCC mice compared with controls and those bearing premalignant dysplastic lesions.

A number of studies have reported differences in the systemic cytokine profiles in patients with HNSCC (Bergmann, 2008). Our department has already found significant increased levels of serum IL-10 in patients with advanced HNSCC, in agreement with other authors, however there is no current published data on intra-tumoural levels of the Th1/Th2 balance in HNSCC. Previous studies investigating the cytokines involved in HNSCC have concentrated on serum levels, or mRNA encoding for the cytokines which may not indicate active molecules. Young *et al* (1996), investigated IL-10, TGF-β,

PGE_2, IFN-γ, and GM-CSF in cultured tumours and found detectable amounts of these cytokines in the supernatant.

Onishi *et al* sampled 35 frozen-stored renal cell carcinomas and used an ELISA technique to assess levels of Th-1 derived cytokines IL-2 and INF-γ, and the Th-2 derived IL-4, IL-5, IL-6 and IL-10. They also measured levels of IL-1α, IL-1β, INF-α, TNF-α, G-CSF, and GM-CSF. The levels of cytokine detected were expressed per milligram of protein (Onishi *et al* 1999). They found no detectable IL-2 in the samples, and only two from thirty five had detectable INF-γ (detection limits >50pg/ml for IL-2, >20 pg/ml for INF-γ).

T regulatory (Treg) cells have been initially demonstrated in maintaining host self-tolerance against auto-immune disease (Sakaguchi *et al* 1995), and have been implicated in direct suppression of effector cytotoxic T cells by Fas-mediated apoptosis (Strauss *et al* 2009, Alhamarneh *et al* 2007). Natural Tregs (nTregs) are derived from the Thymus gland, and a further subset of inducible Tregs (iTregs) can be formed due to the plasticity within the naive Th cell population (Bergmann *et al* 2009).

These cells contain the IL-2 α-chain (CD25+). The Forkhead P3 protein is associated with Forkhead/winged-helix family of transcriptional regulators. Defects in this gene are the cause of immunodeficiency polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), also known as X-linked autoimmunity-immunodeficiency syndrome (Torgerson *et al* 2002). This protein has been identified in Treg cells, and help to classify the group further (Strauss *et al* 2007).

Yates and colleagues found that IL-2 was required for *in vitro* optimal function of human $CD4^+CD25^+$ Tregs and increased expression of FOXP3., and CD25, CTLA-4 (cytotoxic T-lymphocyte antigen-4) and glucocorticoid-induced tumour necrosis factor receptor superfamily member number 18 (GITR). They found that inhibiting phosphatidylinositol-3-kinase using a chemical inhibitor (LY294002) the maximal

suppressive potency was diminished, but had no effect on the up-regulation of FOXPs, CD25, CTLA-4 and GITR. They found the inhibition of the PI3K/AKT-signalling pathway was how the nTreg population were maintained by IL-2 without TCR activation. They demonstrated a redundancy of IL-2 on IL-4, IL-7, and IL-15 (all γ_c type cytokines), which maintained the nTreg suppressive function.

The exact role of IL-17 secreting Th-17 cells in HNSCC is currently incompletely defined, with studies demonstrating both advantageous effect and detrimental effects (Wilke 2011, Kesselring 2010). The study suggested an increase IL-17 production in the early phase dysplastic phase, but reduction in the malignant stage, this was compared to the apparent Th1 response which developed in the premalignant phase and continued in the malignant model. They showed an increase in IFN-γ and CD4 cells and INF-γ and CD8 cells (Tc1) cells. An ideal antitumour immune response has been described as increased Th1 and Tc1 type immunity, and so up-regulation of these types is a goal in immunotherapy (Vesely *et* al, 2011).

One group (Gasparato *et al* 2010) investigated 12 patients with lip and tongue SCC. With PBMCs from 9 patients with OSCC and ten age-matched healthy volunteers, and were stratified according to tumour grade (WHO) as well, moderate or poorly differentiated. They measured IL-10, TGF-β, and IFN-γ (normalised to ng/mg of protein) of tumour tissue supernatants and found statistically significant increased levels of IL-10 and TGF-β compared to control tissue of healthy gingival tissue (removed in orthodontal procedures), and much reduced levels of IFN-γ compared with control tissue, which may be conflicting with the murine results of increased Th1 –like response found by Young *et al* (2012). They also found that tumour infiltrating $CD4^+CD25^+$ T cells isolated from tumour tissue significantly inhibited PHA activated-allogeneic PBMC in a proliferation study. In addition co-culture assays with PHA and demonstrated significantly higher levels of IL-10 and TGF-β when cultured with Treg

($CD4^+CD25^+$ T cells) TILS from oral SCCs. This may well suggest an immuno-suppressive tumour microenvironment in oral cavity SCC.

1.5.7 Immunity, Inflammation and Oncogenesis

The link between inflammation and cancer has been hypothesised for over two centuries following the work of Virchow in 1863 (Balkwill and Mantovani 2001). This was due to the fact that many cancers were found in areas of ongoing chronic inflammatory processes, which initially promote cell proliferation to promote tissue healing, however further recruitment of inflammatory infiltrates, and ongoing DNA damage can lead to neoplastic development (Coussens and Werb 2002). Chronic inflammation has been linked with cancer in a number of human organs, including that of the Helicobacter *pylori* and gastric cancer, inflammatory bowel disease and colon cancer, as well as the ongoing mucosal irritation of smoking and alcohol in the head and neck cancer patient.

Mantovani *et al* describe (2008) two pathways to cancer-related inflammation; the extrinsic pathway, involving chronic infection or inflammation, and the intrinsic pathway via oncogenic activation. They hypothesise that both pathways are linked at the cellular level, as transcription factors are activated (such as NF-KB, or STAT3 - see below) which then produce further cytokines, chemokines and prostaglandins that recruit more inflammatory cells and push the cycle forwards.

The use of non-steroidal anti-inflammatory medication has been associated with reduced risk of developing cancer (e.g. aspirin and cyclooxygenase inhibitors in oesophageal cancer and Barrett's Beales 2013). In HNSCC a recent case-control study, without stratifying by subsite, suggested an association between NSAIDs and a risk reduction of 75% (CI 33-93%) (Ahmadi *et al* 2010).

Another group examined the relationship between chronic periodontal inflammation and HNSCC (Tezal *et al* 2009). Periodontitis is a chronic inflammatory condition associated with periodontal bone loss and epithelial migration. They recruited 473 patients (266 with newly diagnosed HNSCC, and 207 non cancer dental control patients) and assessed the severity of periodontitis according to scores on panoramic dental radiographs. They found a significant association between periodontitis and HNSCC; the strength of the association was greatest with Oral SCC, then oropharynx, then larynx. This also adds evidence to the field change theory of cancer of the head and neck.

1.5.8 Hypoxia, angiogenesis and HNSCC

Hypoxia is a feature of most solid cancers (Wilson and Hay 2011, Le 2007). It can develop due to a number of different factors including the abnormal oxygen diffusion that occurs due to highly replicative tumour cells outgrowing normal tissue vasculature and dysfunctional tumour neo-angiogenesis. Blood flow within the tumour has also been demonstrated to be erratic and can induce intermittent hypoxic regions varying within a tumour (Keith *et al* 2012).

The diffusion distance of oxygen does not exceed 200μm, and is dependent on the local capillary oxygen concentration. Larger tumours are known to have a worse outcome, and also have larger areas of tumour hypoxia, which is also associated with a poor outcome in a number of cancers, furthermore this has been identified as an independent factor in cervical cancer, as well as in HNSCC (Brizel *et al* 1999, Beasley *et al* 2002). Hypoxia is known to have a negative effect on chemoradiotherapy, which is increasingly used in the primary treatment of HNSCC (Adam *et al* 1999, Gee *et al* 2010, Byers *et al* 2010).

Complete anoxia will lead to cell necrosis, which is common in HNSCC. Surrounding areas of necrosis will contain hypoxic cells, which change their transcriptional processes to increase glycolysis, and can alter proliferation and invasive properties of tumour cells (Keith *et al* 2012). Initially measured by intratumoural direct partial pressures of oxygen (pO_2) using a polarographic needle electrode, with partial pressures of O_2 <10 mmHg being suggested as tumour hypoxia, however the largest published series by Nordsmark showed a statistically significant association of an O_2 partial pressure <2.5mmHg with poor prognosis in HNSCC. Further techniques are being developed to measure hypoxia in a less invasive manner (Le 2007).

The hypoxia-inducible factor (HIF) transcription factors mediate the primary transcriptional responses to hypoxic stress in normal and transformed cells. HIF1α has been shown to be increased in HNSCC cells compared with normal surrounding cells using immunohistochemistry (Beasley *et al* 2002).

Biomarkers of hypoxia, which may be detectable within patient blood samples may indicate tumours with more hypoxic regions, which may be associated with a more aggressive tumour type, and therefore benefit from more aggressive therapy. Hypoxic tumours have been shown to be more resistant to radiotherapy and chemotherapy, and are more susceptible to genetic instability (Evans *et al* 2007).

There are a number of important effects of hypoxia on tumour biology. It stimulates survival due to hypoxia-re-oxygenation injury causing TP53 mutations, promotes neo-angiogenesis, the epithelial-to-mesenchymal transition to promote invasion and metastasis, and down-regulation of DNA repair pathways and increased generation of reactive oxygen species (ROS).

The 2-nitroimidazole agent EF5 is used to bind to cells at a rate inversely proportional to the cellular pO_2. High levels of EF5 binding detected by immunohistochemistry,

showing sever hypoxia, in HNSCC was shown to be associated with a shorter survival time (Evans *et al* 2007).

Less invasive measurement via a micro-mRNA has-mir-210 was correlated with the hypoxia-associated immunochemical expression of hypoxia-inducible factor-1 and carbonic anhyrase-9. High levels of has-mir-210 was associated with loco-regional recurrence and short overall survival (p=.008, Gee *et al* 2010).

HIF-1α up-regulation by hypoxic cells is a powerful stimulant of angiogenesis by increased production of VEGF and found to be overexpressed in solid tumours (Zhong *et al* 1999). HIF-1α can also control the switch from oxidative phosphorylation to glycolysis (Semenza 2013).

A network of cytokines are associated with angiogenesis within HNSCC (Montag *et al* 2009). Montag *et al* similarly investigated cytokines in HNSCC tissue homogenates by ELISA, and found a correlation between Hepatocyte-growth factor (HGF), basic fibroblast-growth factor (bFGF) and VEGF in 92% of 41 HNSCC samples; and HGF and bFGF were related to an adverse outcome. They included analysis of tissue from carcinoma of unknown primary patients, by definition nodal metastatic tissue, and noted lower levels of bFGF and G-CSF within these compared with the primary sites of oropharynx, larynx and hypopharynx.

VEGF, although a key cytokine in the induction of neovascularisation of tumour tissue and has been found within the tumour microenvironment, it also has a negative effect on the immune response to the tumour (Zitvogel 2006, Zou 2006). Increased VEGF concentrations within tumour tissue is associated with poorer clinical outcomes (Hendriksen 2008). It has also been implicated in lymphangiogenesis, and is a likely to be a factor in the ability of HNSCC to metastasise to regional lymph nodes.

1.5.9 HPV and HNSCC

In the past decade it has become clear that the Human papilloma virus (HPV) is associated with the aetiology of HNSCC, particularly being linked to increases in the incidence of oropharyngeal SCC, and specifically to tonsillar SCC in younger non-smoking patients (Hobbs et al 2006, Annertz et al 2002). HPV was already known as the agent involved in the development of the majority (97%) of SCCs of the cervix (Evans and Powell 2010), as well as cancers of the vulva, vagina, anus and penis. The national HPV immunisation programme in the UK is aimed at inoculating girls aged 12-13 years old since September 2008, and there is a campaign for it to be extended to boys in the near future. This may well be the most important factor in the reduction in incidence of cervical SCC. Hopefully this may have a positive impact on future incidence of HPV induced oral and oropharyngeal SCC in the UK.

HPV is a non-enveloped, doubled-stranded, circular DNA virus of approximately 7.9 kb (Chang and Califano 2008). The viral DNA of oncogenic subsets HPV 16 and HPV 18 have been found in up to 25% of HNSCC; with HPV 16 in up to 72% of oropharyngeal tumours using in-situ hybridisation (D'ouze et al 2007). This wide range of reported prevalence has led to calls for standardisation of the HPV assay, as currently samples may be processed using frozen or paraffin-fixed material, and varying assays including different PCR-based assays, In-situ-hybridisation, and surrogate $p16^{INK4a}$ IHC detection (Braakhuis *et al* 2009).

HPV-positive HNSCC expressing the viral oncogenes E6 and E7 have been shown to inactivate the p53 and RB-INK4A pathways which trigger M0 and M1 pathways respectively leading to permanent G1 arrest of the cell cycles and therefore senescence (Hunter at al 2005). Patients found to have HPV-positive tumours have an improved prognosis. The exact mechanism behind this is unclear, but may be due to the lack of

field change, the increase of cell senescence, improved radiation susceptibility, and immune recognition and response to viral antigens. A recent US study analysed the association of tumour HPV status and survival amongst stage III or IV oropharyngeal SCC who were enrolled in a trial comparing accelerated-fractionation radiotherapy (with concomitant boost radiotherapy) and standard-fractionation radiotherapy, along with combined cisplatin chemotherapy (Ang *et al* 2010). They assessed paraffin embedded tumour specimens for HPV-16 DNA using in situ-hybridisation-catalysed signal-amplification method for biotinylated probes (Genpoint, Dako). HPV - 16 negative specimens were then assessed using a biotinylated-probe cocktail (For type 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) for further oncogenic HPV types. Any positive specimens of both the above tests were defined as HPV-positive tumours in the analysis. They found HPV DNA was detected in 63.8% of the oropharyngeal tumours assessed (206 of 323 patients). Of these 206 positive samples, 91% (198/206) were positive for HPV-16. The HPV-positive oropharyngeal cancer was more common among non-smokers, and those with a lower number of cumulative pack years of smoking. HPV-positive status was associated with favourable prognostic factors such as younger age, better performance status, smaller primary tumour, white race, and absence of anaemia.

The HPV-positive patients had better overall survival and progression free survival than HPV-negative patients ($p<0.001$), this translated to a 82.4% 3 year survival in HPV-positive group (95% CI, 77.2-87.6) compared with 57.1% (95% CI, 48.1-66.1%) in the HPV-negative group.

Interestingly, one group has demonstrated increased levels of the programmed-cell death-1 receptor (PD-1) and its ligand (PD-L1) within the deep tonsillar crypts of patients with oropharyngeal SCC, and not on the surface mucosa (Lyford-Pike *et al* 2013). The PD-1 receptor was also assessed in TILs and PBMCs of patients, and was

found to have increased expression on the TILs. The PD-1-PD-L1 pathway is a checkpoint control, with the ligand expressed in response to inflammatory cytokines, such as INF-γ to control the inflammatory response. They found increased PD-1 on TILs compared with peripheral T cells. And HPV+ HNSCC were more likely to have higher expression of PD-L1. This may well indicate the ability of HPV to stimulate an immune checkpoint, thus if HNSCC develops, the immune response to reduced.

1.5.10 Immunotherapy and HNSCC

The lack of sustained improvement in treatment of advanced HNSCC in patients has encouraged the search for novel avenues of treatment. The theories of immuno-surveillance and immuno-editing describe the identification of 'abnormal' cancer cells leading to an immune response to eradicate them. The principle of immunotherapy is to harness and improve tumor killing or reducing the immune-modulation that lead to tumour progression.

One of the most successful immunotherapy techniques is that of the allogenic bone-marrow transplant, which can be effective in some leukaemias and lymphomas (Aggarwal and Pittenger 2005). The graft-vs-leukaemia effect uses donor lymphocytes to kill the tumour cells. Donor bone-marrow-derived mesenchymal stem cells (MSCs) have been used to reduce the incidence and severity of graft-vs-host disease. By co-culturing human MSCs with purified subsets of immune cells they demonstrated an anti-inflammatory effect on the cytokine profile produced by dendritic cells (DCs), Th cells, and Natural Killer (NK) cells. The mature type 1 DCs produced significantly less Tnf-α, and the type 2 DCs produced more IL-10. Levels of the Th-1 cytokine INF-γ was reduced in the presence of both Th cells and NK cells. There was a reciprocal increase

in the Th-2 cytokine IL-4. There was also an associated increased in $CD4^+CD25^+$ Treg cells. They found increased levels of immunosuppressive VEGF and $PGE_{2.}$

Wada *et al* investigated patients who had also received immunotherapy in the form of an intravenous injection of $1\text{-}10x10^6$ tumor-pulsed DC-activated lymphocytes (DAK); some patients also received a subcutaneous injection of $2\text{-}30x10^6$ mature DCs loaded with necrotic autologous tumour cells (DC vaccine). Only four patients received bevacizumab (An anti-VEGF Mab therapy). But they found a reduction in Tregs produced by culturing PBMCs in ascitic fluid and the addition of bevacizumab significantly reduced the number of Tregs identified in a trans-well experiment. They also found an increase in VEGFR-2 on the Tregs but only in two patients. In addition they measured levels of VEGF by ELISA from cells cultured from a malignant effusion of a patient with ovarian cancer. They found the concentration of VEGF within the effusion halved during DAK therapy from 1200pg/ml to 600pg/ml over 70 days treatment (Wada *et al* 2009). This suggests a measurable molecular response to immunotherapy by reducing VEGF levels. They also found increased levels of $CD4^+CD25^{high}$ T cells, classed as Tregs, in malignant pleural or ascitic effusions correlated with an increased level of VEGF. These effusions were from a spectrum of cancers, including gastric, pancreatic, lung, breast and ovarian carcinoma.

Initial immunotherapy techniques in HNSCC focussed on the attempted identification of molecules which could be used to identify malignant cells, such as Tumour-associated-antigens (Mellman *et al* 2011). These specific antigens which may be overexpressed on tumour cells, can enable specific targeting of these cells- either by enhancing immune-associated killing of the cells, or by using an inhibitor molecule to block further growth pathways of these particular cells in some way.

Direct cytokine use using recombinant IL-2, as well as the type I interferon-α, has been used with some mixed success, in renal cell carcinoma and melanoma, with response rates up to 15% (DeCosta *et al* 2011). IL-2 has been shown to increase TILs and NK cells in tumour and peri-lymphatic application of rIL-2 in HNSCC and has shown an association with improved disease free survival (Whiteside *et al* 1993). Combination therapy of IL-2 and INF-α has been used with in HNSCC with an 18% response rate, but a significant toxicity was reported (Urba *et al* 1993).

Current immune principles can use targeted principles to utilise the immune system and augment a pro-immune response, by using methods such as inhibition of immune-checkpoints. Two examples of successful use of this avenue are the inhibitors of the co-stimulatory molecule CTLA-4, such as the monoclonal antibody (Mab) ipilimumab, and the PD-1-PD-L1 pathway inhibitors, such as the PD-1 inhibitor Mab Nivolumab.

CTLA-4 is up-regulated following MHC-Tumour-antigen-peptide-TCR activation via an APC expressing B7 co-stimulatory molecules (CD80, CD86). The binding T cell is activated via the TCR and CD28 co-signalling binding to B7. CTLA-4 preferentially binds to B7 and inhibits the T cell response. Ipilimumab blocks the CTLA-4 receptor, and allows enhanced T cell activation and potentially a more effective anti-tumour immune response (Mellman *et al* 2011).

Ipilimumab has shown a clear median survival improvement 10 months in the experimental arm of ipilimumab and gp100 compared to 6.4 months with gp100 alone (Hodi *et al* 2010). There was also a small proportion of patients who have an effective tail in the survival curve, suggesting longer term survival.

The side-effects of ipilimumab are related to the loss of the self-tolerance afforded by the CTLA-4 receptor and give an autoimmune-like array of potential inflammatory toxicity. An interesting point is that the tumour response to ipilimumab may be delayed, and response to treatment have been observed after a 4 cycle course has been given. In

addition, the immune response can increase TILs and give an apparent increase in size of tumour deposits on interval CT scanning.

The PD-1-PD-L1 pathway is an example of targeting the activated T cells during an inflammatory response. PD-1 expression increases on the T cell surface, and when conjugated with its ligand, the activated receptor inhibits T cell activation kinases via SHP2, potentiating a negative immuno-modulatory response and possible tumour escape (Pardoll 2012). PD-1 has also been shown to be heavily expressed on activated Treg cells, and Tregs may be maintained and proliferate in the presence of PD-L1, and so blockade of the ligand may reduce activated Tregs and improve the cytotoxic response.

1.6 Summary

Previous studies have compared plasma and serum levels of cytokines between HNSCC patients and normal healthy control patients (Gunadyin *et al* 2012, Alhamarneh *et al* 2010, Jebreel *et al* 2007, Sparano et *al* 2004). Other studies have focussed on cell culture and *in vitro* attempts at recreating the tumour microenvironment (Kryscek et al 2007, Bergmann *et al* 2007) in HNSCC, and studied immune cytokine levels, and T helper cell subsets by flow cytometry, as well as studying circulating T cells by extracting PBMCs from both mice and human blood (Chikamatsu *et al* 2007, Gao *et al* 2009).

More recent studies have compared plasma and tissue Th1 and Th2 cytokines in HNSCC, and also pathologically normal tissue from the same HNSCC patients and the influence of immune modulation by 1α, 25-dihydroxyvitaminD_3 has been previously investigated (Walker *at al* 2012). This group found increased levels of IL-6 and IL-10, but not IL-2, IFN-γ or TNF-α in the plasma of HNSCC patients; however the levels of

IFN-γ and TNF-α were increased in tumour tissue compared to pathologically normal tissue.

In order to demonstrate the importance of immune function in the tumour microenvironment of HNSCC key cytokines were chosen to illustrate the Th-1/Th-2 axis, the Treg and Th-17 axis. IL-2 was used to demonstrate T cell stimulation, IL-12 and IL-10 were chosen to demonstrate the Th-1 like and Th-2 like response respectively. TGF-β and IL-15 were chosen as a surrogate of the Treg population and IL-17 was used to identify Th-17 cells. VEGF is an important molecule in both angiogenesis and has a potential negative immuno-modulatory effect and completes the cytokine immune panel being investigated.

1.7 Hypothesis

The hypothesis of this thesis is that the HNSCC tumour microenvironment will have increased levels of cytokines that produce an overall negative effect on the cellular cytotoxic immune response against the malignant cells. Specifically, it is hypothesised that a Th-2-like anti-inflammatory response will favour tumour cell progression and be associated with advanced stage HNSCC.

1.8 Aims and Objectives of the Thesis

In order to investigate the cytokine balance within the tumour microenvironment of HNSCC the levels of intra-tumoural Th-1 (IL-12), Th-2 (IL-10), and Th-17(IL-17)-associated cytokines, as well as those associated with Treg development (TGF-β, IL-2, IL-15) will be measured by ELISA. Intra-tumoural levels of VEGF will also be measured as a surrogate marker of hypoxia/neovascularisation and to investigate possible associations with clinical outcome. A robust protein extraction process will be developed to allow an accurate and reproducible ELISA technique to measure lysate cytokines from biopsies obtained from newly presenting HNSCC cancer patients.

These data will be correlated with the overall clinical staging and outcome, including comparisons between primary tumours and metastatic nodal disease in the same patients.

Chapter 2 – Patients and Methods

2.1 Research Participants

2.1.1 Identification of HNSCC Patients

The patients recruited to the study were identified at the Hull and East Yorkshire NHS Trust Head and Neck Cancer Multi-disciplinary team meeting between July 2007 and June 2009. The study was prospectively approved by the Hull and East Yorkshire Research and Development local ethics committee (LREC-05/Q1105/55) and the Hull and East Riding NHS Trust (R0220).

All patients were confirmed to have HNSCC by pathological examination of a biopsy or needle aspiration, and underwent standard radiological staging with CT or MRI of the head and neck region. The treatment options of surgery, radiotherapy, chemotherapy, and combined approaches were discussed at the MDT, and the decision made depending on stage and patient medical comorbidity. Treatment options were not affected by the study.

All patient enrolled on the study gave informed consent to provide pre-treatment and post-treatment blood sample taking. They were also given a standard information fact-sheet.

Patients were excluded from the project if they were known to have a history of previous cancer.

2.1.2 Identification of Control Patients

The patients recruited to the study as the control group were selected from the Hull and East Yorkshire NHS Trust Ear, Nose and Throat waiting list, with informed consent. They were listed for benign oropharyngeal surgery. These cases included uvulopalatoplasty and adult ‘cold’ tonsillectomy for recurrent tonsillitis, but no active inflammation at time of surgery. The standard surgical technique used blunt and sharp dissection to resect the tonsil to reduce tissue disruption from diathermy burns.

2.2 Protein Extraction

2.2.1 Optimisation of protein extraction

Tissue samples from a cohort of twelve patients were used in the optimisation process of protein extraction and cytokine detection. The initial protein extraction kit used was Calbiochem total mammalian extraction kit (Cat. No. 539779, Calbiochem, Merck, Notts, UK). This initial kit was selected because it had been shown to provide a complete proteome extraction from both tissue culture cells as well as tissue.

Tumour and nodal tissue was identified during surgery by the operating surgeon. Primary tumour tissue was taken from the central tumour portion avoiding the macroscopic margin, and overtly macroscopic necrotic areas or tissue charred by diathermy burns. Malignant nodal tissue was taken as en bloc resection, a single macroscopically malignant node was then divided, with half the tissue sent to the pathology lab for confirmation of HNSCC.

2.2.2 Sample Retrieval - Snap Frozen Samples

Initial samples used in the protein and ELISA optimisation process were snap-frozen using liquid Nitrogen in the operating theatre. The samples were taken from the primary tumour, or macroscopically identified involved lymph nodal tissue; then trimmed of overt necrosis, fat or muscle tissue. The tissue was double wrapped in tin-foil and clearly marked with an anonymous identifier on both layers prior to being placed in the liquid nitrogen dewar.

The Snap frozen sample retrieval was used initially in early protein extraction assessment and feasibility studies in cytokine detection, but was replaced by the fresh tissue sample method due to problems maintaining the liquid nitrogen supply in theatre.

2.2.3 Sample retrieval – Fresh Tissue Samples

The fresh tissue was immediately placed in a 10ml vial of sterile RPMI 1640 (RPMI-1640 Medium with sodium bicarbonate, without L-glutamine, liquid, suitable for cell

culture, Sigma-Aldrich, Haverhill, Suffolk, UK) for transfer to the laboratory. Each 50 ml of RPMI 1640 was supplemented with 10µl of protease inhibitor cocktail III (supplier?). The protease inhibitors are provided as a solution in DMSO at the following concentrations: 100 mM AEBSF, HCl, 80 mM Aprotinin, 5 mM Bestatin, 1.5 mM E-64, 2 mM Leupeptin Hemisulfate, and 1 mM Pepstatin A. A volume of 1 ml is sufficient for 20 g of tissue. (Calbiochem, Merck, Notts, UK). The vial was then placed on ice and transferred to the laboratory for protein extraction.

2.2.4 Initial Protein Extraction – Calbiochem Complete mammalian protein extraction kit (C-PEK).

The manufacturer's protocol for complete proteome extraction from tissue was used. The sample was weighed and the wet weight noted. The sample was then placed in a cooled mortar (on dry ice) to minimise any thaw effect, and the sample was divided initially with an 11 blade disposable scalpel (Swann-Morton, Sheffield, UK) into small pieces approximately 2 mm^3. The cooled pestle was used to finely crush the tissue into powder. Small amounts of liquid nitrogen were applied to the tissue until it was macroscopically powdered, taking approximately 5 minutes.

Aliquots of 50-100 mg of the powdered sample were finally transferred to a 2 ml microcentrifuge tube (Eppendorf, Hamburg, Germany) and stored at -80˚C in a freezer until analysis.

To perform the extraction 150µl of ice-cold resuspension buffer (Imidazole/Sucrose buffer, ready to use) was added to a microcentrifuge tube containing the ground, frozen HNSCC tumour biopsy tissue and vortexed for 1 minute.

The extraction reagent (Urea/detergent 25ml vial) was reconstituted as per the manufacturer's instruction by adding 11ml of double-distilled water to the vial provided, mixed by vortexing. Next the contents were warmed to 25˚C for complete

solubilisation (an endothermic reaction was noted by the vial becoming cold to the touch). The extraction reagent solution was then stored at 4˚C until required for use.

To continue protein isolation 800µl of the extraction reagent was added to the sample at room temperature, followed by 50 µl of reducing agent (freshly reconstituted by adding 2 ml of double-distilled water to vial provided, then vortexed at room temperature, giving 2.27 ml of 1M DTT). Next 0.5 ml of a glass bead solution was added, and the mixture was then agitated by vortex for 1 min and put on ice for 1 min; this process was repeated four times.

The glass bead solution was allowed to settle at the bottom of the tube under gravity for 5 minutes after which the supernatant was carefully aspirated and transferred into a fresh microcentrifuge tube, leaving the beads behind. An aliquot of Benzonase (a non-specific nuclease, 1.5 µl) was added and mixed to the volume of cell/tissue lysate. The extract was incubated at room temperature under gentle agitation for 1 hour.

The sample was centrifuged at 15,000g for 30 minutes and the supernatant was separated into 30 µl aliquots and stored at -80˚C, until analysis.

The first eight patient tumour samples were used in the initial protein extraction. Protein concentration was assessed using the Coomassie (Bradford Assay) described in section 2.2.6 to confirm protein had been extracted.

2.2.5 Second Protein Extraction Method – ProteoJET™ Mammalian Cell lysis reagent

A fresh tissue sample was decanted from the 10 ml vial of RPMI 1640 into a petri-dish under sterile conditions in a class II Biological Safety cabinet. The sample was weighed (wet weight) prior to homogenisation.

Initial sample processing was performed by slicing the tissue using two size 11 blade scalpels (Swann-Morton, Sheffield, UK). The sample was repetitively sliced until no tissue pieces were larger than $1mm^3$; this took approximately 5 minutes to complete.

The sample was transferred to a 10ml polypropylene centrifuge tube (or 1.5ml microcentrifuge tube depending on sample size (Eppendorf, Cambridge, UK), and the appropriate volume of cell lysis reagent was added by pipette (Cat no. K0301, ProteoJET^{TM} Mammalian cell lysis reagent, Fermentas, UK) depending on the weight of the sample according to the manufacturer's instruction (5µl of extraction reagent per 1mg of tissue).

The tissue was resuspended by vortexing, and then incubated for 10 minutes at room temperature. Protease inhibitor Cocktail III (Calbiochem) was then added by pipette depending on initial weight (0.05µl per 1mg of tissue).

The protein extract solution was homogenised by sonication using a microtip for 10 seconds whilst the sample was kept in an ice-bath to avoid overheating (50% pulse power, maximum microtip power, Cole-Parmer Mod. 4710 Ultrasonic Homogenizer, Vernon Hills, IL, USA)(Onishi *et al* 1999). This process was repeated once, for larger samples, when macroscopic tissue was still evident.

Sonification utilises the piezoelectric effect to disrupt the tissue cell membranes. By rapidly alternating the electrical current a piezoelectric crystal rapidly contracts and expands and thus creates a mechanical vibration (20-30 kHz). This effect was used to increase the likelihood of cytokine detection by disrupting all the cells within the tumour microenvironment of HNSCC (Houze and Gustavsson, 1996).

The lysate was then clarified by centrifugation at 12,000 x g for 15 minutes. Multiple aliquots of 200µl of supernatant were taken by pipetting and transfer to microcentrifuge tubes. These were uniquely labelled and stored at -80˚C until analysis. The volume of supernatant depended on the initial wet weight of tissue sample taken. There was a wide variation in sample sizes, and so for smaller tissue samples aliquots of 50µl or 100µl were taken to allow for further analysis.

2.2.6 Protein concentration analysis – Coomassie Plus Assay™

Two aliquots of each tissue sample were analysed for total protein concentration. The Coomassie Plus (Bradford) Protein Assay (Pierce, ThermoFisher Scientific, Loughborough, UK) was used according to the manufacturer's instructions. Plain flat bottomed 96-well microplates were used for analysis (Biorad, UK).

An 8-point standard curve of albumin in phosphate-buffered-saline (PBS) was used (5000mg/ml, 2000, 1000, 500, 250, 125, 62.5, 31.25mg/ml) (Pierce, UK), and each extracted aliquot was assessed by doubling dilutions in triplicate.

Standard or sample (10µl) were added to each well. Then 300µl of Coomassie Plus reagent was added to each well and mixed on a plate shaker for 30 seconds, before incubation at room temperature for 10 minutes.

The plate was finally read for absorbance at 595nm and measurements for the blank wells subtracted from these values, and the samples measured against the eight point standard curve, using a four-parameter logistic curve fit. A mean was taken of the triplicate analysis for each sample and recorded.

In order to allow comparison between the tissue samples the concentration of cytokine detected by ELISA in pg/ml was converted to pg/mg of total protein extracted.

2.3 General Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) technique

2.3.1 ELISA Technique

Individual Duo-set developmental commercial sandwich ELISA kits were purchased (R&D systems, Abingdon, UK) for a panel of human cytokines (IL-2, IL-10, IL-12 p70, IL-15, IL-17, TGF-β1, and VEGF-A).

The kits supply the constituent capture antibody and detection antibody in a lyophilised form. The capture antibody was reconstituted with 1 ml of PBS to give the original capture Ab concentrations listed in the table below. The working concentration was used to coat flat-bottomed 96-well plates. The detection antibody was reconstituted with 1 ml of the reagent diluent used for the particular cytokine, then further diluted with reagent diluent to the working concentration as listed in table 2.1.

Table 2.1. Reagent diluent and Blocking buffer used for each cytokine

Cytokine	Reagent Diluent (RD)	Blocking Buffer
IL-2	0.1% BSA, 0.05% Tween 20 in PBS, pH 7.2-7.4	1%BSA in PBS with 0.05% NaN_3
IL-10	1% BSA in PBS, pH 7.2-7.4	RD
IL-12 p70	1% BSA in PBS, pH 7.2-7.4	RD
IL-15	1% BSA in PBS, pH 7.2-7.4	RD
IL-17	1% BSA in PBS, pH 7.2-7.4	RD
TGF-β1	1.4% delipidised bovine serum, 0.05% Tween 20 in PBS, pH 7.2-7.4.	5% Tween 20 in PBS with 0.05% NaN_3
VEGF	1% BSA in PBS, pH 7.2-7.4	RD

2.3.2 Plate Preparation

Nunc MaxiSorp™ high protein-binding capacity polystyrene 96 well ELISA plates (ebioscience) were used. Capture antibody (100µl) at the recommended concentration was added to each well and the plates were covered and incubated at room temperature overnight, the antibodies used are listed in Table 2.2. The reagent diluents were purchased from R&D systems where available, or made up from molecular grade chemicals (Sigma).

Table 2.2. Capture antibodies and working concentrations

Cytokine	Capture Ab type	Capture Ab Conc	Working CA Conc.
IL-2	Mouse anti-human IL-2	720µg/ml in PBS	4 µg/ml in PBS
IL-10	Mouse anti-human IL-10	360µg/ml in PBS	2 µg/ml in PBS
IL-12 p70	Mouse anti-human IL-12 p70	720µg/ml in PBS	4 µg/ml in PBS
IL-15	Mouse anti-human IL-15	360µg/ml in PBS	2 µg/ml in PBS
IL-17	Mouse anti-human IL-17	720µg/ml in PBS	4 µg/ml in PBS
TGF-β1	Mouse anti-human TGF- β1	360µg/ml in PBS	2 µg/ml in PBS
VEGF	Mouse anti-human VEGF	180µg/ml in PBS	1 µg/ml in PBS

2.3.3 Plate blocking

The coated plates were washed using an automated plate washer (ELx50 Microplate Strip Washer, Biotek, UK) three times with 400µl of wash buffer per cycle (wash buffer - 0.05% Tween 20 in PBS, pH 7.4). Any remaining binding sites were blocked by addition of 300µl of the recommended blocking buffer (reagent diluent depending on cytokine – see Table 2.2).

2.3.4 Standard seven point dilution curves

Following a further wash-cycle, 100 µl of the standards and samples were added per well, and incubated for 2 hours at room temperature. The standards supplied in the kits were reconstituted with 0.5ml of double distilled water, and the working concentrations are listed in Table 2.3. Doubling dilutions in PBS of standard were used in duplicate each time a plate was run for a particular cytokine, to give a seven point standard curve.

Table 2.3 Working concentrations of Recombinant Human Standard cytokines

Cytokine	Standard Reconstituted	High Standard Conc	Detection Limit Conc
IL-2	50 ng/ml	1000 pg/ml	7.8 pg/ml
IL-10	210 ng/ml	2000 pg/ml	15.6 pg/ml
IL-12 p70	190 ng/ml	2000 pg/ml	15.6 pg/ml
IL-15	150 ng/ml	1000 pg/ml	7.8 pg/ml
IL-17	220 ng/ml	1000 pg/ml	7.8 pg/ml
TGF-β1	140 ng/ml	2000 pg/ml	15.6 pg/ml
VEGF	120 ng/ml	2000 pg/ml	15.6 pg/ml

2.3.4 Lysate Sample dilutions and Analysis

The lysate samples were each analysed in duplicate, and had two doubling dilutions in order to analyse for appropriate dilution of the sample. Therefore 6 wells of each flat-bottomed plate were used for each sample analysed per cytokine. The samples were diluted in the appropriate reagent diluent for the cytokine being analysed. The lysate aliquots were diluted up to 500µl with reagent diluent. Two x 100µl of this was used for the most concentrated sample dilutions. Then a further 300µl of reagent diluent was added to the remaining sample to make the first doubling dilution, and two x 100µl of this was pipetted into the plate. The final dilution was made by adding 400µl of reagent diluent to the remaining lysate solution to make the final doubling dilution. This method was used to ensure accurate, reproducible volumes of 100µl were placed into the wells and to allow for volume loss in the pipetting process or coating on the microcentrifuge tubes.

The lysate aliquots were either 50µl, 100µl or 200µl sample sizes. Because of the dilution technique, a dilution factor was considered depending on how much lysate was available. The dilution factor was increased by the activation of the immunoreactive TGF-β1. To activate latent TGF-β1 0.1ml 1N HCL was added to 0.5ml sample and

incubated at room temperature. This was neutralised by adding 0.1ml 1.2 N NaOH/0.5 M HEPES and mixing. This increased the dilution factor by x1.4.

The dilution factors were recorded for each sample, and used in the final analysis when combining the overall protein concentration of each sample to give pg cytokine/mg protein. The lysate concentrations were determined during the washing and blocking period. Once each 100µl of sample and standard had been accurately applied to the plate, the plate was covered with an adhesive strip and incubated at room temperature for 2 hours. The wash cycle was then repeated as above.

Each tissue sample was analysed in duplicate, and at two further doubling dilutions. In addition, each cytokine had repeated plates analysed at least once for each tumour sample.

2.3.5 Detection Antibody

The appropriate concentration detection antibody was added to each well (100µl) for a further 2 hours, and the wash cycle was repeated, to ensure no free detection antibody remained in the wells to interfere with the results. The type of detection antibody used for each cytokine tested and the working concentration dilution in the respective reagent diluents used for the analysis are listed in Table 2.4.

Table 2.4 Detection Antibody type and concentration

Cytokine	Detection Antibody	Detection Ab Conc	Working Conc. in RD
IL-2	Biotinylated goat anti-human IL-2	31.5µg/ml in RD	175 ng/ml
IL-10	Biotinylated goat anti-human IL-10	54µg/ml in RD	300ng/ml
IL-12 p70	Biotinylated goat anti-human IL-12 p70	18µg/ml in RD	100 ng/ml
IL-15	Biotinylated mouse anti-human IL-15	90 µg/ml in RD	500ng/ml
IL-17	Biotinylated goat anti-human IL-17	27µg/ml in RD	150 ng/ml
TGF-β1	Biotinylated chicken anti-human TGF-β1	54µg/ml in RD	300ng/ml
VEGF	Biotinylated goat anti-human VEGF	18µg/ml in RD	100 ng/ml

A total of 10 ml of detection antibody at the working concentration was used for each of the 96 well plates, the biotinylation of the detection antibody allows binding of the streptavidin-HRP reagent.

2.3.6 Streptavidin-HRP

A 1:200 dilution of Streptavidin-HRP (streptavidin conjugated to horseradish-peroxidase) was added to each well (100µl), and incubated under cover for 20 minutes, then the wash cycle was repeated. For a 96 well plate a 1:200 solution was made up using 50µl of streptavidin-HRP added to 9950µl of the particular reagent diluent.

2.3.7 Substrate Solution

Substrate solution was added to each well using TMB as per the manufacturer's instructions (100µl). TMB - 3, 3', 5, 5'-tetramethylbenzidine was made up by adding 6 drops of Buffer stock solution, 6 drops of TMB stock solution, and 6 drops of Hydrogen peroxide solution to 10ml of ddH20 (Vector, Burlingame, US). This was incubated with gentle agitation in the dart at room temperature to avoid light activation of the substrate for up to 20 minutes, or until the top standards were approaching a deep azure colour.
The reaction was then stopped by adding 50µl of 1N H_2SO_4 to each well, this stopped any further reaction of the TMB and can increase the absorbance of TMB substrate by 2-4 times when measured at 450nm.

2.3.8 Automated absorbance measurement

The absorbance was then measured using a plate reader (Synergy-HT Microplate Reader, Bio-Tek, Potton, Bedfordshire, UK) at 450nm and subtracting the values at 540nm to correct for optical imperfections of the plate. The absorbance measured in the blank wells were averaged and subtracted from the sample wells. An analysis programme (Gen-5 software, BioTek) was used to create the standard curve using a 4-PL line of best fit.

2.4. Statistical Analysis

The data were analysed using SPSS v.20 (SPSS inc, IBM, Illinois, USA). Each data set for each cytokine tested were initially tested for normality using the Kolmogorov-Smirnov test.

The data were stratified according to the TNM stage, grouping T1 and T2 into early stage and T3 and T4 into late stage (Yuan *et al* 2006). Each dataset were also analysed by absolute results, and a binomial detectability assessment. These were analysed using the Mann-Whitney U-test and Chi-squared test for non-gaussian populations. Survival analysis was calculated using Kaplan-Meier survival curves and log rank analysis.

2.5 Peripheral Blood Samples

2.5.1 Serum Samples

Following patient consent, 10ml of venous blood was collected in two 7ml commercial clotting/serum separation tubes (BD Bioscience).The samples were centrifuged at 725g for 10 minutes, and the serum supernatant was pipetted in 500µl aliquots into 1.5ml microtubes (Eppendorf). These were stored at -80˚C until required for analysis.

2.5.2 Peripheral Blood Monocyte Cell Retrieval

Venous blood (50ml) was obtained using a 50 ml syringe, which was previously heparinised with 10000 IU heparin (Mucous, 5000IU/ml, Leo-Pharma, Princes-Risborough, UK), by drawing 2ml into the syringe.

Within a Category II Biosafety cabinet, the blood samples were first diluted to a 1 in 2 dilution in 50ml of PBS and 20ml samples were then carefully layered onto 20 ml of lymphocyte separation medium in a 50 ml tube (PAA, LSM 1077, Yeovil, UK) using a Pasteur pipette, utilising a Ficoll^{TM} density gradient to separate the lymphocytes. The tubes were centrifuged at 400g for 30 minutes at room temperature (Whiteside *et al*, 2012).

The lymphocyte layer between the plasma and Ficoll^{TM} layers was then carefully pipetted off, and washed in 20 ml of PBS in five separate fresh 50 ml tubes. The tubes

were centrifuged again at 400g for ten minutes, and the supernatant carefully poured off into waste. The cells from all five tubes were combined by resuspension in a further 20 ml of PBS and centrifuged at 400g for 5 minutes. The supernatant was poured off once more. The remaining cells were resuspended in 5 ml of FBS/10% dimethylsulphoxide (DMSO) solution to reduce intra-cellular ice crystal formation, and the solution was aliquoted in 1ml cryovials and placed in a temperature controlled reducing vessel 'Mr Frosty' reducing the temperature by 1 degree per minute down to -80˚C, then transferred to liquid nitrogen storage. Each aliquot was labelled with its unique identifying number and date.

Chapter 3

3.1 Optimisation of Protein Extraction

Aim – To develop a reproducible method to extract protein from tissue biopsies and subsequent assessment of cytokine levels within the HNSCC biopsy lysates.

3.1.1 Introduction

The study of immunological cytokines *in vitro* regularly involves the use of cell culture with the use of certain subsets of immune cells (Skibinski *et al* 1997) developed in controlled environments with specific concentrations of stimulant cytokines. The tumour microenvironment involves a highly variable network of these cytokines, which are produced from all the cells within the malignant mass and surrounding stromal cells in addition to those recruited to the site, such as tumour infiltrating lymphocytes (Uppaluri *et al* 2008).

Multiple ways of studying the cytokine response within a tumour mass exist, all of which have their own advantages and disadvantages, e.g. level of sensitivity and ability to identify production of specific cytokines by specific cell subsets. In the case of measuring protein levels it is important to be able to extract these cytokines as efficiently as possible within tissue so as to be able to measure these reliably. The method needs to be reproducible and not alter the structure of the cytokines during isolation, and so alter antibody detection via ELISA.

3.2 Methods and Materials:

A cohort of twelve patients with pathologically confirmed HNSCC had tumour tissue excised at surgical intervention, which was processed as previously described (section

2.2). It was debrided of necrotic or connective tissue, double wrapped in two pieces of tin-foil and uniquely marked before being snap-frozen in liquid nitrogen (section 2.2.2). The first method used for protein extraction utilised the Complete Mammalian Protein Extraction Kit (CPEK) (Calbiochem, Merck, Notts, UK), following the manufacturer's protocol for complete proteome extraction from tissue.

3.3 Initial ELISA

3.3.1 Cytokine detection feasibility study

In order to demonstrate the feasibility of ELISA to detect the presence of cytokines within the tumour microenvironment individual commercial sandwich ELISA kits were purchased (R&D systems, Duo-set Development ELISA kits, Abingdon, UK). The first cytokines investigated were IL-1β, a pro-inflammatory TH-1 type cytokine (Guo et al, 2009), IL-4 and IL-10 which are TH-2 like cytokines, (Manetti et al, 1993, O'Garra et al, 2008) and TNF-α a cytokine implicated in cell-death via activation of intracellular proteases and caspases (Duffey et al, 2000).

3.3.2 Analysis of lysates

The ELISA protocol in chapter 2 was followed. For each well, 100 µl was required for analysis. The standard volume of lysate stored per aliquot after protein extraction (See methods) was 30 µl. In order to duplicate the analysis, and make serial doubling dilutions to confirm consistent results, the sample aliquots were diluted in the appropriate reagent-diluent dependent on the ELISA method used. Thus all 30 µl

aliquots were made up to 300 µl in total volume in reagent diluent. This dilution factor was noted for cytokine level to be expressed as pg/mg of protein.

3.3.3 ELISA Optimisation

The initial ELISA method was followed as per the manufacturer's recommended instructions (see Methods 2.3, R&D systems). The ELISA plates were used as per methods section 2.3.

The cytokine standard curves are listed (Table 2.3), and according to manufacturer's guidance a seven point scale was devised using 2-fold serial dilutions.

Initially, as part of the ELISA optimisation a 3 point standard curve was used in duplicate and two tumour lysates (GDT (18), TLT (58)) were assessed against this also using two-fold serial dilutions also in duplicate (see fig). The initial Calbiochem CPEK samples were in 30µl aliquots. The micro-plates required 100 µl per well of sample/standard.

These were diluted in PBS up to a volume of 300ul, in order to take 2x100ul top samples, then two further doubling dilutions, both in duplicate were analysed, to ensure correct readings (Table 3.1a and b)

Table 3.1 Example ELISA test plate for IL-1β (Values in pg/ml), GDT and TLT are sample identifiers

IL-1β 250 HS (pg/ml)	IL-1β 250 HS	GDT (18) 1	GDT (18) 1	TLT (58) 1	TLT (58) 1
IL-1β 125	IL-1β 125	GDT (18) 1/2	GDT (18) ½	TLT (58) 1/2	TLT (58) ½
IL-1β 62.5	IL-1β 62.5	GDT (18) 1/4	GDT (18) ¼	TLT (58) 1/4	TLT (58) ¼
Blank	Blank	Blank	Blank	Blank	Blank

Table 3.2 Example ELISA test plate for IL-4 (Values in pg/ml), GDT and TLT are sample identifiers

IL-4 2000 HS (pg/ml)	IL-4 2000 HS	GDT (18) 1	GDT (18) 1	TLT (58) 1	TLT (58) 1
IL-4 1000	IL-4 1000	GDT (18) ½	GDT (18) ½	TLT (58) 1/2	TLT (58) ½
IL-4 500	IL-4 500	GDT (18) ¼	GDT (18) ¼	TLT (58) 1/4	TLT (58) ¼
Blank	Blank	Blank	Blank	Blank	Blank

3.3.4 Initial Elisa Results

The first standard 3 point curves showed good reproducibility, however the optical density readings from the initial Calbiochem CPEK were unreliable, with most cells reading equivalent to, or even lower than, the blank optical density (See Table 4.2). There were also random wells which gave abnormally high readings above the high standard, which showed no dilution effect, or serial recovery. This suggests that the sample matrix may be masking the detection, or an element of the Calbiochem CPEK was interfering with the assay.

The ELISA was repeated for IL-1β and the same extraction samples, but increasing the serial dilutions by a factor of ten. This was to assess the lower detection level, and assess if further dilution could correct for the apparent false positive ELISA result of the CPEK kit (Table 3.3a and 3.3b)

Table 3.3a Example plate of Calbiochem CPEK IL-1β, 1:10 serial dilutions (pg/ml)

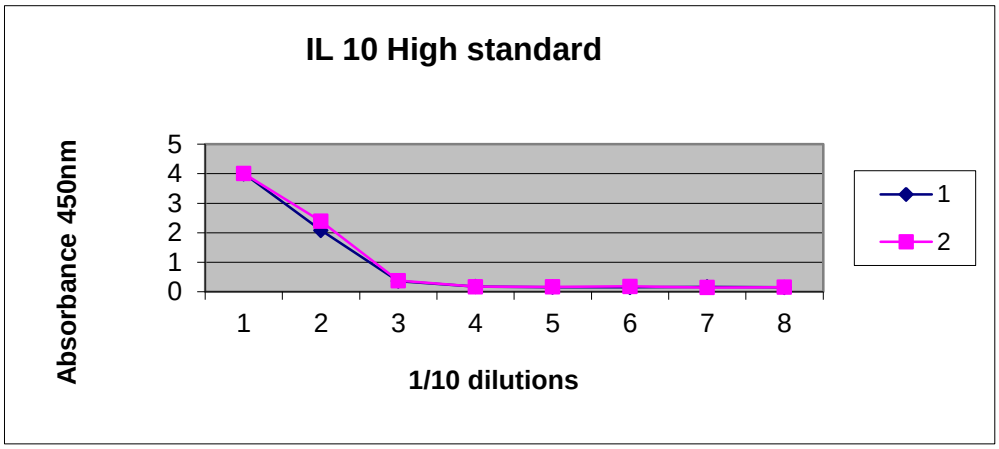
IL-1β 250 HS (pg/ml)	IL-1β 250 HS	GDT (18) 1	GDT (18) 1	TLT (58) 1	TLT (58) 1
IL-1β 125	IL-1β 125	GDT (18) 1/2	GDT (18) ½	TLT (58) ½	TLT (58) ½
IL-1β 62.5	IL-1β 62.5	GDT (18) 1/4	GDT (18) ¼	TLT (58) ¼	TLT (58) ¼
Blank	Blank	Blank	Blank	Blank	Blank

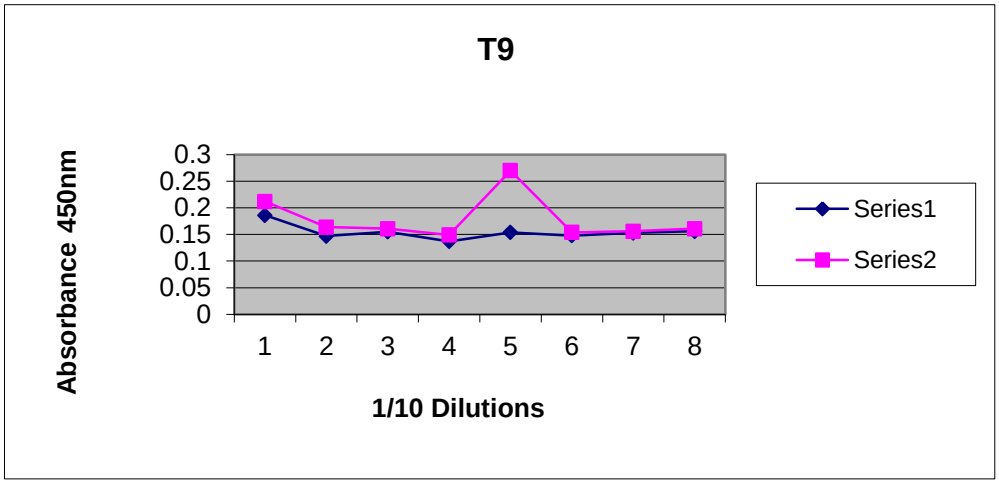
Table 3.3b Example plate of Calbiochem CPEK IL-1β, 1:10 serial dilutions (Absorbance at 450nm)

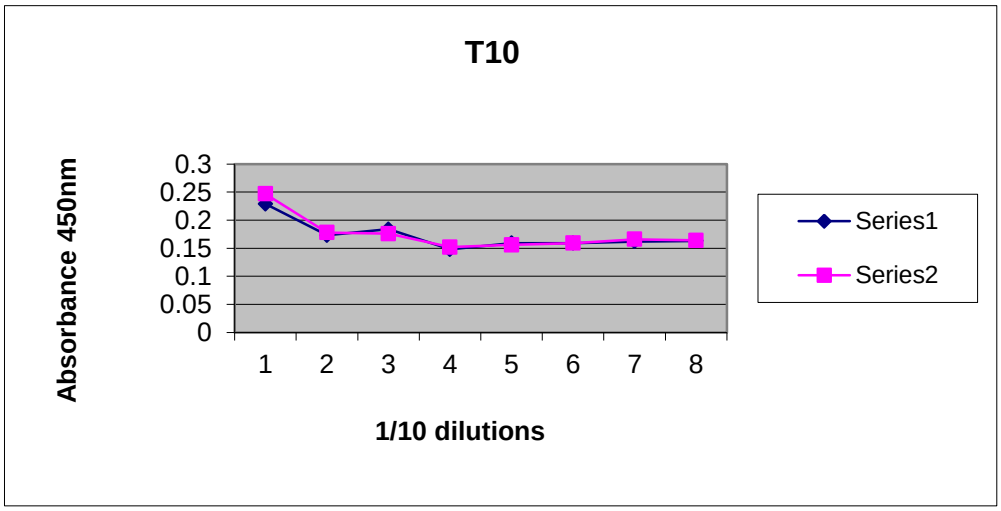
3.499	3.611	0.15	0.179	0.521	0.591
1.926	2.076	0.172	0.204	0.407	0.468
0.633	0.622	0.322	0.339	0.438	0.454
0.348	0.325	0.331	0.345	0.394	0.397

The standard curves for IL-1β became non-detectable after the second dilution. There was no dilutional effect observed with the tumour samples, nor a dilutional correctional effect This was repeated with further doubling dilutions using further protein extraction samples (T9 and T10, Fig. 3.1). The Rows 1-8 represent doubling dilutions.

Fig 3.1 Example absorbance *vs* doubling dilution graphs for IL-10 standards and two tumour samples (T9 and T10).







3.4. Dialysis of Calbiochem CPEK samples

3.4.1 Dialysis of samples

In an attempt to remove suspected interfering molecules involved in the protein extraction process dialysis of the CPEK samples was performed. To provide a model control, cytokine standards were mixed with the extraction reagent and treated as biological samples. The aliquots were dialysed using 0.5-3 ml 10kDa slide-A-lyzer™ Dialysis cassettes (Pierce, Rockford, US). A 10kDa cut-off membrane was used as all the cytokines being analysed had greater molecular weights (Table 3.3).

Table 3.4 Molecular weights (kDa) of cytokines tested

Cytokine	Molecular Weight (kDa)
IL-1β	17
IL-2	15
IL-4	17
IL-10	36 Homodimer
IL-12	70
IL-15	13
IL-17	31 Homodimer
TGF-β	25
TNF-α	17
VEGF	45 Homodimer

Five recombinant human cytokines were mixed with the Calbiochem CPEK extraction reagent at the following concentrations: IL-1β - 250pg/ml, IL-4, IL-10, IL-12 - 2000pg/ml, TNF-α - 1000pg/ml. Two tumour sample extracts and one normal tissue extract were also dialysed. Each sample was made up to 1ml using the CPEK extraction reagent and reagent-diluent of each cytokine as per the Duoset manufacturer's instructions.

The cassettes were hydrated initially for 2 minutes in 500 ml of dialysis buffer (PBS). The test sample (1ml) was taken up in a syringe and the sample was injected via a corner gasket into the cassette using an 18 gauge needle (BD). The remaining volume of air was then aspirated to ensure full contact of the membrane with the solution.

The cassettes were placed in buoys and then floated in 500 ml glass beakers of fresh PBS as dialysis buffer. The samples were dialysed at 4˚C using a magnetic flea for gentle agitation for 2 hours. The PBS was then replaced with a further volume of 500ml, and dialysis continued overnight. A second change of 500ml PBS was made the following morning, and a further dialysis period of 2 hours, again at 4˚C, was performed. The cassettes were then aspirated of sample and the dialysate aliquoted and analysed immediately, or stored at -80˚C.

3.4.2 Dialysis Results

All samples had increased in volume, suggesting osmotic change and dilution of the sample. This was confirmed by Coomassie analysis showing an equivalent dilution in protein concentration (range of 2-3 fold dilution).

The dialysed samples were then analysed by ELISA for the relevant cytokine, with doubling dilutions as described in section 3.3.3, but these were negative and no cytokine detectability was demonstrated.

3.4.3 Summary

The Calbiochem CPEK kit was not useful in reproducibly identifying cytokine levels in human tumour lysates. There appeared to be a factor within the kit that interfered with the assay results. This factor was not removed by dialysis. Multiple dilutions of the samples also failed to deliver accurate detectable reproducible results.

3.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

3.5.1 Introduction

Western blotting was used to try and confirm cytokine content within the Calbiochem CPEK extracted lysate samples. The initial ELISAs (section 3.3.4) had suggested a problem in the detection of particular cytokines, and a possible contaminant to produce abnormal high results, which could not be diluted away.

In order to assess whether the Calbiochem CPEK was effectively extracting functional cytokines SDS-PAGE and Western blotting were used to assess the presence of the cytokines. This was used to assess aliquots of protein extracted tumour and normal samples, and dialysates.

3.5.2 SDS-PAGE Materials and Methods

Running Gel Preparation:

A 10% polyacrylamide resolving gel was made up using 3.33ml acrylamide/bis, 2.6ml Tris-HCL (pH 8.8), 4ml dH_20, 50µl 10% (w/v) ammonium persulphate (APS) and 10µl Tetramethylenediamine (TEMED). The solution was poured between two glass plates assembled with 1mm spacers, leaving a 1-2cm space at the top. A layer of butanol was then carefully pipetted onto the surface and the gel was allowed to polymerise.

Further 12% and 15% (w/v) gels were also made up by altering the concentration of acrylamide/bis and H_2O appropriately (12% - 3ml dH_20/4ml Acryl/bis, 15% - 2.3ml dH_20/5ml Acryl/bis) in order to differentiate between the smaller molecular weight proteins which electrophorese quicker through the gel.

The stacking gel was prepared using 1.3ml acrylamide/bis, 1.35ml Tris-HCL (pH 6.8), 7.4ml dH20, 50µl 10% (w/v) APS, and 20µl TEMED. The layer of Butanol was

removed by blotting once the resolving gel was set, and the stacking gel was added on top, with a well-forming comb inserted. The set gel was then placed into an electrophoresis tank and covered with running buffer (The running buffer was made up with 12.1g Tris base, 23.8g HEPES, and 1g SDS dissolved in 1L ddH20).

The sample buffer used 35µl of lysate or standard, and 10µl of SDS sample buffer (13ml 1M Tris-HCL (pH 6.8), 6.5ml 20% (w/v) SDS, 5.2ml 100% glycerol and 260µl 0.5% (w/v) bromophenol blue). β-mercapto-ethanol (5µl) was added for reducing samples.

Precast Gels

Precast 10% (w/v) polyacrylamide mini gels were also obtained (Pierce, Rockford US). The gels were rinsed with Tris-Hepes-SDS running buffer within the electrophoresis tank to remove air bubbles. The running buffer was as previously used.

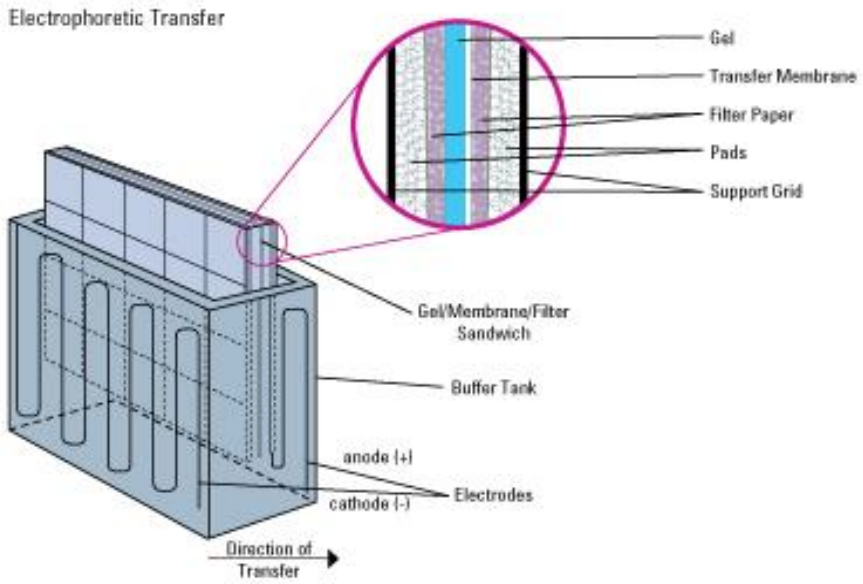
Benchmark Prestained Protein ladder (10 µl, 10-200kDa, Invitrogen) or PageRuler prestained protein ladder (10 µl, 10-170kDa, Fermentas) were used to determine protein size.

3.5.3 Western Blotting – General Method:

The electrophoresis procedure was run at 120W for 1 hour at 4ºC. Once complete the gel was placed in transfer buffer (3g Tris base, 4.08g Bicine and 100ml Methanol added to 900ml dH_20) at 4˚C. An equivalent piece of nitrocellulose membrane to the size of the gel was also added and allowed to equilibrate for five minutes (5x7cm approx.).

The proteins were transferred from the gel to the membrane by using a transfer sandwich (Fig 3.2).

Fig 3.2 Diagram of Electrophoresis equipment.



Cathode (-ve)
Scotch-Brite Pad
4x filter paper sheets
Gel
Transfer membrane
4x filter paper sheets
Scotch-Brite Pad
Anode (+ve)

The transfer sandwich was placed in a Biorad cassette and correctly orientated in the transfer apparatus. Transfer buffer was then gently poured into the apparatus, until covering the top of the sandwich. The apparatus was run at 100V for 1 hour, at 4˚C, under gentle stirring with a magnetic flea.

Once transfer was complete, the membrane was blocked in 20ml 2% (w/v) non-fat milk/1% (w/v) BSA in PBS for 1 hour at room temperature.

The primary antibody was then added to the blocking buffer and incubated for 2 hours. The membrane was then washed 3 times for five minutes per wash (0.05% (v/v) Tween 20 in PBS). Then the secondary biotinlyated antibody was diluted with blocking buffer and incubated for 1hour. A second series of three washes as described above was then performed to remove any excess secondary antibody.

The membrane was finally placed in equal volumes of ECL detection reagents (GE Healthcare) for 1 min, with gentle agitation to ensure total coverage. After blotting excess ECL reagent, the membrane was then placed between a folded acetate sheet, marked to show the appropriate lanes, and put in a X-omatic fine cassette (Kodak, Rochester, US) and exposed onto a photographic film (Amersham) for 1-30 minutes depending on the antibody used.

After exposure, the film was placed in developer solution for 1 minute, then quickly rinsed in 1% acetic acid, before being placed in fixer solution for a further minute. The film was then rinsed in water and air dried. The lanes were then analysed and the ladders marked.

3.5.4 Western Blotting Results

The initial gels made in the laboratory using the above method in section 4.5.2 gave inconsistent results, with both curvature of the samples, and lateral spread during the electrophoresis process. Samples were run on three separate occasions.

Another problem was non-uniform separation of the samples. This was initially remedied by using higher strength gels of 12% and 15% (as described in section 4.5.2). However this increased the curvature of the samples in the gel, and the lateral spread.

Therefore, pre-cast gels were then used for further analysis. The cytokines IL-1β, IL-4, IL-10, IL-12, and TNF-α were reassessed using the above general western blotting method.

In order to avoid the previous ELISA problems encountered in section 4.4.3, the Duoset cytokine standards were used as control samples, aliquots of the Calbiochem CPEK kit extracted samples were also used.

The standards and samples were assessed with and without β-mercaptoethanol (β-ME), to assess both reduced and native proteins; the standards detailed above were used at the second highest level. The blots were repeated in duplicate, and also repeated with the highest concentration of the standards available (IL-1β – 100ng/ml, TNF-α – 290ng/ml, IL-12 – 190ng/ml).

Initial Western Blotting Results

The initial western blots had no detectable cytokines in either of the standard lanes, or the samples, despite three-fold repetition. When analysing IL-12 p40 without β-ME it was noted that the solution precipitated on boiling so further analysis was impossible.

Because the highest concentration of cytokine within the standard solutions were in nanograms per ml, further recombinant IL-1β and TNF-α were sourced (Biosource, Camarillo, Ca, USA). These provided 2 µg (IL-1β) and 10 µg (TNF-α), allowing a reconstituted concencentration of 0.1-1mg/ml.

These were analysed at a concentration of 1µg/ml and 500 ng/ml with and without the addition of 10µl Calbiochem CPEK extraction reagent (ER). The ER may have been the factor which had interfered with the previous ELISAs in section 3.3.3. Only IL-1β was detected. Tumour samples were also tested for both IL-1β and TNF-α, with no positive result.

Fig. 3.3/Table 3.5 Diagram of example Western blot film demonstrates the detection of recombinent IL-1β only without CPEK extraction reagent (lane 9 and 10 positive at ~15 kDa protein ladder).

1	2	3	4	5	6	7	8	9	10
Lad	T9 10µl	T9 Double dil.	T9 + ER 10µl	T9 + ER Double-dil.	IL-1β 1ug/ml + ER	IL-1β 500ng/ml + ER	Lad	IL-1β 1ug/ml	IL-1β 500ng/ml

3.5.5 Summary

The only detected cytokine using the western blot method was IL-1β at concentrations of 1µg/ml and 500ng/ml. The addition of extraction reagent stopped this cytokine being detected.

Because of the problems that seemed to be caused by the extraction process, a second extraction reagent was sourced and assessed (See chapter 2).

3.6 Discussion

The extraction of detectable cytokines from the lysates of tumour tissue that could be reliably detected proved problematic. The initial extraction process using the Calbiochem complete mammalian protein extraction kit was initially created for SDS-PAGE analysis. SDS is known to alter protein quaternary structure, and both SDS-Page and western blotting is dependent on pH, solubility, and sample preparation (Celis and Gromov 1999). Further immunoblotting work, and multiplex analytical kits all use ELISA with various multiplicative techniques in the detection of cytokines (Lin *et al* 2003). The methods illustrated above could not accurately identify key cytokines from tissue lysates, this was despite using dialysis to reduce levels of probable interfering molecules. The ability to detect higher concentrations of IL-1β of 500ng/ml and 1µg/ml was demonstrated, but this was beyond the ELISA detection upper range of 2000 pg/ml, and was demonstrated to be disrupted by the addition of the initial extraction reagent used. Previous work has shown plasma levels of a range of cytokines to be in the picogram range between 6-12 pg/ml by ELISA (Saparano *et al* 2004). If equivalent levels are present in the lysate of tumour tissue, these would not be detected using the Calbiochem kit. Therefore the Calbiochem protein extraction kit was abandoned.

4. Chapter 4 - Results

4.1 Patient Clinico-pathological Data

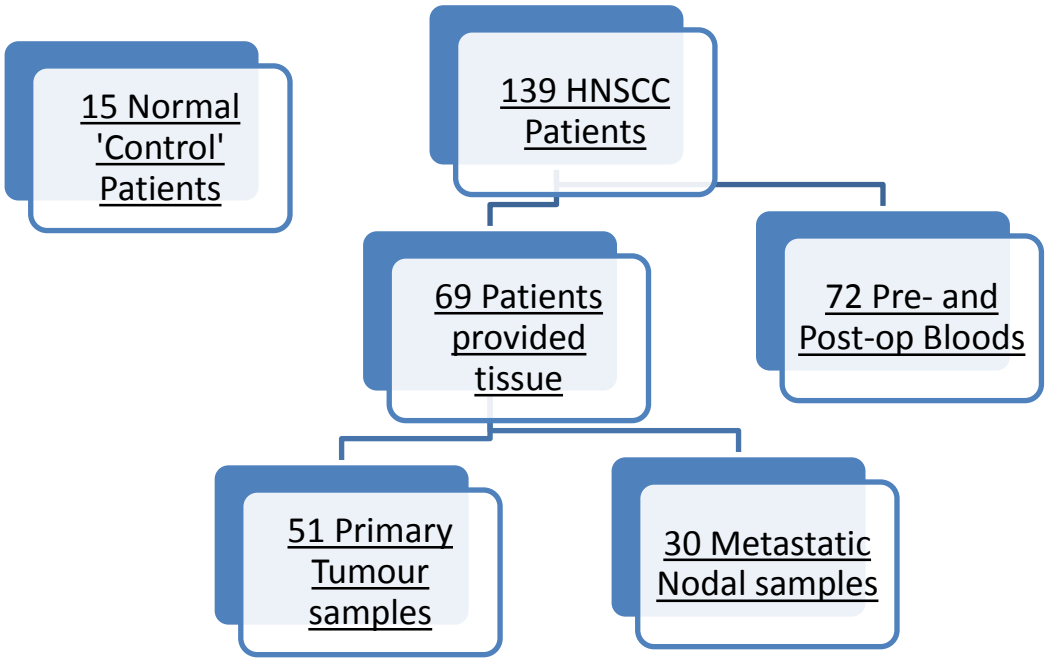
4.1.1. Introduction

During the period between July 2007 and June 2009 one hundred and thirty-nine patients were recruited to the study as described in chapter 2. Of these patients, Seventy-two had pre and post treatment blood samples taken, and sixty-nine patients had tissue samples taken. These sixty-nine patients provided a total of eighty-one samples; Fifty-one tumour tissue samples and thirty nodal tissue samples were used in the final analysis, after protein extraction and ELISA optimisation (See Fig 4.1). Paired tissue samples from the primary tumour and metastatic nodal tissue of the same patient were retrieved from nine patients to explore potential differences in cytokine levels between primary tumour and immune-cell rich metastatic lymph nodes.

Fifteen patients undergoing benign oral surgical procedures (see chapter 2) were recruited to the study to provide tissue to be used as 'normal' controls. They consented to donate small pieces of normal mucosa and connective tissue dissected from uvulo-pharyngo-palato-plasty or tonsillar surgery which would have otherwise been discarded. Of these samples, seven were uvular mucosa and eight samples were palatal/upper tonsillar pole mucosa and connective tissue.

The patients enrolled were followed up via the HEY NHS trust online computer system clinicom. The longest follow-up period was 63 months. The endpoints used were time to recurrence (months), and time to death of all causes (months).

Fig. 4.1. Schematic diagram of recruited patients



4.1.2 Patient Demographics

Tumour tissue:

The tumour tissue cohort of sixty-nine patients was skewed towards the male sex: fifty-nine males and ten females. The median age at diagnosis of this cohort of HNSCC patients was 62.12 years (Range 36.77 – 87.43 years, Q1 - 54.44, Q3 – 70.3 years). The complete patient tumour group demographics are listed in Appendix 1.

4.1.3 Patient Stage (TNM)

The patient cohort was stratified by T stage from the TNM classification. The tumour microenvironment of HNSCC is thought to have at least immuno-modulatory properties (Whiteside 2012), and these may have immune suppressive effects. The first hypothesis to investigate was to assess whether tumour size had an effect on different cytokine expression. The patients were divided into two groups, T1 and T2 tumours were compared with T3 and T4 primary tumours (See Table 4.1). The patients were also grouped according to overall TNM stage, however patients with distant metastatic

disease were excluded, so any patients presenting with stage IVC were not included in the study.

Table 4.1 Patient T stage and Overall Stage

T stage	T1	T2	T3	T4	Unknown	Total
Patients	17	19	11	20	2	69
Overall Stage	I	II	III	IV	Unknown	Total
Patients	7	7	7	46	2	69

4.1.4 Subsites of Disease

The tissue samples were stratified as per the subsite of the HNSCC origin. HNSCC seems to behave in a heterogeneous fashion, with different subsites having slightly different pathophysiological chronology and patterns of spread. The different anatomical regions, and lymphatic drainage can explain some, but not all of the differences (see intro).

The subsites of HNSCC are dependent on the anatomical site of origin of the primary tumour. The anatomical boundaries are described in section 1.3. For the purposes of this study, the primary tumour sites were characterised as Oral, Oropharyngeal, Laryngeal, and Hypopharyngeal origin (See Table 4.2).

Table 4.2 Number of patients recruited by HNSCC Subsite

Subsite	No. of Patients
Oral SCC	12
Oropharyngeal SCC	17
Laryngeal SCC	32
Hypopharyngeal SCC	3
Unknown primary	5

Five patients had biopsy histology proven metastatic SCC nodal disease, of most probable head and neck origin, but in whom a primary was not found on panendoscopy or imaging. These are classed as carcinoma of unknown primary, presumed HNSCC.

4.1.5 Paired Tumour and Nodal Tissue Comparison

Paired primary tumour tissue and nodal tissue was retrieved from nine patients. These were compared to look for any difference in the tumour microenvironment of the primary tumour, compared with that of the metastatic lymph nodes, which as part of the lymph-reticular system have an inherent strong immune cell presence (see table 4.3).

Table 4.3 Paired Tumour and Node Samples

Patient No.	T-stage	N-Stage	Overall Stage	Subsite
1	4	N2b	4A	Oral
2	3	N2b	4A	Oral
3	1	N2c	4A	Oral
4	2	N2c	4A	Oropharynx
5	2	N2b	4A	Oropharynx
6	1	N2b	4A	Larynx
7	3	N2c	4A	Larynx
8	2	N2c	4A	Hypopharynx
9	3	N2c	4A	Hypopharynx

Table 4.3 illustrates the high overall stage once lymph nodes become involved with metastatic HNSCC.

4.1.6 Control samples:

The control samples were recruited from normal ENT operating theatre lists undergoing benign oropharyngeal surgical procedures (See table 4.4). Blood samples for PBMC and serum were also taken with consent. There were 15 patients recruited, 8 males and 7 females. Median age at time of surgery was 42 years old (range 17-67 years, Q1 – 24, Q3 – 56).

Table 4.4 Normal Control Patients

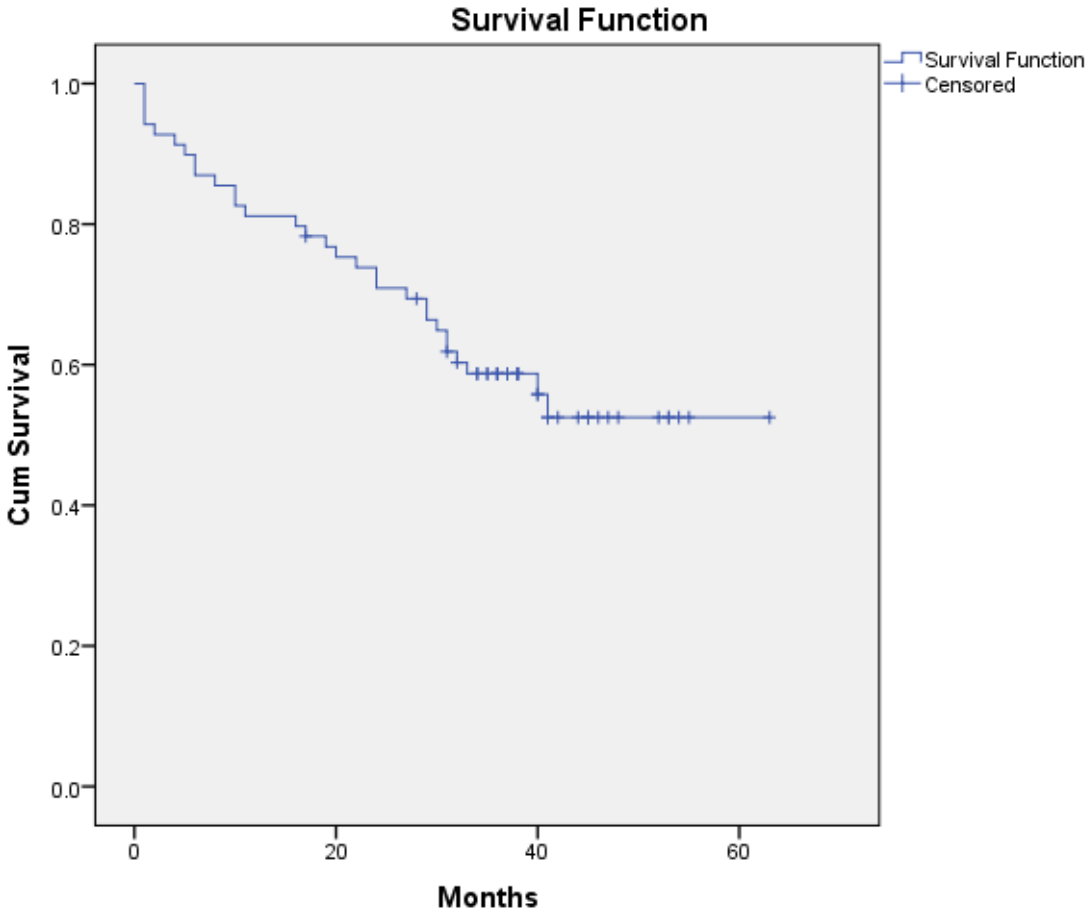
Patient	Sex	Site
1	F	Uvula
2	F	Uvula
3	F	Tonsil/palate
4	F	Tonsil/palate
5	F	Tonsil/palate
6	F	Tonsil/palate
7	F	Tonsil/palate
8	M	Uvula
9	M	Uvula
10	M	Uvula
11	M	Uvula
12	M	Uvula
13	M	Tonsil/palate
14	M	Tonsil/palate
15	M	Tonsil/palate

4.2 Survival

4.2.1 Overall survival

Of the 69 of patients recruited to the study, 30 patients died within the follow-up period. The estimated mean survival time was 42.2 months (S.E. 2.93, C.I. 36.6-48.1, see Fig 4.2). The median survival could not be defined because the end point of death has not been reached by 50% of the study population in the follow-up time frame.

Fig. 4.2 Overall Survival Kaplan-Meier curve

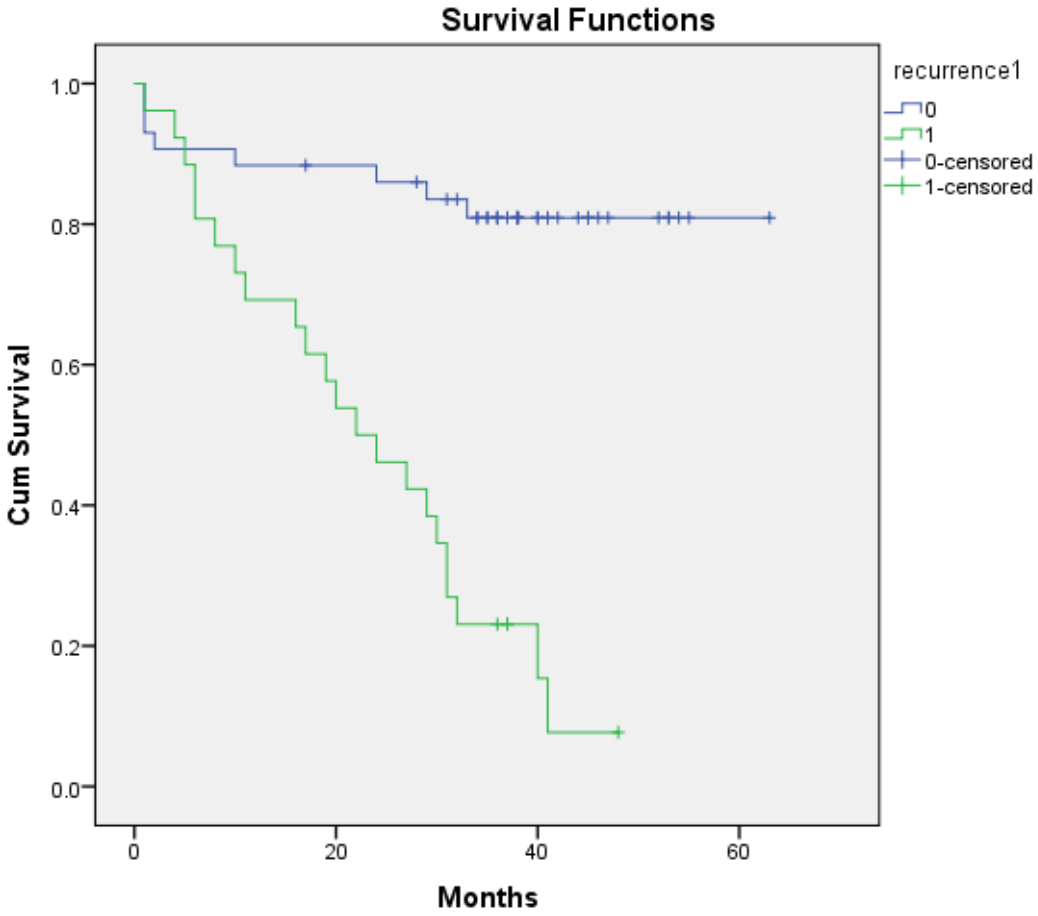


4.2.2 Recurrence, and recurrence free survival

The cohort follow-up also recorded recurrence of the HNSCC. This was classed as either loco-regional recurrence of biopsy proven HNSCC, or metastatic disease picked up on imaging such as pulmonary metastases on CT scan.

The median survival time for patients who recurred was 23.3 months, and log rank comparison for survival between those patients with recurrence and those without was statistically significant ($p<0.005$, see Fig 4.3).

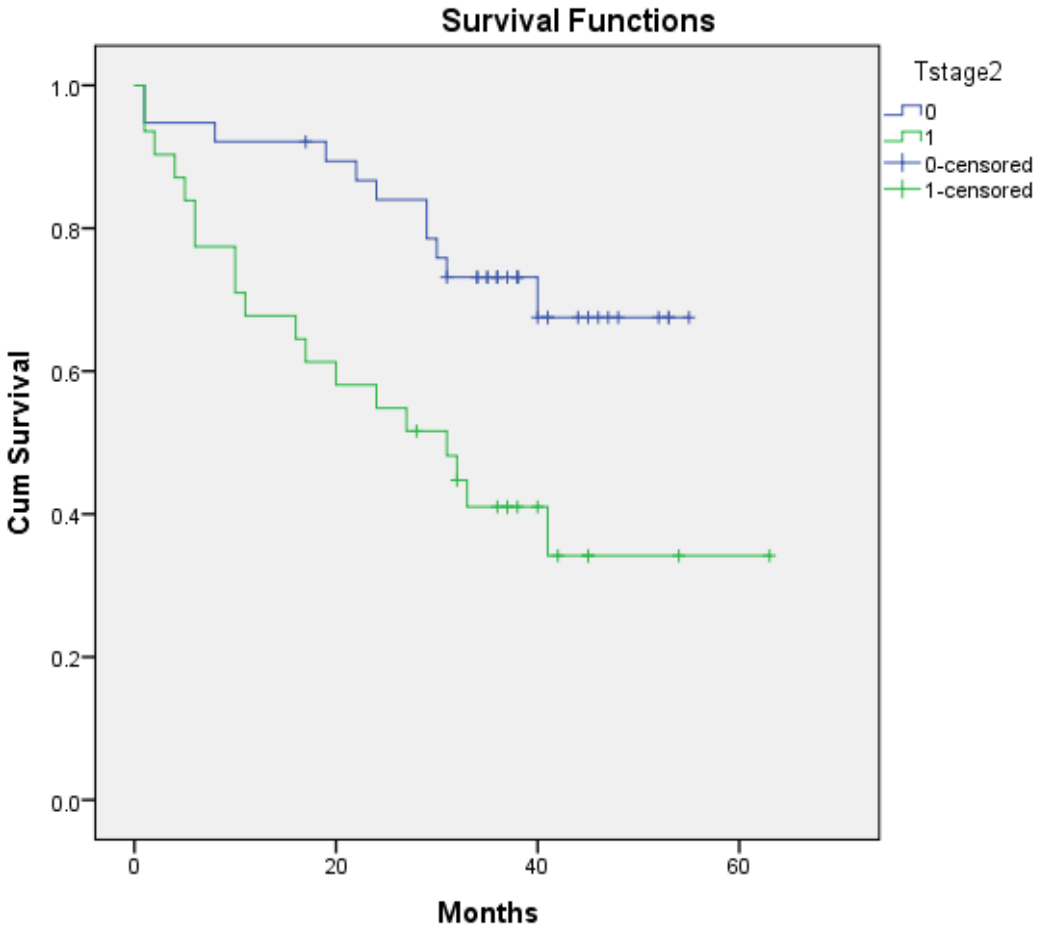
Fig 4.3 Survival and recurrence Kaplan-Meier Curve



4.2.3 Survival stratified by T-stage and Overall stage

The cohort was analysed for survival and stratified for T-stage, grouping into T1/T2 (38 patients, group 0 on graph) tumours and T3/T4 (31 patients, group 1 on graph) tumours. Eleven patients died in the T1/T2 group and 19 patients died in the T3/T4 group.

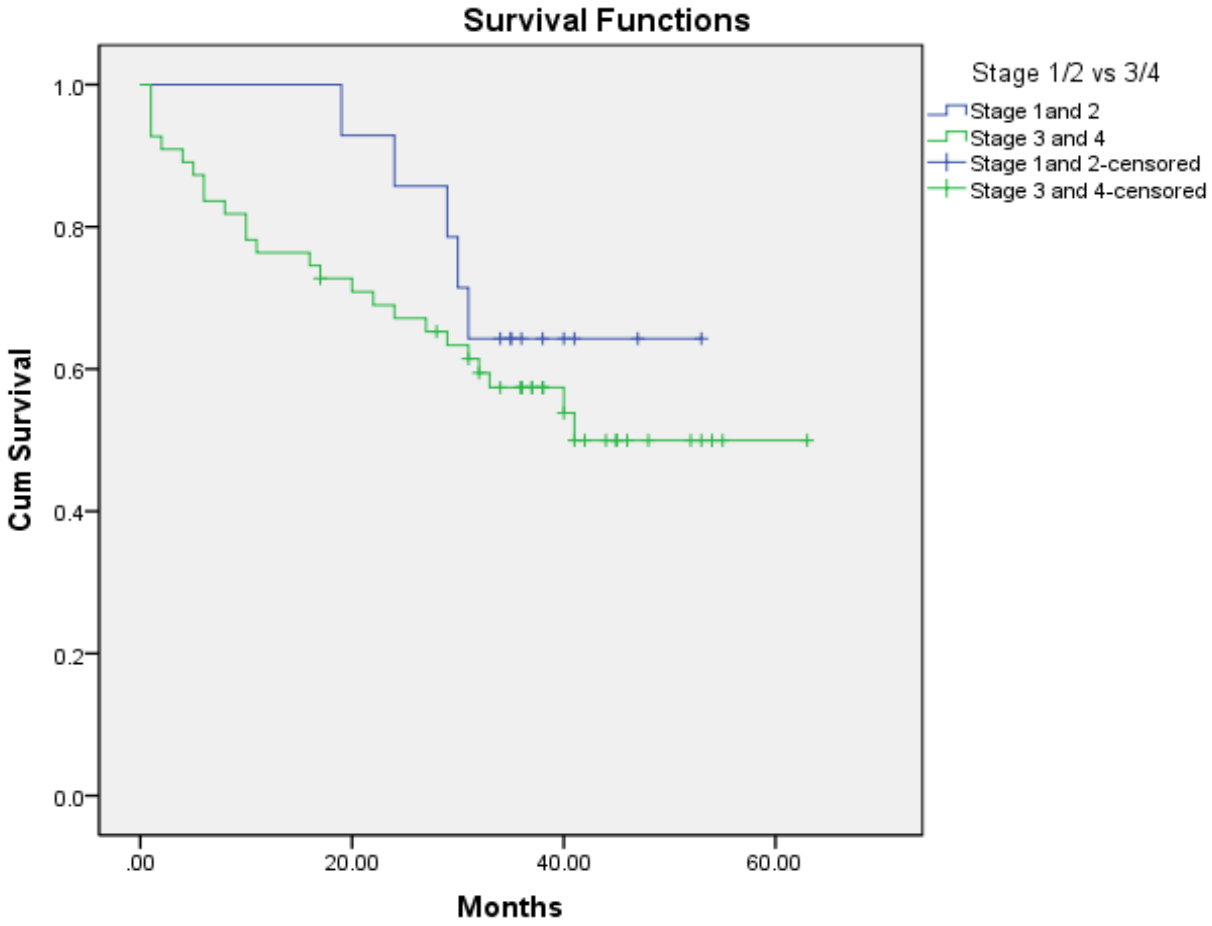
Fig 4.4 Kaplan-Meier Survival Curve by T-stage (T1/T2-Blue vs T3/T4-Green)



The estimate mean survival for the T1/T2 group was 44.6 months (S.E. 2.77, 95% C.I. 39.2-50.1), compared with a mean of 32.9 (S.E. 4.5, 95% C.I. 24.1-41.6) months in the T3/T4 group. Log rank analysis showed the difference was statistically significant (p= 0.004).

The group was stratified by overall stage, and group into stage 1 and 2 compared with stage 3 and 4, because the more advanced disease including any N-status stages the patient at stage 3 at least. Only 14 patients were stage 1 or 2, with 55 patients staged 3 or 4.

Fig 4.5. Kaplan-Meier Survival curve by Overall Stage 1-2 (Blue) vs 3-4 (Green)



There was no statistical difference found by log-rank analysis between the two overall stage groups (1+2 vs 3+4). This may have been due to the difference in the group sizes, with the more advanced stage 3 and 4 being much larger.

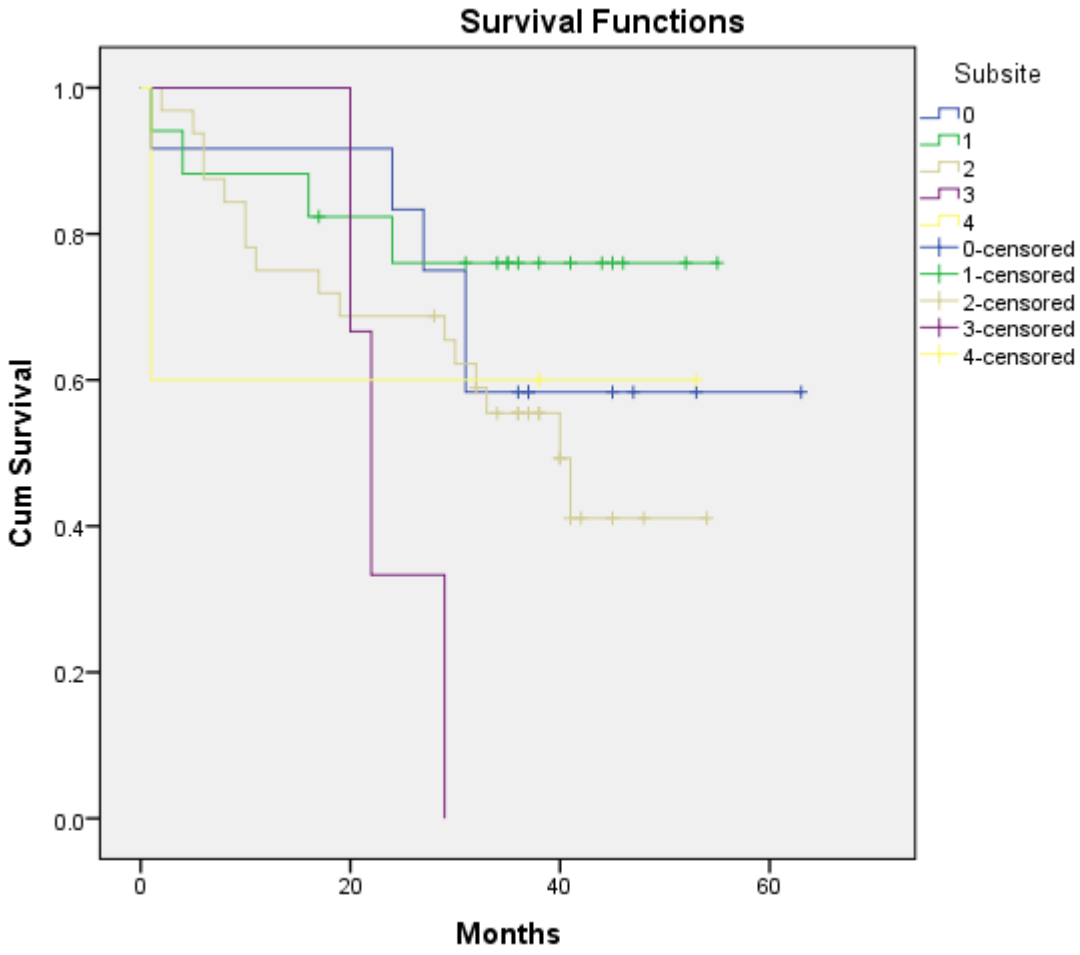
4.2.4 Survival stratified by subsite

The cohort was also stratified by subsite of the HNSCC primary and overall survival in months (Table 4.5).

Table 4.5 Survival stratified by Subsite

Subsite	Number	Total No.	No. of Deaths	Survival Mean	CI
Oral	0	12	5	46.3	34.3-58.1
Oropharynx	1	17	4	44.6	35.4-53.7
Larynx	2	32	16	35.1	28.2-41.9
Hypopharynx	3	3	3	23.7	18.3-29
Unknown	4	5	2	32.2	9.8-54.5
Overall		69	30	42.3	36.5-48.1

Fig 4.6 Survival by HNSCC Subsite



The survival curves for the different subsites do not show an obvious difference between the groups in this cohort. The Log rank analysis did not show a statistical difference between the five groups.

4.3 Discussion

The most striking point from the demographics of the HNSCC patients was the skew towards the more extensive tumours within both the T-stage and overall TNM stage cohorts. This has resulted from sample bias in obtaining tumour tissue, biopsy samples were taken as fresh samples from surgical specimens, and this method excludes analysis small primary tumours. Smaller tumours with lower stages would be more accessible after histological fixation, but the processing involved in this may have an effect on the true cytokine levels.

HNSCC has been shown to be more common in males, probably due to the increased incidence in tobacco and alcohol consumption in this group (Section 1.4). More recently the increase incidence in HPV related oral and oropharyngeal SCC which has affected younger patients apparently does not follow this trend with a male to female ratio approaching 50:50 in younger patients (Ang *et al* 2010, Annertz *et al* 2002). The cohort of patients analysed hade a male:female ratio of around 6:1. The control group was not age-matched, with a ratio of 1:1.

The nodal status of the patients was also inherently biased. Assessment of normal nodes in a patient with HNSCC could be unethical due to morbidity of sampling, although fine needle aspiration could be used as a modality with low morbidity. The use of sentinel node biopsy (SNB), using methods of dye and radioactive molecules injected into the tissue surrounding the primary tumour in order to identify the first node draining that area, which is then biopsied as a surrogate marker for involvement. This method has been used successfully in breast cancer in order to decrease patient morbidity from axillary clearance or nodal regional adjuvant radiotherapy. It has been studied extensively in other cancers such as colo-rectal adenocarcinoma and SCC of the vulva. In HNSCC the technique is still considered investigational, with selective neck dissection the recognised treatment technique, although there are currently randomised controlled trials investigating SNB in HNSCC (Stoeckli *et al* 2009, Antonia *et al* 2012).

The survival of this cohort of patients is similar to those found elsewhere (Myers *et al* 2001), and follow the principle of the TNM staging system of more advanced stage gives a poorer prognosis. The subsite analysis of survival did not show a statistically significant difference between the groups, however the Kaplan Meier curve seems to show a poorer outcome for hypopharyngeal patients, as others have found (Le 2007, Warnakulasuriya 2009). Data by subsite had too few patients per group to demonstrate a statistically significant difference.

Chapter 5 – Characterisation of cytokine levels affecting the immune response in the tumour microenvironment of HNSCC.

5.1 Introduction

The major hypothesis of this thesis is that the HNSCC tumour microenvironment would have higher concentrations of cytokines that inhibit the cellular cytotoxic immune response in favour of tumour survival. Furthermore it is hypothesised that the cytokine levels within these tumours would reflect a TH2-like response and a regulatory T-cell rich environment which would be associated with reduced patient survival.

The tumour microenvironment has previously been shown to have increased levels of immune cells with suppressive functions, such as increased Tregs (Bose *et al* 2008, Whiteside *et al* 2008) and $CD34^{+}$ progenitor macrophage cells (Young *et al* 1997). A number of studies report the Th1/Th2 immune response balance may have been pushed towards Th2 dominance, leading to the reciprocal decrease in Th1 action (Bose *et al* 2008, Lathers 2003).

The exact role of Th17 cells within the HNSCC tumour microenvironment has not yet been clearly defined, but they are known to be pro-inflammatory stimulating surrounding cells such as stromal fibroblasts and endothelial cells to produce IL-6, IL-8, MMP-1, and NOS-2 within non-malignant inflammatory arthitides (Agarwal *et al* 2008). It is not clear whether this pro-inflammatory action has an overall anti-tumour effect, or a pro-tumour effect allowing tumour escape.

In order to demonstrate the importance of immune function in the tumour microenvironment of HNSCC key cytokines were chosen to investigate the Th-1/Th-2 axis, as well as the Treg and Th-17 axis. IL-2 was used to demonstrate T cell stimulation. IL-12 and IL-10 were chosen to demonstrate the Th-1 like and Th-2 like response respectively. TGF-β levels were used to assess Treg population and IL-17 was used to identify Th-17 cells. It is hypothesised that the tumour microenvironment would

have an immune suppressive slant, with increased levels of IL-10 and reduced IL-12 indicating a Th-2 –like skewed response, with increased levels of TGF-β and reduced levels of IL-17 indicating a skew towards Treg predominance over TH-17-like response.

5.2 Investigating immuno-modulatory Cytokines in primary HNSCC tumour tissue compared to 'normal' control tissue.

5.2.1 Patients and Methods

The HNSCC tumour lysates and normal control tissue were measured for levels of the immune cytokines IL-2, IL-10, IL-12, Il-15, IL-17, TGF-β, and VEGF by quantitative ELISA. The methods used for ELISA are as described in chapter 2 Patients and methods (Section 2.1). The patient demographics are as described in Chapter 4 (Section 4.1, figure 4.1).

The optical density of each well of the micro-plate was measured using wavelength correction by subtracting readings at 540nm from those at 450nm to allow for optical imperfections in the micro-plate (Nunc). Each sample was measured in duplicate, and the average of both readings taken. Each sample was also diluted by 50% to confirm accurate readings, as the measured absorbance should also reduce by approximately half if no contaminants are present within the sample.

The standards were also analysed in duplicate and a seven-point standard curve was drawn using the plate-reader software (generated using a 4-PL curve fit). The optical density of the lysate samples were read against the standard curve to give a cytokine level in pg/ml. This figure was then normalised per tumour sample depending on the initial samples overall protein concentration as detected by Coomassie staining (Section 2.2.6). The values were then corrected any dilution factor, used when the sample was prepared for ELISA. The final results are given in pg of cytokine/mg total protein.

5.2.2 Results: Tumour tissue *vs* Control Tissue

The total numbers of the HNSCC patients and control patients are listed in Table 5.1, with the statistical significances displayed in Table 5.2. There was a statistically significant difference found between tumour tissue and control tissue in the levels of IL-17, TGF-β, and VEGF using the Mann-Whitney U-test between groups. This was confirmed using the Kruskal-Wallis analysis comparing the distribution of the groups of cytokines between all tumour samples and controls confirmed the above result (IL-17 $p=0.016$, TGF-β $p<0.001$, and VEGF $p=0.011$). There was no significant difference between levels of IL-2, IL-10, IL-12, or IL-15 between the HNSCC samples and the normal tissue controls.

Table 5.1 Normalised cytokine levels in HNSCC tumour samples compared with normal control tissue samples

Cytokine Group	HNSCC No. Samples	Median Conc. pg/mg	Interquartile Range	Control Number	Median Conc. pg/mg	Interquartile Range
IL-2	61	0	0-0	15	0	0-0
IL-10	79	10.8	0-51.7	15	0	0-53.9
IL-12	71	0	0-19.6	15	5.9	0-8.9
IL-15	72	4.7	0-14.8	15	4.8	0-8.1
IL-17	69	4.2	0-21.4	15	18.6	12.3-22.9
TGF-β	61	1044	720-1984.3	13	678.9	607.9-877
VEGF	67	83	18.18-210.8	14	27.6	13.7-46.4

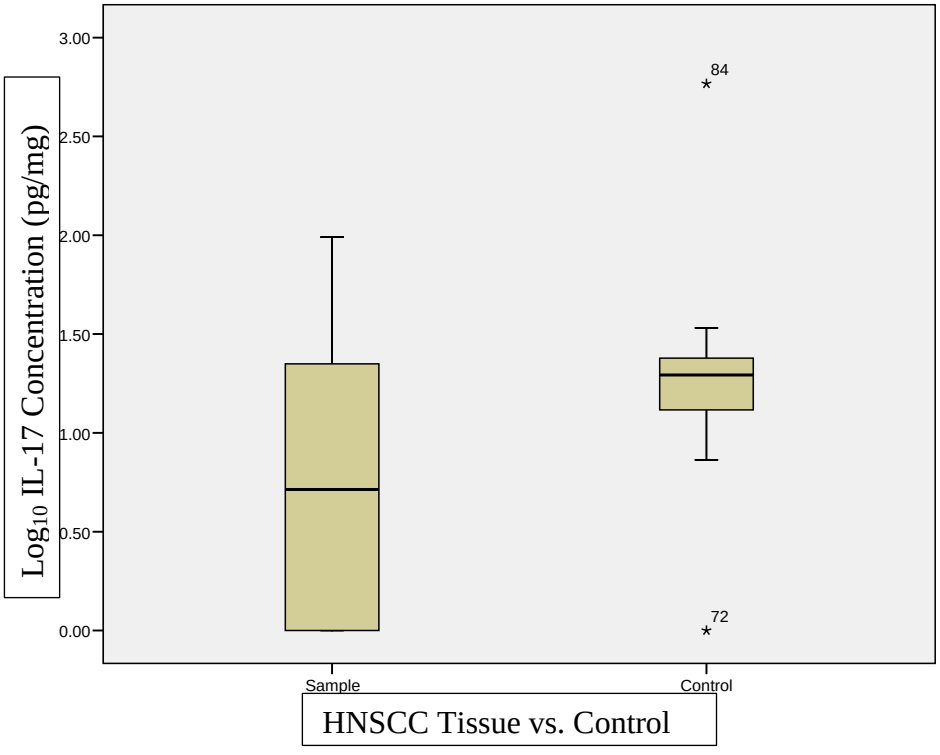
Table 5.2 Summary of Mann-Whitney-U statistical analysis effect size (r) of Tumour tissue compared to normal controls.

Cytokine Group HNSCC vs Control	Mann-Whitney U significance (2-tailed)	R (effect size est.)
IL-2	0.876	-0.01
IL-10	0.899	-0.01
IL-12	0.773	-0.03
IL-15	0.858	-0.02
IL-17	0.009*	-0.28
TGF-β	0.004*	-0.33
VEGF	0.026*	-0.25

* Indicates significance level below 0.05.

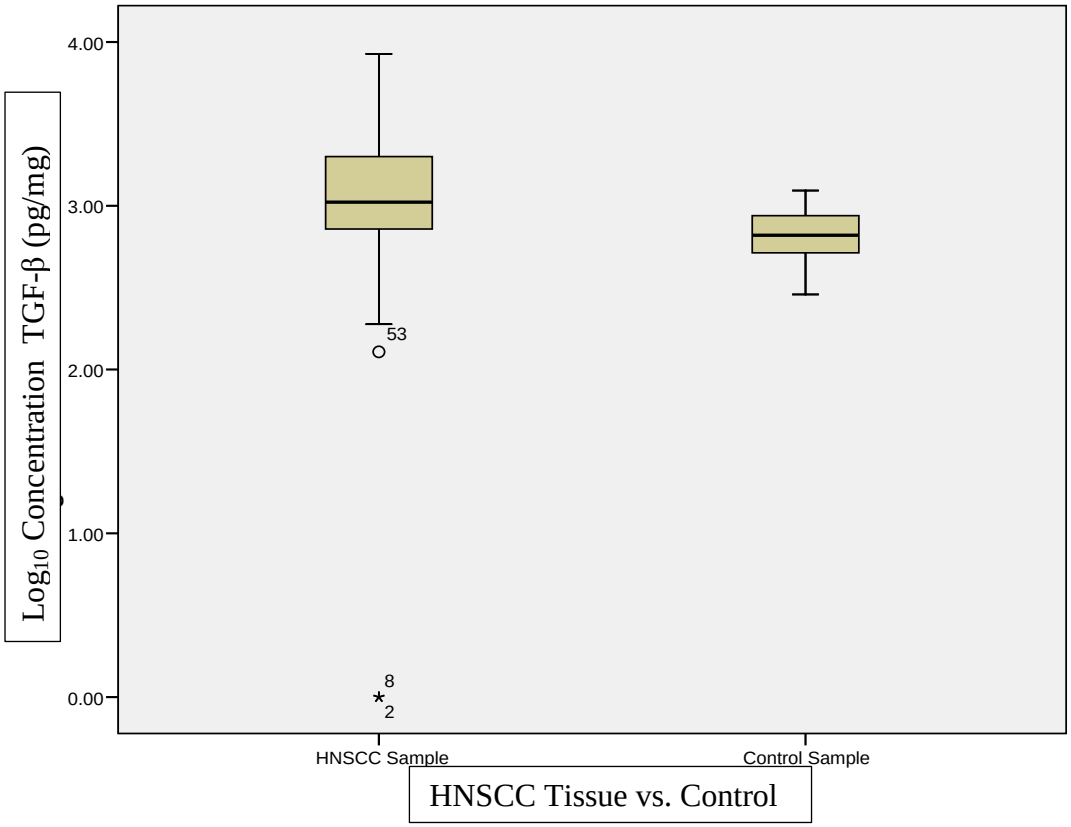
There was a significantly higher level of the concentration of IL-17 per mg of protein found in the control cohort (median 18.64pg/mg), compared with the HNSCC sample cohort (See fig 5.1), with a small to moderate effect size.

Fig. 5.1 Log_{10} Concentration of IL-17 in HNSCC tissue vs. Control tissue (pg/mg)



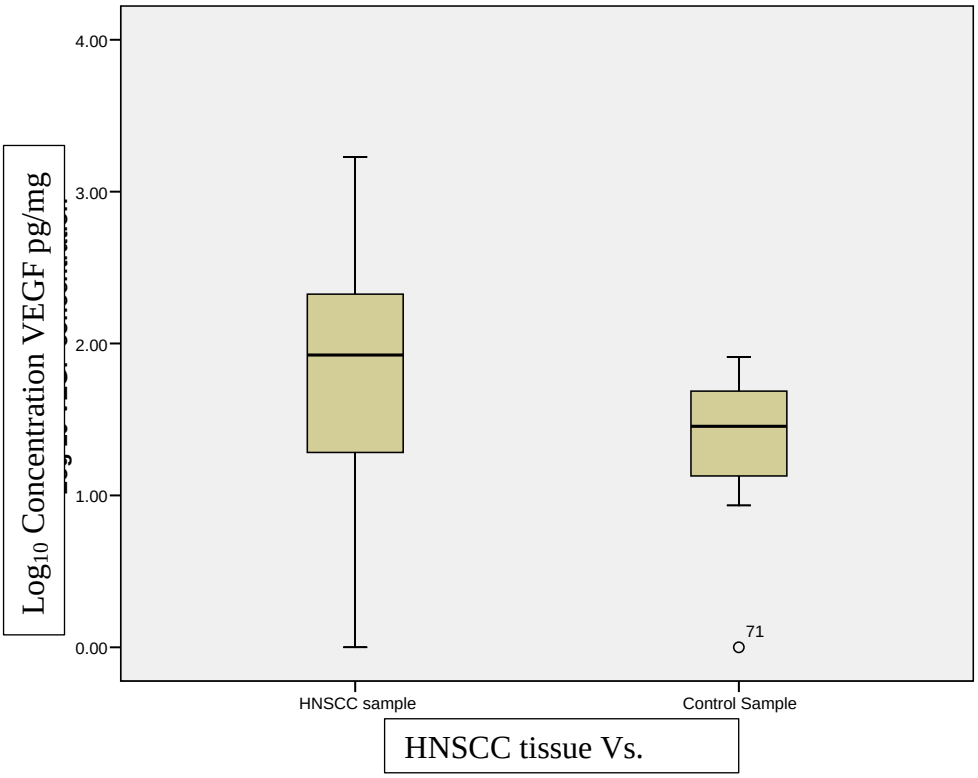
The concentration of TGF-β was also found to have a significant difference between HNSCC (median 1051 pg/mg) and control cohorts (median 659 pg/mg) which signifies a moderate effect size (Fig. 5.2).

Fig. 5.2 Log_{10} Concentration of TGF-β in HNSCC tissue vs. Control tissue.



There was also a significant difference between the higher concentrations of VEGF found in the HNSCC sample (median - 83pg/mg) compared with the Control samples (median - 27.5 pg/mg), which indicates a small to moderate effect size (Fig. 5.3). A log_{10} scale was used to correct for outlier values.

Fig. 5.3 Log_{10} Concentration of VEGF in HNSCC tissue vs. Control Tissue.



5.3 Immuno-modulatory cytokine levels in paired primary HNSCC tissue and nodal metastatic disease.

5.3.1 Introduction

HNSCC commonly metastasises to lymph nodes in areas of the neck depending on the site of the primary tumour, as discussed in Section 1.4. The lymph nodes are an essential part of the lympho-reticular system, and are an integral part of the immune system. They are the tertiary organs of the immune system to which antigen-presenting cells migrate in order to present antigen and activate T cells via the TCR. This induces the adaptive immune response, and activates T cells to migrate and infiltrate the tumour microenvironment (Vesely *et al* 2011).

A total of nine patients had paired samples taken from both the primary tumour and the involved nodal tissue taken during the same operation. The levels of IL-2, IL-10, IL-12, Il-15, IL-17, TGF-β, and VEGF were assessed by ELISA as the methods previously described.

5.3.2 Results

All nine patients were stage IV, indicating the advanced nature of head and neck cancer once nodal metastatic disease is present. One patient had positive ELISA detection of IL-2. The concentration of IL-2 detected within the tumour tissue was higher (182.49 pg/mg) than that detected within the nodal tissue (4.55 pg/mg). The other eight paired samples had no detectable IL-2 within the tumour tissue or the nodal tissue. The other cytokines measured had variable detectability within the tumour tissue and the nodal tissue. There was no significant statistical difference found between the levels of cytokines within the tumour tissue and the nodal tissue (See Table 5.3).

Table 5.3 Summary of Cytokine levels in Paired Tumour and Nodal tissue (pg/mg)

Sample	Stage	Site	IL-10	IL-12	IL-15	IL-17	TGF-β	VEGF
1	4	T	0	0	19.8	0	0	0
		N	81.31					182
2	4	T	51.3	0	0		1634	0
		N	35		20.7		0	
3	4	T	0		0			360
		N	0		0			
4	4	T	0	0	9.52	4.17		
		N	0	0	9.88	13.84		
5	4	T	19.2	10.24	5.28	25.4	1995.36	200
		N	15.48	0	3.42	0	1168.74	18
6	4	T	16	5.76	7.68	32	840.16	96
		N	12.8	22		56.4	1153.8	42.4
7	4	T	0	0	4.07	5.95	2657.34	19.98
		N	10.56	0	3.52	10.63	2511.08	26.4
8	4	T	99.76	44.51	0	12	937.4	17.97
		N	51.2	18.22	7.96	0	2501.6	808.2
9	4	T	184.4	36	28	65.9		48.3
		N	1411.4	53.1	25.4	40.3		220.3

5.4 Comparing levels of immuno-modulatory cytokines between T-stage and Overall Stage of HNSCC

5.4.1 Introduction:

The immuno-modulatory effects of HNSCC have implicated a more pro-tumour microenvironment prevalent within increasingly advanced disease (Young *et al* 2006, Allen *et al* 2011). This implication may be reflected in the raised levels of those cytokines associated with increased immuno-suppression in more advanced tumours. Although different for each subset in HNSCC, the T-stage is based upon gross size of the tumour and local invasion through normal anatomical structures, whereas the overall Stage includes loco-regional metastatic disease spread to lymph nodes, and distant metastatic spread. The levels of IL-2, IL-10, IL-12, IL-15, IL-17, TGF-β, and VEGF were compared in groups stratified to T1/T2 or T3/T4 depending on local spread, and also overall stage 1-3 or stage 4(a or b) depending on loco-regional nodal spread. Stage 4c characterises distant metastatic spread of disease, and these patients are not suitable for curative surgery, and therefore no stage 4c patients at presentation were recruited.

5.4.2 Patients and Methods

The Patients and methods used are those described in Chapter 2. The staging was assessed from clinical and radiological assessment, and time of surgery, as well as analysis of final pathological staging of the specimen and multi-disciplinary team (MDT) discussion.

5.4.3 Results

The number of patients in each group is summarised in Table 5.4. The levels of IL-2 showed no difference between the groups due to low levels of detectability (data not shown).

Table 5.4 Summary of Patient numbers in each cohort of T1/T2, T3/T4, OS 1-3/OS 4

Cytokine Group	T1/T2 Group	T3/T4 Group	Overall Stage 1-3	Overall Stage 4	Mann-Whitney U	R (effect)
IL-10	40	36	21	56	0.38	-0.1
IL-12	38	31	22	47	0.7	-0.05
IL-15	33	37	20	50	0.49	-0.08
IL-17	37	30	23	44	0.22	-0.15
TGF-β	30	30	20	40	0.77	-0.03
VEGF	34	31	23	42	0.403	-0.1

* Statistically significant at the 0.05% level.

There was no statistical difference in the concentration of any of the 6 cytokines found at detectable levels when the tumours were stratified into T1/T2 and T3/T4.

The Overall stage according to the UICC TNM staging classification takes into account the T-stage as well as the progressive nodal involvement. They were stratified into Stage 1-3 and stage 4, although there are some differences across anatomical sites of HNSCC, if a tumour is locally advanced and classed as a T4, or the nodal metastatic disease involves more than a single node, or a single node greater than 3cm in diameter, the cancer is staged as 4. Table 5.5 summarises the differences between the overall stage 1-3 group of tumours with the more advanced stage 4 tumours, and table 5.6 summarises the statistical analysis between the two groups.

Table 5.5 Summary of the comparison between overall stage 1-3 and stage 4 tumours

Cytokine Group	Stage 1-3 Samples	Median Conc pg/mg	Interquartile Range	Stage 4 Samples	Median Conc pg/mg	Interquartile Range
IL-2	17	0	0-0	44	0	0-33.7
IL-10	23	50.4	0-91.6	56	0	0-53.9
IL-12	21	18.22	0-37.5	50	0	0-9.1
IL-15	20	0	0-7.8	52	5.8	0-19
IL-17	23	0	0-10	46	5.3	0-22.6
TGF-β	19	937.4	655.2-1121.6	42	1149.9	782.6-2150.7
VEGF	22	104.6	21.8-296.4	45	72.5	18.4-198

Table 5.6 Summary of significance between overall stage 1-3 and stage 4 tumours

Cytokine Group Stage 1-3 vs Stage 4	Mann-Whitney U significance (2-tailed)	R (effect size est.)
IL-10	0.15	-0.16
IL-12	0.01*	-0.3
IL-15	0.03*	-0.26
IL-17	0.17	-0.04
TGF-β	0.3	-0.13
VEGF	0.89	-0.01

* Statistically significant at the 0.05% level, IL-2 data not shown, as levels were not detectable

When the cytokines were compared between the stage 1-3 group and the stage 4 group, there was no statistically significant difference in IL-2, IL-10, IL-17, TGF- β, or VEGF. The levels of IL-12 was significantly lower in the more advanced stage 4 samples when compared with those staged 1-3 (median stage 4 0 pg/mg, Stage 1-3 16.5, p=0.013) r = -0.3 demonstrating a moderate size effect.

Furthermore the levels of IL-15 were significantly higher in the stage 4 group compared to the stage 1-3 (medians 5.8 pg/mg and 0 pg/mg respectively, p=0.032, r=-0.26 demonstrating a mild size effect.

5.5 Comparing immuno-modulatory cytokines between different anatomical subsites of HNSCC

5.5.1 Introduction

The site and subsite of origin of HNSCC are important factor in the determining prognosis (Zigon *at al* 2011). The complex anatomy of the head and neck region influences local progression, invasion, and metastasis of the primary tumour. The HNSCC cohort was divided into 5 groups of samples depending on their anatomical site of primary origin, and associated immune modulatory importance, and the levels of cytokines were compared.

5.5.2 Patients and Methods

The same cohort of patients were analysed by tissue subsite and cytokine concentration as detected by ELISA and described in the methods used as Chapter 2.

5.5.3 Results

Analysis of the groups revealed no statistical difference in the levels of IL-2, IL-10, IL-15, TGF-β and VEGF between the subsites of HNSCC and the normal controls. The data are displayed graphically in Figures 5.4 and 5.5. There was a difference found between the levels of IL-12 (Kruskal-Wallis test. Sig 0.025) and IL-17 (Sig. 0.043) between the groups.

Fig 5.4 Comparing median levels of TGF-β and VEGF across HNSCC subsites and normal tissue (pg/mg). No significant difference between the subsites.

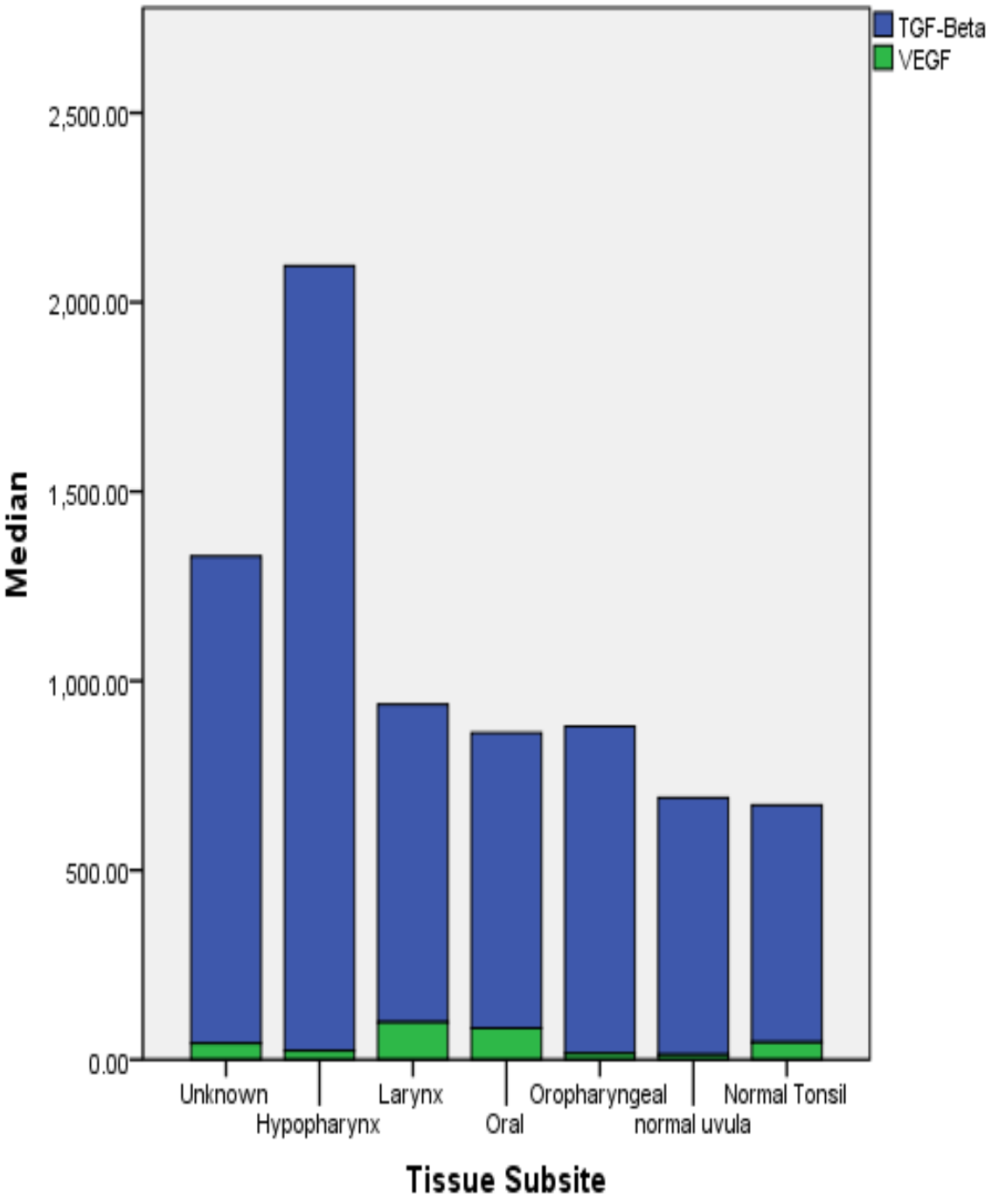


Fig 5.5 Comparing medians of IL-2, IL-10, IL-12, IL-15, and IL-17 across subsites and normal tissue (pg/mg)

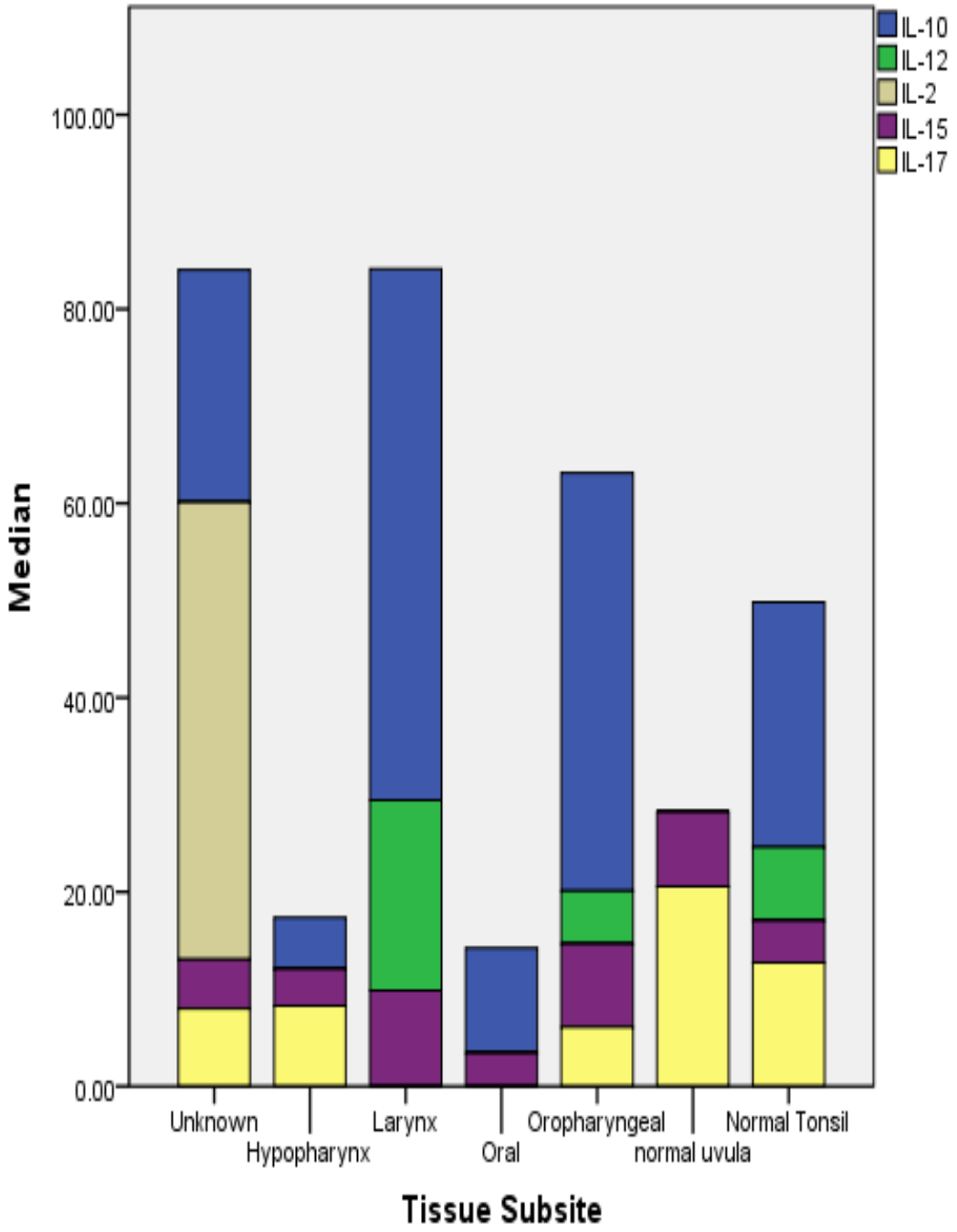


Fig 5.5 illustrates the reduced detected levels of IL-17 (Yellow bars) within the laryngeal and oral HNSCC tissue, and the reduced detected levels of IL-12 (Green bars) in the unknown primary site (therefore metastatic lymph node tissue), hypopharynx and oral subsites.

5.6 Discussion

This study has shown increased levels of TGF-β and VEGF in HNSCC tissue when compared with healthy control tissue. TGF-β is known to have a crucial role in immune modulation of many cellular proliferation mechanisms, however it is also essential in developing both nTreg and iTreg cells (Bergmann *et al* 2009). This difference could be indicative of an immuno-suppressive microenvironment of the HNSCC. The VEGF family of cytokines are an important element of the molecular network involved in neovascularisation and therefore oxygen and nutrient delivery to the tumour, and inducing hypoxia-driving factors in tumour cell growth(Montag *et al* 2009, Homer *et al* 2003). It also has roles in lymphangiogenesis and has been implicated in tumour cell metastasis although the exact role needs further study (Stacker *et al* 2001). The small cohort of patients with both primary tumour and nodal tissue did not show a significant difference in VEGF or the other immune cytokines studied. An increased level of VEGF in the nodal tissue cohort may have indicated increased metastatic potential of a tumour. However, the cohort was small, and analysis of larger numbers of paired samples may identify a statistically significant difference in cytokine levels. Comparing cytokine levels within samples of uninvolved lymph nodes draining the primary tumour could also be useful.

On comparison of T1/T2 and T3/T4 tumours, it was hypothesised that the more advanced the tumour, the more immuno-suppressed the tumour microenvironment, as per Sparano *et al* 2004, who analysed the plasma levels of these factors. The current study did not find a difference between any of the cytokines from the tissue samples between the T1/T2 and T3/T4 groups. This may have been due to the data skew towards greater numbers in the more advanced T3/T4 tumours and the relatively small sample size.

The T-staging of primary tumours is primarily based on anatomical spread, as well as function impairment due to the primary tumour, for example progressive invasion of a primary cancer within the larynx, causing vocal cord impairment, will upstage the disease. The overall staging of HNSCC takes into account the local nodal spread and distant metastases and has a greater bearing on overall prognosis (Carvalho *et al*, 2005). Metastatic disease confirms that primary tumours cells have gained the ability to invade mesenchymal and lymphovascular tissue. This may be indicative of more advanced tumour-induced methods of immune escape. This study confirmed significantly lower levels of IL-12 in the more advanced stage IV patients, which could be indicative of fewer immune cells in the microenvironment producing a Th-1-like tumouricidal response. IL-15 was also significantly higher in stage IV group and has been shown to be important in the development of iTregs (Bergmann *et al* 2007) in addition to the increased TGF-β concentration within advanced HNSCC tumours, this may be indicative of higher levels of iTregs within the microenvironment.

This aim of this study was to identify immune cytokines which can potentially suppress the immune response in HNSCC. Analysing tumour tissue lysate may be useful for identifying cytokine profiles within individual HNSCC tumours, but it does not identify the type of cells which may be producing them and further work will be needed in this area. Flow-cytometry has been used to identify subsites of TILs (Uppaluri *et al* 2008), and further molecular identification has been used in fixed immunohistochemistry to identify these cells further (Pretscher *et al* 2009). There has been demonstration of plasticity of the cellular immune-infiltrate as well as tumour HNSCC cells (Zhou *et al* 2009, Tong *et al* 2013), which may demonstrate that a fluid changes in cytokine levels and the cellular make-up of the tumour microenvironment could change outcome. Targeting these cytokine pathways to stabilise the immune infiltrate and encourage a more tumoricidal outcome.

Chapter 6 - Concluding Remarks – Immuno-regulation within the Head and neck tumour microenvironment.

This thesis has shown that ELISA can be used to determine levels of immune cytokines within HNSCC tumour tissue lysates and that there are statistically significant variations, with increased concentrations of TGF-β and VEGF, and reduced levels of IL-17 detected, between tumour tissue and normal control tissue. There was also statistically significant increased levels of IL-15 and reduced IL-12 in Stage IV patients when compared to the earlier stage I-III group.

The initial challenge within this thesis was to demonstrate a reproducible protein extraction method to allow analysis of cytokines by ELISA from HNSCC tissue samples. ELISA, western blotting and immunohistochemistry have been used to demonstrate cytokines within HNSCC and other cancers (Montag *et al* 2009, Strauss *et al* 2005, Chang *et al* 2005, Chen *et al* 1996). The use of western blotting to demonstrate the detection of cytokines within the initial Calbiochem (Merck, UK) protein extraction lysate could have been simplified by the use of immunoblotting, for example. The detection of cytokines via ELISA from tissue and fluids has rapidly advanced since this thesis has been written.

This thesis has demonstrated in the quantitative ELISA analysis of tumour tissue lysates a potential avenue to add to prognosis in newly diagnosed HNSCC patients. Further analysis of circulating T-helper cells, and correlation with serum cytokine levels and tumour lysate levels in the same patients could demonstrate more robust data. Multiple commercial ELISAs are now available to test for a wide range of individual cytokines from a single sample. Multiplex ELISA arrays can now analyse multiple human biomarkers simultaneously (Eg Luminex Screening Assay, B&D, Abingdon, UK). Wide ranges of immune cytokines can be detected this way. This would allow a much more

efficient analysis of both lysates from the smaller tumour biopsies and tumour-involved lymph-nodes. With these new techniques, the number of patients recruited to this study could have provided much improved data.

Previous research has identified serum and plasma levels of cytokines and also compared cell-cultures of HNSCC tumour cells from different subsets of HNSCC. These have been stratified according to tumour size and stage via the TNM staging protocol (Sparano *et al*, 2004, Jebreel *et al*, 2004). There has also been work on simulating the tumour microenvironment and monitoring immune cell response (Bergmann *et al* 2007).

This thesis examined excised tumour tissue samples, in addition to metastatic lymph node tissue to assess the levels of IL-2, IL-10, IL-12, IL-15, IL-17, TGF-β, and VEGF. By taking a 'cytokine snap-shot' of the tumour microenvironment prior to any previous treatment it was envisaged that a more advanced SCC of the head and neck may contain a milieu of cytokines which may be representative of a negatively-modulation of the immune system. This may allow a tumour profile to be made in conjunction with standard histopathological techniques to identify high risk patients on the initial tumor biopsy sample. This may help identify a cohort of patients with potentially more aggressive disease in which more aggressive treatments, such as induction chemotherapy prior to chemoradiotherapy may be required, and in addition potentially spare some patients with less aggressive tumours the extra toxicities of treatment.

Personalised cancer treatments depending on additional prognostic indicators are something to aspire to in the clinical management of HNSCC. It is already known that HNSCC patients with oropharyngeal cancer, whose tumours are positive for the HPV surrogate marker P16, are more responsive to chemoradiotherapy and have a better survival compared to P16-negative patients (Rautava *et al* 2012). However, it is also recognised that different assays assessing the HPV status of HNSCC tumours, such as

PCR or immunohistochemical analyses, and can lead to false positives which may affect further treatment regimens if current clinical trials prove beneficial in terms of reduced toxicity (Braakhuis *et al* 2009). For example De-escalate is comparing standard chemoradiotherapy with Cisplatin 100mg/m^2 with the investigative arm concurrent EGFR monoclonal antibody Cetuximab with the same dose of radiotherapy (70Gy in 35#) (Trial no. NCT01874171).

As previously described in the introduction (chapter 1) the relationship between the Th1 and Th2-like immune response and the relationship between Th-17 and Treg cells have both been described as reciprocally regulated (Yang, Nurieva, *et al* 2008, Whiteside *et al* 2007). The complete relationship between infiltrating immune cells, and their regulation is not completely understood but it will be a cornerstone in the future treatment strategies devised to treat HNSCC. Identification of truly immune-suppressive cells that allow cancer progression will give opportunities to develop therapeutic immunological targeted therapy, This would be comparison to the rather blunderbuss approach with ipilimumab and other anti-CTLA-4 molecules, in which the brakes are effectively removed from cytotoxic T cells, and patients are at risk of potentially life-threatening auto-immune like toxicities, such as hypophysitis and bowel perforation.

The significantly higher levels of TGF-Beta within the tumour tissue compared to control tissue could be indicative of an immune suppressant tumour microenvironment and favour development of FoxP3 Treg cells. The significantly higher levels of VEGF within the tumour samples when compared with the normal samples could also indicate increased neo-vascularisation in response to tumour hypoxia, as well as increased propensity to metastasise (Strauss *et al*, 2010). The increased concentration of IL-15, an important mediator in Treg cell propagation, was found in tumours of a higher overall stage.

Understanding the profile of immune factors within the tumour microenvironment is required if new immunotherapies will be able to fulfil their potential. An individual's immune competence, or dysfunction, is important in both the development and progression of cancer. Immune therapies, such as the monoclonal anti-CTLA4 inhibitor ipilimumab, are now in clinical use and have been shown to improve survival in progressive melanoma (Hodi *et al*, 2010). Targeted therapies, such as PD-1 ligand-1 inhibitors as well as direct anti-PD-1-recpetor inhibition have shown encouraging evidence of clinical efficacy in a number of cancers, such as the immune sensitive melanoma and ovarian cancer in phase-1 trials (Brahmer *et al* 2012, Topalian *et al* 2012). There have also been promising objective responses demostrated in the historically more resistant metastatic non-small-cell lung cancer. Further larger studies are being undertaken to assess clinical benefit however this is not by any means the complete treatment.

Other therapies such as dendritic cell vaccines in HNSCC are in development (Whiteside, 2007, Mellman *et al* 2011). However, the interactions of such a complex vaccine, utilising similar immune modulators to those produced the tumour itself, or induced by infiltrating cells, requires more detailed explanation. Sipuleucel-T (Provenge) is an example of clinical cell-based therapy currently in use (Kantoff *et al* 2010). A phase three trial involved leucopheresis at 0, 2 and 4 weeks, followed by cell-culture incubation of 36-44 hours, at 37ºC, with prostatic acid phosphatase (PAP) and the cytokine GM-CSF before re-infusion to the patient. The control arm incubated the patient's leucopheresis sample was incubated at 2-8ºC without the PAP. Thus the control arm was processed differently and without the presumptive immune activation. The trial results did not show significant tumour response or delay in progression, but did demonstrate a 4.1 month median survival improvement.

The tumour microenvironment of head and neck cancer is made up of a complex interaction between tumour cells, surrounding epithelial and mesenchymal cells, vascular epithelia, and both immune and inflammatory infiltrates. The effects of immune infiltrate and cytokine production by both these cells and interactions with the cells of the tumour microenvironment is becoming better understood. However the precise action of TGF-β and IL-17 producing Th-17 cells and their interaction with Treg cells within cancer, specifically HNSCC, is still unclear (Young, 2012).

The importance of cross-talk between infiltrating immune cells, stromal cells and cytokine production by tumour cells still needs to be accurately quantified. There are important questions to be answered, such as is there an element of deterministic factor or a threshold switch in the development pathway of effector T-cells? Cytokines have a pleiotropic effect, for example TGF-Beta has been shown to be necessary for both Treg production and Th-17 cell production, but are different concentrations of TGF-beta engage the plasticity switch and produce preferential Tregs from pleuri-potent T cells?

The understanding of the transcription factor cascades and intracellular messenger molecules involved in the plasticity of the human immune cell response will be important in clarifying how the HNSCC tumour micro-environment gains immuno-suppressive function, and may open avenues for treatment strategies to reverse this process.

The complex nature of the tumour microenvironment and the difficulty in replicating the large number of cellular and molecular interactions in HNSCC makes it difficult to identify potential treatment targets that will be effective. It is human nature to try and simplify pathways, and easy to make assumptions that inhibiting the pathway will result in favourable responses. These molecular pathways within the cell are better thought of as molecular systems, with multiple redundant routes, which could be an explanation for treatment resistant disease.

It will be essential to identify the mechanisms of immune response at the proteomic level, specifically the transcriptional control of the genome, in order to successfully identify further molecular therapeutic targets in order to switch the immune response to a more favourable setting to eradicate HNSCC.

I believe that further expansion within immunological interventions will be an important step in improving outcomes in the treatment of HNSCC in the future.

Appendix 1 – Clinico-pathological Data

Pt No.	Type	Age	Hist	Subsite	T	N	Stage	Sub-stage
11	T&N	54	Mod Diff	Hypopharynx	T3	N2b	4	A
13	T&N	62	Mod Diff	Oral	T3	N2b	4	A
14	T&N	61	Mod-poor	Oro	T2	N2b	4	A
32	T&N	68	Mod Diff	Oral	T1	N2b	4	A
33	T&N	84	Mod Diff	Larynx	T1	N2b	4	A
45	T&N	61	Mod Diff	Hypopharynx	T2	N2c	4	A
49	T&N	59	Poorly Diff	Oro	T1	N2b	4	A
56	T&N	47	Mod Diff	Larynx	T3	N2a	4	A
1	T	53	Mod Diff	Oral	T2	N0	2	
2	T	67	Mod Diff	Oral	T4	N1	4	A
3	T	68	Mod Diff	Oral	T4	N0	4	A
5	T	57	Early Invasive	Larynx	T1	N0	1	
6	T	48	Mod Diff	Oro	T1	N1	3	
7	T	62	Mod Diff	Oro	T4	N2b	4	A
8	T	52	Mod Diff	Larynx	T2	N2b	4	A
9	T	56	Mod Diff	Oral	T2	N1	3	
10	T	67	Mod Diff	Larynx	T4	N2b	4	A
12	T	56	Mod Diff	Larynx	T4	N0	4	A
17	T	58	Mod Diff	Larynx	T3	N2c	4	A
18	T	80	Poorly Diff	?	T2	N0	2	
19	T	77	Mod Diff	Larynx	T3	N2b	4	A
20	T	50	Mod Diff	Oral	T4	N0	4	A
21	T	54	Mod Diff	Oral	T2	N0	2	
23	T	58	Mod Diff	Oro	T1	N2a	4	A
28	T	74	Mod Diff	Larynx	T4a	N0	4	A
29	T	60	Mod Diff	Oral	T4a	N1	4	A
34	T	66	Mod Diff	Larynx	T1b	N0	1	
35	T	77	?	?	T2	N0	2	
37	T	78	Mod Diff	Larynx	T1	N0	1	
40	T	70	Mod Diff	Larynx	T4	N0	4	A
41	T	64	Mod Diff	Larynx	T1	N0	1	
42	T	70	Mod Diff	Oral	T2	N2b	4	A
43	T	69	Mod Diff	Larynx	T4a	N0	4	A
44	T	61	Mod Diff	Larynx	T2	N0	2	
46	T	57	Mod Diff	Larynx	T4	N1	4	A
47	T	51	Mod Diff	Larynx	T4	N0	4	A

51	T	72	Mod Diff	Larynx	T2	N0	2	
52	T	87	?	Larynx	T1	N0	1	
53	T	59	?	Larynx	T1	N0	1	
54	T	53	Basaloid	Oro	T2	N0	2	
55	T	72	Mod Diff	Larynx	T4a	N0	4	A
58	T	54	Mod Diff	Larynx	T3	N1	3	
60	T	62	Mod Diff	Larynx	T3	N0	3	0
Pt No.	**Type**	**Age**	**Hist**	**Subsite**	**T**	**N**	**Stage**	**Sub-stage**
61	T	37	Mod Diff	Larynx	T4a	N0	4	A
62	T	55	Mod Diff	Larynx	T4a	N0	4	
64	T	82	Mod Diff	Larynx	T3	N1	3	
65	T	64	Mod Diff	Larynx	T3	N2b	4	A
66	T	63	Mod Diff	Larynx	T1b	N0	1	
69	T	80	Poorly Diff	Larynx	T4	N1	4	A
36	N R+L	52	?	Oral	T4	N2c	4	A
4	N	86	Mod Diff	Oral	T4	N2b	4	A
15	N	68	Poorly Diff	Oro	T1	N2b	4	A
16	N	65	Mod Diff	Oro	T2	N2b	4	A
22	N	53	Poorly Diff	Larynx	T4	N2	4	A
24	N	48	Mod Diff	Oro	T1	N2a	4	A
25	N	52	Poorly Diff	Oro	T3	N2b	4	A
26	N	70	Mod Diff	Larynx	T1	N2b	4	A
27	N	51	Poorly Diff	Oro	T1	N2a	4	A
30	N	55	Poorly Diff	Oro	T2	N2a	4	A
31	N	65	Mod Diff	Oro	T2	N2b	4	A
38	N	58	Mod Diff	?	Tx	N2b	u	
39	N	68	Poorly Diff	?	Tx	N2b	u	
48	N	68	Poorly Diff	Oro	T2	N1	3	
50	N	79	Mod-poor	?	T1	N3	4	B
57	N	79	Mod Diff	Larynx	T3	N1	3	
59	N	55	Mod Diff	Oro	T2	N2b	4	A
63	N	81	Well-Mod	Hypopharynx	T2	N3	4	B
67	N	84	Anaplastic	?	T4b	N2	4	B
68	N	49	Mod Diff	Oro	T2	N2a	4	A

Appendix 2

UICC TNM Classification of Malignant Tumours 7th Edition. (International Union Against Cancer 2010, Blackwell publishing)

Lip and Oral Cavity

TNM Stage Grouping Lip and Oral Cavity			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1, T2, T3	N1	M0
Stage IVA	T4a	N0, N1	M0
	T1, T2, T3, T4a	N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

TNM Summary Lip and Oral Cavity	
T1	≤ 2cm
T2	>2-4cm
T3	>4cm
T4a	Oral Cavity: through cortical bone, deep intrinsic muscle of tongue, maxillary sinus, skin of face
T4b	Masticator space, pterygoid plates, skull base, ICA
N1	Ipsilateral single ≤ 3cm
N2	a) Ipsilateral single >3-6cm b) Ipsilateral multiple ≤ 6cm c) Bilateral, contralateral ≤ 6cm
N3	>6cm
M0	No distant metastasis
M1	Distant metastasis

Pharynx (Oropharynx and Hypopharynx)

TNM Stage Grouping Oropharynx and Hypopharynx			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1, T2, T3	N1	M0
Stage IVA	T1, T2, T3	N2	M0
	T4a	N0, N1, N2	M0
Stage IVB	T4b	Any N	M0
	Any T	N3	M0
Stage IVC	Any T	Any N	M1

Pharynx	
Oropharynx	
T1	≤ 2 cm
T2	> 2-4 cm
T3	> 4 cm
T4a	Invasion Larynx, deep/extrinsic muscle of tongue, medial pterygoid, hard palate, mandible.
T4b	Lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base, carotid artery
Hypopharynx	
T1	≤ 2cm and limited to one subsite
T2	> 2-4cm or more than one subsite
T3	> 4 cm or with hemi-larynx fixation
T4a	Thyroid/cricoid cartilage, hyoid bone, thyroid gland, oesophagus, central compartment soft tissue
T4b	Pre-vertebral fascia, carotid artery, mediastinal structures
Oropharynx and Hypopharynx	
N1	Ipsilateral single ≤ 3cm
N2	a) Ipsilateral single > 3-6cm b) Ipsilateral multiple ≤ 6cm c) Bilateral, contralateral ≤ 6cm
N3	> 6cm

Larynx

Larynx Stage Grouping			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1, T2 T3	N1 N0, N1	M0 M0
Stage IVa	T4a, T4b T1, T2, T3	N0, N1 N2	M0 M0
Stage IVb	T4b Any T	Any N N3	M0 M0
Stage IVc	Any T	Any N	M1

Larynx - Subgroups	
Supraglottis	
T1	One subsite, normal mobility
T2	Mucosa of more than one adjacent subsite of supraglottis or glottis, or adjacent region outside the supraglottis, without fixation
T3	Cord fixation or invades post-cricoid area, pre-epiglottic tissues, paraglottic space, thyroid cartilage erosion
T4a	Through the thyroid cartilage; trachea, soft tissues of the neck: deep/extrinsic muscles of tongue, strap muscles, thyroid, cartilage
T4b	Pre-vertebral space, mediastinal structures, carotid artery
Glottis	
T1	Limited to vocal cord(s)
a)	One cord
b)	Both cords
T2	Supraglottis, subglottis, impaired cord mobility
T3	Cord fixation, paraglottic space, thyroid cartilage erosion
T4a	Through the thyroid cartilage; trachea, soft tissues of the neck: deep/extrinsic muscles of tongue, strap muscles, thyroid, cartilage
T4b	Pre-vertebral space, mediastinal structures, carotid artery
Subglottis	
T1	Limited to subglottis
T2	Extends to vocal cord(s) with normal/impaired mobility
T3	Cord Fixation
T4a	Through cricoid or thyroid cartilage; trachea, soft tissues of the neck:

	deep/extrinsic muscles of tongue, strap muscles, thyroid, cartilage
T4b	Pre-vertebral space, mediastinal structures, carotid artery
All Sites	
N1	Ipsilateral single ≤ 3cm
N2	a) a) Ipsilateral single > 3-6cm b) Ipsilateral multiple ≤ 6cm c) Bilateral, contralateral ≤ 6cm
N3	> 6cm

Appendix 3 – Reagents Used

Methods to make required Solutions

PBS
(137 mM NaCl, 2.7 mM KCl, 10 mM Sodium Phosphate dibasic, 2mM Potassium Phosphate monobasic, pH 7.4)
For 1 litre of 1X Phosphate-buffered saline (1X PBS buffer): - Dissolve in 800ml of distilled H2O: 8.0g NaCl 0.2g KCl 1.44g Na2HPO4 0.24g KH2PO4
- Adjust the pH to 7.4 with HCl or NaOH
- Add H2O to 1 litre.

Tris-Buffered Saline Tween 0.05%
(20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4)
For 1 litre of 1X Tris-buffered saline with Tween-20 (1X TBST buffer) -Dissolve in 800ml of distilled H2O: 8g NaCl 0.2g KCl 1.2g Tris base

-Add 500ul Tween-20
-Adjust the pH to 7.4 with HCl
-Add distilled H2O to 1L

Tris-Buffered Saline (TBS)
(20 mM Tris, 150 mM NaCl, pH 7.4)
For 1 litre of 1X Tris-buffered saline (1X TBS buffer) -Dissolve in 800ml of distilled H2O: 8g NaCl 0.2g KCL 1.2g Tris base
-Adjust pH to 7.4 with HCl
-Add H2O to 1L.

0.05% Tween
500µl Tween detergent up to 1L PBS

Appendix 4

Patient Consent forms and Information Sheets

PATIENT INFORMATION SHEET

Measurement of intra-tumoural cytokines and systemic T-helper cells in Head and Neck Squamous Carcinoma

1. Introduction:

We invite you to take part in a research study which is designed to develop possible new treatments for head and neck cancer. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

*The Academic Surgical Unit of the University Of Hull is involved in many cancer research projects we rely on patients' good will to make **blood and tumour** samples available to us so we can continue our work.*

Take time to decide whether or not you wish to take part.

Thank you for reading this.

2. What is the purpose of the study?

*Many cancers cause changes in the **immune system.** Some cells and chemicals are made more in response to tumours, some are not. We aim to investigate the levels of certain cells called **Regulatory T-cells** and immune system chemicals called **cytokines**. Regulatory T-cells have been investigated recently for their role in fighting tumour cells in Head and Neck cancer patients. Different **cytokines** have been associated with **fighting tumours** and others with **stopping the body fighting tumours** in studies on other cancers. The relationships have not been investigated in Head and neck cancer specimens. Better understanding of this relationship would potentially lead to the development of new treatments*

This study is conducted jointly by the Department of Head and Neck Surgery and Academic Surgical Unit, University Of Hull. The **blood and tumour** specimens will be stored at the Medical Research Laboratory at the University Of Hull, which is also where the laboratory work with the specimens will take place.

3. Why have I been chosen?

The patients who will participate in this study are patients who have been scheduled to undergo **surgery or radiotherapy** to treat their head and neck cancer. Also included in this study are healthy volunteers whose results will be compared to the cancer group.

4. Do I have to take part?

Taking part in this study is entirely voluntary, and it is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive, and will not alter the plan of treatment already scheduled (i.e. you will still have your operation or radiotherapy to treat your head and neck cancer).

5. What will happen to me if I take part?

If you agree to take part in the study, then excess tumour tissue removed at operation will be sent to the laboratory for testing and destroyed afterwards. We will also take blood samples from you **before and after any kind of treatment i.e. surgery and/or radiotherapy.** This will involve a venous puncture on the arm or back of hand.

6. What do I have to do?

Apart from reading this information sheet, signing the consent form and having your blood taken **before and after each treatment**, you need not do anything else. Your treatment will take place as scheduled (whether or not you agree to participate in this study).

7. What are the possible benefits of taking part?

This study is one of the first steps towards our better understanding of a complex cancer. However, it is hoped that the results of this study will inspire and lead to further research by researchers, both nationally and internationally, to develop treatments which will eventually benefit patients with head and neck cancer.

8. What are the possible risks of taking part

Apart from the minor risks associated with having your blood taken, which include bruising and transient pain at the needle entry site, there are no significant risks associated with taking part in this study. Please note that your blood will need to be taken in any case for routine tests before you undergo an operation, irrespective of whether you participate in this study or not.

9. Will information in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any of your medical records may be inspected by responsible and authorised individuals officially engaged in the Head and Neck research from the Department of Otolaryngology and Head and Neck Surgery and Academic Surgical Unit, University Of Hull, for purposes of analysing the results. However, any information about you which leaves the hospital WILL NOT have your name and address so that you cannot be recognised from it.

10. What will happen to the results of the research study?

It is hoped that the results of the study will be published in peer reviewed scientific/medical journals once the results have been analysed and collated at the end of the study period. However, you will not be identified in any publication.

11. Who is organising and funding the research?

The research is organised and funded by the Academic Surgical Unit, University Of Hull. The researchers in this study conduct research on a full-time basis and are paid a fixed salary which is independent of whether you participate in the study or not.

12. Who has reviewed the study?

The study has been reviewed and approved by the South Humber Local Research Ethic Committee and the Hull and East Yorkshire Hospitals NHS Trust Research and Development Consortium.

13. What if I have other concerns?

If you have any problems, concerns or other questions about this study, you should preferably contact us as follows:

Prof N Stafford	(Lead Researcher)	01482 464213
Dr J Greenman	(Research Supervisor)	01482 466032
Mr P F Murray	(Researcher)	Ward 11, Hull Royal Infirmary
Miss S O'Donnell	(Researcher)	Ward 11, Hull Royal Infirmary
Mr A Prasai	(Researcher)	Ward 11, Hull Royal Infirmary

If you have any complaints about the way the investigator has carried out the study, you may contact the hospital's complaints department on 01482 328541.

If you decide to take part, please ensure you keep a copy of this information letter and signed consent form(s).

Thank you very much for considering taking part in our research
Please discuss this information with your family, friends, or GP if you wish.

Affiliated to the University of Hull

Patient Identification Number:

PATIENT CONSENT FORM

Title of Project; **Measurement of intra-tumoural cytokines and systemic T-helper cells in Head and Neck squamous cell carcinoma.**

Name of Researcher: Prof N Stafford FRCS, Dr J Greenman PhD,
Mr P Murray MRCS, Miss S O'Donnell MRCS, Mr A Prasai MRCS.
Department of Otolaryngology and Head and Neck Surgery
Medical Research Laboratory, University of Hull

If you agree with each sentence below, please INITIAL the box:

1. I confirm that I have read and understand the information sheet *dated December 2006* for the above study and have had the opportunity to ask questions. ☐

2. I agree to give a sample of blood for research in this project. I consent to a specimen of tumour being analysed in the laboratory exclusively for the purpose of this project.
I understand how the sample will be collected, that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I know how to contact the research team if I need to, and how to get information about the results of the research. ☐

4. I understand that sections of any of my medical notes may be looked at by responsible individuals from the Department of Head and Neck Surgery and Otolaryngology, Hull Royal Infirmary, University of Hull and Department of Histopathology, Castle Hill Hospital where it is relevant to my taking part in research. I give permission for these individuals to have access to my record. ☐

5. I understand that I will not benefit if this research leads to the development of a new treatment or medical test but it is hoped that the results will be used to benefit future patients ☐

6. I agree to take part in the above ethics committee approved study. ☐

_____ _____ _____

Name of Patient (Block letters) Date Signature

_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature

_____	_____	_____
Researcher	Date	Signature

1 copy for patient; 1 for researcher; 1 to be kept with hospital notes

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