THE UNIVERSITY OF HULL

Regulatory T cells and circulating cytokines; novel prognostic markers in squamous cells carcinoma of the Head & Neck

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by

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide and is considered a highly immunosuppressive tumour. Advances in the treatment modalities for HNSCC over the last 20 years involving surgery, radiotherapy, chemotherapy and immunotherapy are not fully reflected in increases in the 5 year survival rates, mainly due to locoregional recurrences and to a lesser extent, distant metastasis.

The role of anti-tumour immunity in relation to HNSCC has been intensely studied in the past few years. It is now becoming clear that the complex interaction between HNSCC and immune cells plays an important part in determining tumour growth and progression.

HNSCC patients were found to have an imbalance in Th1/Th2-type cytokines and elevated levels of $CD4+CD25^{high}$ Regulatory T cells (Treg). In this thesis, the circulating levels of serum IL10, IL12 and Treg cell levels in PBMC were determined and their association with clinical outcome in HNSCC patients were investigated.

Serum cytokine levels were determined by ELISA in patients pre-treatment (n=107), 4-6 weeks post-treatment (n=43) and in a cohort of non-tumour controls (n=40). Treg cell levels were determined by flow cytometry in these groups.

IL10 detectability was significantly higher in patients than controls. Pre-treatment IL10 levels in all anatomical sub sites, except oral cavity, were significantly elevated in stages III/IV, N+ patients and in T3/4 tumours. The detectability of IL10 significantly correlated with poorer survival after a maximum follow-up of 36 months. Treg cell levels were significantly higher pre-treatment in patients vs. controls, which did not change significantly 4-6 weeks post-treatment and did not correlate with any clinical parameters.

In conclusion, serum IL10 was found to be an independent factor in predicting a poor clinical outcome in newly-presenting tumours of laryngeal and pharyngeal origin. The

role of circulating Treg cells as predictors of clinical outcome requires further investigation. Future work to correlate findings regarding IL10, IL12 and Treg cells in the peripheral circulation of HNSCC patients with their levels and function in tumour tissue and TIL is recommended.

Abbreviation list

APC	Antigen Presenting Cells
BSA	Bovine Serum Antigen
CRT	Chemoradiotherapy
CSC	Cigarette Smoke Condensate
CT	Computed Tomography
CTLA-4	Cytolytic T lymphocyte associated antigen 4
DC	Dendritic cells
EBNA1	Epstein-Barr Nuclear Antigen 1
EBV	Epstein-Barr virus
EGFR	Epidermal Growth Factor Receptor
EGFR	Epidermal Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
FOXP3	Forkheadbox Transcription Factor P3
GITR	Glucocorticoid-Induced Tumour-Necrosis Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GVHD	Graft Versus Host Disease
HER	Human Epidermal growth factor Receptor
hiFCS	Heat Inactivated Foetal Calf Serum
HLA	Human Leukocyte Antigen
HNSCC	Head and neck squamous cell carcinoma
HPV	Human Papilloma virus
HRP	Horse Radish-Peroxidase
IARC	International Agency for Research in Cancer
IFN-γ	InterFeron gamma
IL10	Interleukin 10
IMRT	Intensity-Modulated Radiotherapy
LMP	Latent Membrane Protein
LPR	Laryngopharyngeal reflux

mAb	Monoclonal Antibody
MALDI	Matrix-Assisted Laser Desorption and Ionization
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
MS	Mass Spectroscopy
NK cells	Natural Killer cells
NPC	Nasopharyngeal Carcinoma
OR	Odds Ratio
PAH	Polynuclear Aromatic Hydrocarbons
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PET	Positron Emission Tomography
PGE2	Prostaglandin E2
pRB	Retinoblastoma Protein
RIC	Radiation-Induced Cancers
RR	Relative Risk
SEER	Surveillance Epidemiology and End Result
SELDI	Surface Enhanced Laser Desorption and Ionization
SLNB	Sentinel Lymph Node Biopsy
TAA	Tumour-Associated Antigens
Tc cells	T cytotoxic cells
TDLN	Tumour-Draining Lymph Nodes
TGF-β	Transforming Growth Factor Beta
Th cells	T helper cells
TNF-α	Tumour Necrosis Factor Alpha
TSA	Tumour-Specific Antigens
UK	United Kingdom
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation

Thesis associated Publications and Presentations

Publications

1. Regulatory T-Cells: What role do they play in the depressed antitumour immunity observed in patients with Head and Neck cancer?

O. Alhamarneh, S. Amarnath, N.D. Stafford, J. Greenman

Head & Neck, 2008 Feb 30(2):251-261

2. Head and neck tumour immunology: basic concepts and new clinical implications

KP Topping, LM Fletcher, FO Agada, O Alhamarneh, ND Stafford, J Greenman

J Laryngol Otol. 2009 Jan; 123(1):9-18

3. Immunotherapy in head and neck cancer: current practice and future possibilities

F.O. Agada, O. Alhamarneh, N.D.Stafford, J. Greenman

J Laryngol Otol. 2009 Jan; 123(1):19-28

4. Serum IL10, but not circulating $CD4^{+}CD25^{high}$ regulatory T cell numbers, predicts clinical outcome and survival in head and neck squamous cell carcinoma patients

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Presentations

1. Interleukin 10, an independent predictor of clinical outcome and survival in HNSCC patients"

Oral presentation at The American Association of Otolaryngologists Head and Neck Surgeons Meeting, Chicago, USA, September 2008

Oral presentation at Joint RSM/ENTUK Annual Meeting, London, September 2007

2. Pre&Post Treatment Levels of IL10&IL12 in HNSCC Patients"

Poster presentation at The American Association of Otolaryngologists Head and Neck Surgeons Meeting, Washington, USA, September 2007

3. IL-10, but not regulatory T cells, correlates with clinical outcome in head and Neck Cancer"

Oral and Poster presentation at the Society of Academic Research in Surgery SARS, Cambridge, UK, January 2007

4. A comparison of pre- and post-operative levels of IL-10 and IL-12 in patients with head and Neck Cancer"

Oral and Poster presentation at the Society of Academic Research in Surgery SARS, Cambridge, UK, January 2007
Poster presentation at The North of England Otolaryngology Society Meeting, Newcastle, March 2007

5. Correlation between regulatory T cells and serum levels of IL10 and IL12 with disease outcome in HNSCC patients"

Oral presentation at The North of England Otolaryngology Society Autumn Meeting, Manchester, September 2006
Poster presentation at the Society of Surgical Oncology Meeting SSO, Washington, USA, March 2007

Contents

1. Introduction - Head and Neck cancer

1.1 Overview

Head and Neck cancer is a general term used to describe a heterogeneous group of tumours which develop in the upper aerodigestive epithelium including the mouth (oral cavity), throat (pharynx), voice box (larynx), salivary glands and the nose and paranasal sinuses (Argiris et al., 2008). When all the subtypes are grouped together, head and neck cancer is the sixth most common cancer worldwide (Parkin et al., 2005).

Greater than 90% of malignant tumours arising within the head and neck region are squamous cell carcinomas. Tumours arising in this area from other histological types are less common and include lymphomas, adenocarcinomas, sarcomas and a collection of extremely rare tumours arising mainly from minor salivary glands (Daley & Darling, 2003).

The risk of developing Head and Neck Squamous Cell Carcinoma (HNSCC) is greatly increased by tobacco smoking and alcohol consumption, an effect which is multiplicative (Hashibe et al., 2009). Other associations include viruses such as the human papillomavirus (HPV) and the Epstein-Barr virus (EBV), genetic and familial causes, diet, occupational carcinogens and extra-oesophageal reflux.

Treatment of HNSCC often requires multiple modalities. The complex anatomy and vital physiological role of the tumour-involved structures dictates that the goal of treatment is not only to improve survival outcomes but also to preserve organ function. Advances in the treatment modalities over the last 20 years involving surgery, radiotherapy and chemotherapy have not been fully reflected in increases in the 5-year survival rates, mainly due to locoregional recurrences and to a lesser extent, distant metastasis (Young, 2006). Recent advances in incorporating systemic agents in curative therapies i.e. the epidermal growth factor receptor (EGFR) inhibitors, have shown promising results with

improved locoregional control and overall survival in a multinational randomised phase III clinical trial (Bonner et al., 2006).

Further clarification of the molecular events in HNSCC is expected to yield novel pathways of intervention and prognostic biomarkers. Recent advances in cancer immunology facilitate better understanding of the mechanisms tumours use to implement their effect and also of the relationship between the tumour and the host immune cells. This knowledge is likely to be critically vital for the personalised selection of the available modalities of treatment and ultimately for improving clinical outcome.

1.2 Epidemiology

Head and Neck cancer is the sixth most common cancer worldwide, representing an estimated 650,000 new cases and 350,000 cancer related deaths every year (Parkin et al., 2005). In the United Kingdom, the latest figures for which data are available showed that approximately 7,120 new cases of head and neck cancer and 2,500 cancer related deaths were registered in the year 2005; accounting for nearly 2.3% of all cancers diagnosed annually. In Yorkshire and the Humber area, 808 new cases were registered out of a total of 36,291 cancers diagnosed (2.2%) (www.statistics.gov.uk).

Oral cancer includes tumours of the lip, mouth, tongue, oropharynx, piriform fossa, hypopharynx, but excludes nasopharyngeal cancers (NPC) which are dealt with as a separate clinicopathological entity, due to their unique histology and strong association with Epstein-Barr virus infection (Wei & Sham, 2005). Two thirds of oral cancer cases occurr in developing countries and thus is thought to be closely linked to tobacco consumption (Parkin et al., 2005). In high-risk countries such as Sri Lanka, India, Pakistan and Bangladesh, oral cancer is the most common cancer in men and accounts for up to 30% of all new cases of cancer compared with 3% in the UK or 6% in France. In the UK, the incidence is also rising. From 1995 to 2004 the number of new diagnoses of oral cancer rose from 3,696 to 4,769 (six cases in every 100000 people in the UK population), an increase in age standardised incidence of 23% (www.statistics.gov.uk).

This increase in oral cancer incidence reflects a trend in the developed countries over the past decade and has been attributed to an increase in prevalence of HPV infection (Shiboski et al., 2005).

Laryngeal cancer, in contrast, has shown a slight decrease in incidence with over 2,150 new cases and 800 deaths each year in the UK. Rates over the past 20 years are steadily falling. From 1993 to 2004, the age standardised incidence rates for laryngeal cancer in men in the UK have fallen from 7 to 5 cases per 100,000 population. (www.statistics.gov.uk).

Laryngeal cancer is predominantly more common in men with a male to female ratio of 4.8:1 in the UK. This ratio is similar to tumours found in the piriform fossa, but for the rest of oral cancers the male to female ratio is 2:1. The sex ratio of oral cancers however was almost 5:1 fifty years ago (www.cancerresearchuk.org), and the change is thought to be due to alteration in smoking habits, with the vast increase in numbers of females taking up the habit (Gallus et al., 2003).

The overall 5-year survival when all stages of HNSCC are combined on the basis of Surveillance Epidemiology and End Result (SEER) data are about 60%; survival is worse for specific primary sites such as the hypopharynx while it is better for lip tumours (Argiris et al., 2008).

1.3 Aetiology

Smoking and alcohol are by far the most common risk factors for developing HNSCC and are implicated in 75% of all HNSCC with a multiplicative combined effect (Pelucchi et al., 2008). Following is a list of risk factors associated with HNSCC development, each of these will be discussed in turn.

- Tobacco: smoking, chewing

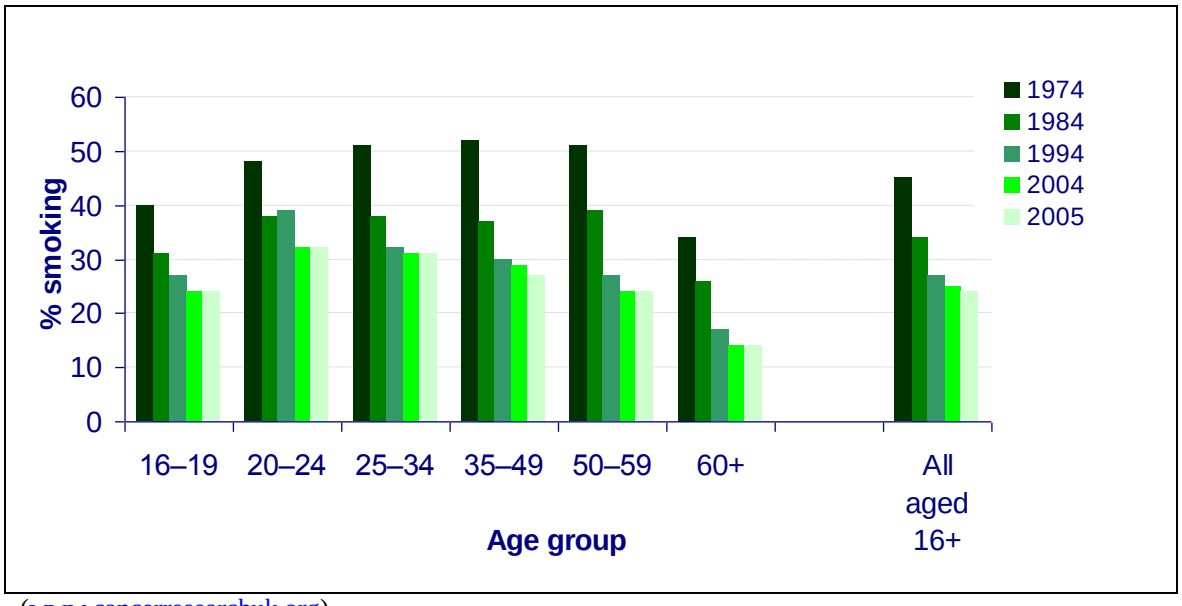
- Alcohol consumption: ethanol
- Viruses: Human papilloma virus (HPV), Epstein-Barr Virus (EBV)
- Genetic and familial
- Diet and socioeconomic background
- Occupational: chemicals, radiation
- Extra-oesophageal reflux (Laryngopharyngeal reflux)

1.3.1 Tobacco

Different forms of tobacco consumption have been linked with the development of Head and Neck Cancer for over 50 years (Mills & Porter, 1950). Since this early study, a wealth of evidence has accumulated relating tobacco smoking or chewing to different types of cancers; accounting for some 30% of cancer deaths in developed countries, that is 46,000 deaths in the UK in 2005 (www.cancerresearchuk.org).

Both cigarette smoking and smoking other forms of tobacco, including bidi, pipe, and cigars, can cause cancers in multiple organs (Vineis et al., 2004). Smoking cigarettes is the commonest form of tobacco consumption or "addiction" in the developed world. In the UK, the prevalence of smoking in adults has been steadily declining since the mid seventies (Figure 1). However, the latest figures for 2005 still show that around a quarter of the British population aged 16 and over smoke cigarettes, equating to approximately 11 million people. A further one million people smoke pipes/cigars (www.cancerresearchuk.org).

Figure 1: Prevalence of cigarette smoking by age, persons aged 16 and over in Great Britain, 1974-2005



(www.cancerresearchuk.org)

"Tar" is the term used to describe the toxic chemicals found in cigarettes. It contains more than 69 chemicals which behave as a complex mixture of interacting cancer initiators, promoters, and co-carcinogens. Cigarette smoke condensate (CSC) has been shown to be mutagenic and genotoxic (Zhang et al., 2007). CSC works through DNA strand breaks, oxidative damage, microsatellite instability, chromosomal aberrations and the formation of micronuclei. Tumours of smokers contain a high frequency and unique spectrum of gene mutations, reflective of the Polynuclear Aromatic Hydrocarbons and possibly other compounds in the smoke (DeMarini, 2004).

Lower tar cigarettes, i.e. less than 22mg tar content, and filters to trap tar were introduced to reduce the consumption. In a case control study of 1,034 patients, the risk of developing lung and laryngeal cancer was consistently lower among long-term smokers of filter cigarettes than among smokers of non-filter high-tar cigarettes, irrespective of the quantity smoked (Wynder & Stellman, 1979). Kabat and colleagues, in a large study of more than 1,500 patients, also demonstrated that the risk of developing oral and pharyngeal cancer was nearly 50% less in smokers with filtered cigarettes compared with non-filtered cigarettes (Kabat et al., 1994). Another large case control study of 741 patients with HNSCC from a variety of different sites and 1,272 controls reported a significantly increased risk with smoking cigarettes of tar content above 22mg compared with cigarettes with tar content less than 22mg with doubling of the odds ratios for developing oral, pharyngeal and laryngeal tumours (La Vecchia et al., 1990). Although less tar and the use of filters reduced the overall risk of developing SCCHN, they have not succeeded in substantially eliminating the adverse effects of tobacco on smokers. On the contrary, few reports have shown the elusiveness of such measures (Pollay & Dewhirst, 2002) and have debated the motives behind supporting them, their marketing and indeed their effectiveness in tackling the adverse effects of smoking.

The risk of developing laryngeal and hypopharyngeal cancer in smokers compared with non smokers was found to be increased by a factor of 10 in a well-designed, large, multicentre, case controlled study involving 1,147 patients and 3,057 controls (Tuyns et al., 1988). In a more recent large-scale case-controlled study, the odds ratios between

smokers/ never smokers and smokers/ ex-smokers to develop SCC of the larynx was found to be 19.8, and 7.0 respectively (Talamini et al., 2002). The latter study included 527 patients with laryngeal SCC and 1,297 age and sex matched controls. This study also showed that the risk increased in relation to the number of cigarettes smoked and for how long patients have been smoking them for, with odds ratios of 42.9 for $\geq$ 25 cigarettes/day and 37.2 for $\geq$ 40 years, respectively.

Heavy smokers have also been shown to have a 5 to 25-fold higher risk of developing HNSCC than non-smokers with the risk rising as high as 40-fold when combined with heavy drinking (Goldenberg et al., 2004). A recent publication by the International agency for research in cancer (IARC) investigated the independent association between HNSCC and cigarette smoking among "never drinkers" by performing a meta-analysis from 15 case–control studies that included 10,244 head and neck cancer cases and 15,227 controls. The results showed that among never drinkers, cigarette smoking was significantly associated with an increased risk of head and neck cancer (Odds Ratio: OR for Ever versus Never smoking = 2.13, 95% CI = 1.52 to 2.98), and there were clear dose – response relationships for the frequency, duration, and number of pack-years of cigarette smoking. Among non-drinkers in this meta-analysis, approximately 24% (95% CI = 16% to 31%) of head and neck cancer cases would have been prevented if these individuals had not smoked cigarettes (Hashibe et al., 2007b). The risk in this study was stronger for laryngeal cancer than for oral cavity and pharyngeal cancers, which is consistent with a previous estimate from a meta-analysis of over 35 studies which showed the larynx to have the highest average relative risk (RR= 10.0) compared with oral cavity and pharynx (RR= 4.0-5.0) of developing HNSCC in smokers (Vineis et al., 2004). These results suggest that the larynx is most susceptible site in the head and neck to the effects of cigarette smoking. However, the mechanism for this increased susceptibility is unclear.

The results from the above mentioned large scale, well-designed studies as well as a wealth of other studies over the past 40 years, confirm that cigarette smoking is a risk factor in Head and neck cancer with the highest risk being in laryngeal cancer. Although

longer duration and a higher number of cigarettes smoked were found to be directly associated with HNSCC, cessation of smoking was associated with a sharply reduced risk of this cancer, with no excess risk detected among those having quit for 10 or more years, suggesting that smoking affects primarily a late stage in the process of tumour carcinogenesis (Blot et al., 1988), (Vineis et al., 2004).

1.3.2 Alcohol

The risk of developing HNSCC from alcohol, whether alone or in conjunction with smoking has been shown to be related to almost every kind of the beverage and most crucially to the amount consumed. Among "never" users of tobacco, alcohol consumption was associated with an increased risk of HNSCC only when alcohol was consumed at high frequency i.e. three or more drinks per day (OR for three or more drinks per day versus never drinking = 2.04, 95% CI = 1.29 to 3.21). In this meta-analysis of data from pooled case-control studies (n=15), the association with high-frequency alcohol intake was limited to cancers of the oropharynx, hypopharynx and larynx (Hashibe et al., 2007b). The finding of an increased cancer risk only at high-frequency alcohol intake in "never" smokers is consistent with results from two other earlier large studies (Ng et al., 1993), (Schlecht et al., 1999).

The risk of developing HNSCC in alcohol consumers increases with concomitant tobacco smoking, each agent approximately multiplying the effect of the other (Hashibe et al., 2007a). In a large case-control study of 527 patients with laryngeal cancer and 1,297 controls, the combined alcohol and tobacco consumption showed a multiplicative effect (OR=177) with the supraglottic region showing the highest risk for current smokers and drinkers (OR 54.9 and 2.6, respectively, (Talamini et al., 2002)). A more recent meta-analysis of 20 studies conducted in North America, Europe, Japan and Korea showed that the multivariate relative risks for the highest levels of alcohol consumption in patients with laryngeal cancer were about 2 for 50 g (approximately 4 drinks)/day and about 4 for 100 g/day compared with "non-drinkers", in the absence of evidence of a threshold. This study also demonstrated that the risk increased with concomitant tobacco smoking, each agent approximately multiplying the effect of the other, but in the absence of smoking the risks are small for moderate alcohol consumption (La Vecchia et al., 2008). Other conclusions from this large meta-analysis showed that the role of age at starting and stopping drinking in relation to cancer risk is still unclear, and that the most commonly used alcoholic beverage appears to be the most associated with cancer risk, suggesting that no meaningful difference exists for different types of alcoholic beverages. Out of all

the anatomical sub-types in the head and neck, the pharynx seems to be the site most affected with alcohol consumption. A meta-analysis by Zeka and colleagues, including 14 studies estimating the effects of alcohol consumption on the risk of HNSCC, confirmed the multiplicative effect of tobacco and alcohol on the relative risk (RR) scale, and showed that alcohol consumption appeared to have a much stronger effect on the pharynx than on any of the other head and neck sites (Zeka et al., 2003).

Although the exact mechanisms by which chronic alcohol ingestion stimulates carcinogenesis are not known, evidence has accumulated that the substance acetaldehyde, a metabolite of ethanol, is predominantly responsible for the carcinogenic effect (Poschl & Seitz, 2004). Acetaldehyde is both carcinogenic and mutagenic, binds to both DNA and proteins, destroys the folate molecule and results in secondary cellular hyper regeneration. Other mechanisms by which alcohol induces cancer include the induction of cytochrome P-4502E1 which leads to free radicals production, procarcinogen activation and nutritional deficiencies such as riboflavin, zinc, folate and retinoic acid which hamper anti-tumour immune responses. Local mechanisms are believed to be involved in cancer induction such as tissue injury, through direct contact or solvent action or even perhaps by enhancing the effects of tobacco or other environmental carcinogens. All these factors are believed to be, in some part at least, actively modulating alcohol-related carcinogenesis (Poschl & Seitz, 2004) .

It is believed that 25-68% of the upper aerodigestive tract cancers, including oral cavity, pharynx, larynx and upper oesophagus, are influenced due to alcohol drinking and around 80% of such tumours can potentially be prevented by abstaining from alcohol and smoking (La Vecchia et al., 1997). However, even with rigorous advertising campaigns this message does not appear to be reaching people, although initial results after the smoking ban took place in Great Britain in public places, pubs and bars seem to be encouraging with a yet, unquantified, reduction in the number of smokers and the number of cigarettes smoked by current smokers (www.scotland.gov.uk).

1.3.3 Viruses

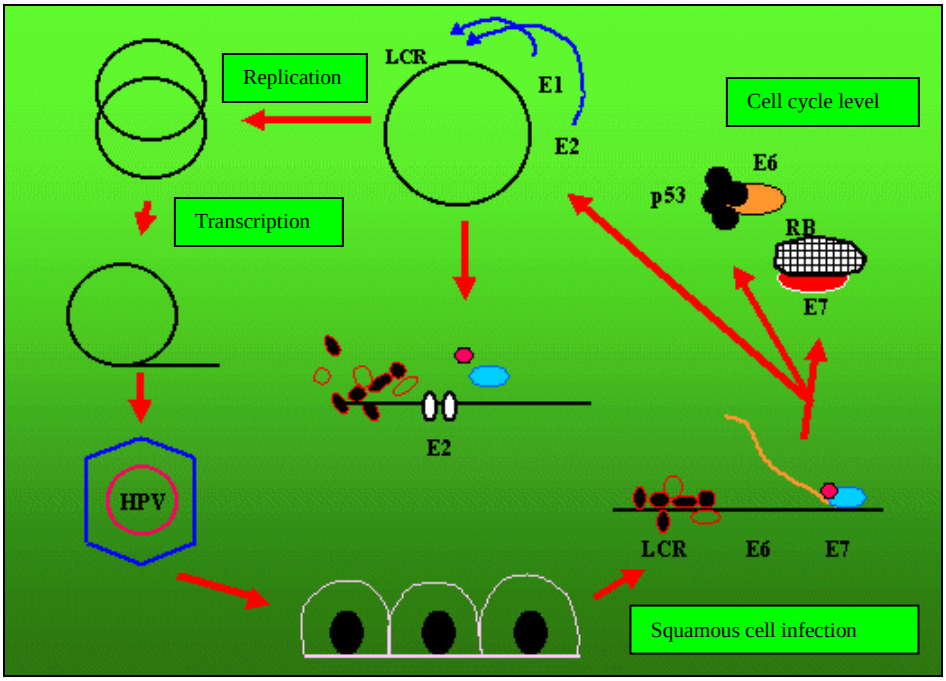
Two groups of viruses have long been associated with HNSCC; Human papilloma virus (HPV) and Epstein-Barr virus (EBV). HPV have been recently found to work not only synergistically with other co-factors, but also as an independent agent in the aetiology of certain HNSCC subsets (Hennessey et al., 2009). Oral HPV infection is strongly associated with oropharyngeal cancer among subjects with or without the established risk factors of tobacco and alcohol use and is regarded as a separate epidemiological, clinical and molecular entity (Smith et al., 2004). In a systematic review and meta-analysis, the association between HPV16 and cancer was strongest for tonsil (OR: 15.1, 95% CI: 6.8-33.7), intermediate for oropharynx (OR: 4.3, 95% CI: 2.1-8.9) and weakest for oral (OR: 2.0, 95% CI: 1.2-3.4) and larynx (OR: 2.0, 95% CI: 1.0-4.2) (Hobbs et al., 2006). Another meta-analysis of epidemiological studies and multi-centre case-control studies, confirmed HPV type16 and to a lesser extent type18, as independent risk factors for oral cancer (OR between 3.7 and 5.4) again with the highest prevalence in tonsil tumours (Syrjanen, 2005). With in-situ hybridisation, HPV16 genomic DNA can be detected in up to 72% of oropharyngeal tumours and interestingly patients with such tumours, regardless of their tobacco and alcohol use, seem to have a better overall- and disease-specific survival, as compared with the HPV-negative group (D'Souza et al., 2007), (Gillison et al., 2008). HPV 16 seropositivity has also been stongly associated with increased risk of HNSCC in a case control study of 486 patients and 548 controls. This association was also independent of heavy smoking or drinking (Furniss et al., 2008).

A number of oncogenic HPV types, i.e. 16 & 18 are considered “high-risk” in HNSCC development due to the prevelance of their carcinogenic effect through E6 and E7 viral oncoproteins. E6 inactivates P53, which regulates cell growth as well as tumour growth suppression. The P53 protein suppresses cell growth by transcriptionally activating p21, which inhibits the cell-cycle kinases critical for G1 progression. E7, on the other hand, inhibits the retinoblastoma tumour suppressor protein pRB which can inhibit cell-cycle progression through its regulation of members of the E2F family of transcription factors

that are able to induce DNA synthesis and cellular proliferation (Munger & Howley, 2002), (Figure 2).

The association between HNSCC and HPV has potential implications in the management of these tumours. Due to the favourable prognosis of HPV-positive tumours and better responsiveness to standard treatments, such tumours might be more amenable to immune surveillance of tumour-specific antigens than HPV-negative tumours and may well benefit from future vaccination protocols to prevent HNSCC from occurring (Argiris et al., 2008).

Figure 2: The mechanism of action of HPV through E6 and E7 viral oncoproteins



When the HPV DNA is released within the nucleus, multiple cellular transcription factors interact with the non-coding viral regulatory region (LCR) starting transcription of the two HPV16 transforming early genes (E6 and E7). After translation, the transforming proteins interact with the cellular antioncogenic regulators P53 and RB thus disrupting the cell cycle and inhibiting growth arrest. At the same time, the viral genes E1 and E2 are expressed, promoting viral DNA replication and transcription. Adapted from (http://www.cinvestav.mx)

On the other hand, EBV is almost exclusively associated with nasopharyngeal carcinoma (NPC) (Burgos, 2005). EBV-encoded RNA signal is present in all NPC cells, and early diagnosis of the disease is possible through the detection of raised antibody titres against EBV antigen. The quantity of EBV DNA load detected in blood indicates the stage of the disease and thus a useful prognostic marker especially in endemic areas (Wei & Sham, 2005). NPC is a relatively rare tumour but is endemic in certain regions; southern China, Northern Africa and Alaska. Genetic, ethnic and environmental factors are all believed to have a role in this type of cancer; however, there is an increasing body of evidence that among all these factors, EBV appears to be the strongest and most consistently related factor (Vasef et al., 1997), (Chang & Adami, 2006). Specific EBV latent genes, such as latent membrane protein1&2 (LMP) and Epstein-Barr nuclear antigen 1 (EBNA1), are consistently expressed in NPC and early dysplastic lesions and have profound effects on cellular gene expression and growth resulting in a highly invasive and malignant growth

of the tumour, with the exact mechanism of action still poorly understood (Raab-Traub, 2002), (Zheng et al., 2007).

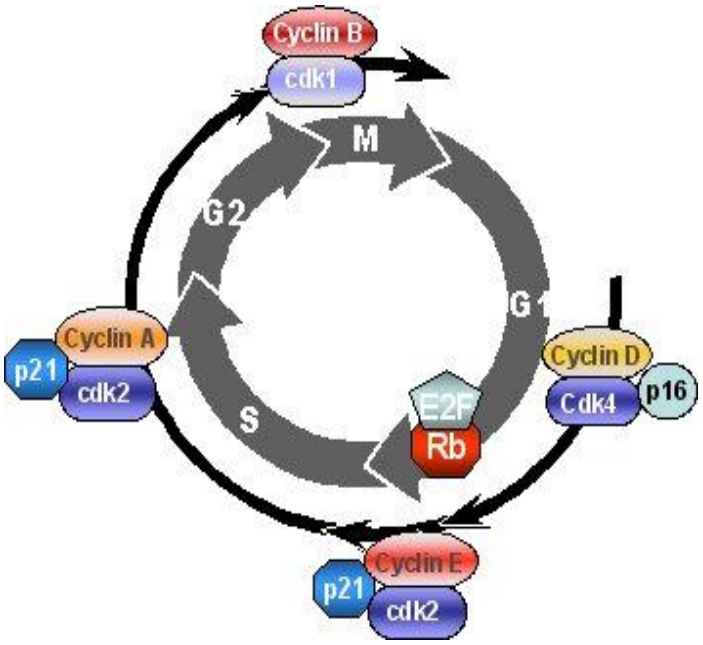
Chemoradiotherapy (CRT) is now considered the current mainstay of treatment for NPC (Ferrari et al., 2008). Novel treatments targeting EBV-associated NPC such as gene therapy, immunotherapy and vaccines are promising but still limited (Li et al., 2002), (Duraiswamy et al., 2003), (Hui et al., 2009).

1.3.4 Genetic and Familial Factors

The continuous exposure of the upper aerodigestive tract tissue to carcinogens drives tumourogenesis by specific genetic alterations, leading to the inactivation of tumour-suppressor genes or activation of proto-oncogenes leading to the development of HNSCC. These effects very much depend upon the underlying genetic background that varies from patient to patient.

The cell cycle mechanism underpins normal cell growth and division. Progression is controlled by a family of protein kinases and cyclin-dependant kinases, whose activity is tightly regulated through a variety of biochemical events (Figure 3). In cancer, these control mechanisms are often defective, resulting in uncontrolled growth. Disruptions of some of these control mechanisms or “the checkpoints”, such as P53 and pRb inactivation by HPV described previously, provides cancer cells with a growth advantage.

Figure 3: The cell cycle

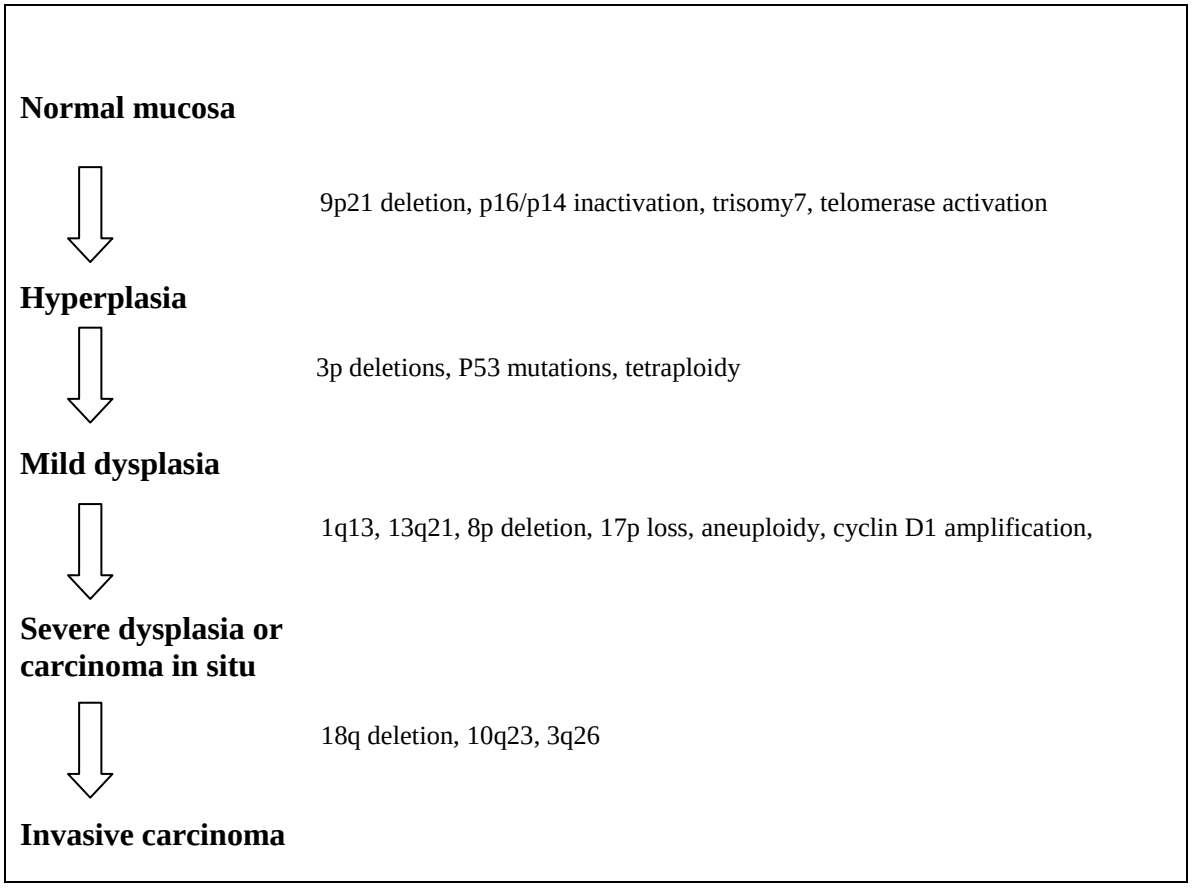


During a normal cell cycle, the completion of mitosis (M phase) is followed by the G1 phase, in which a regulated series of events take place to prepare the cell for division before entry into the S (DNA synthesis) phase. These events include elevations in D- and E-type cyclin levels, activation of cyclin-dependent kinases (CDKs), phosphorylation of the retinoblastoma protein (pRb), and subsequent activation of the E2F transcription factor family. Once activated, E2F stimulates the transcription of genes whose protein products are required for S-phase entry and transition. P53, the tumour suppressor gene, can detect genetic mistakes during the G1 phase and does not allow the cell to enter into the S phase and copy its DNA. After S phase, cells enter the G2 phase, in which additional cyclin/CDK complexes are activated, in particular, the cyclin B1/CDC2 complex that stimulates mitotic entry (www.di.uq.edu.au).

One of the early steps commonly seen in the progression of HNSCC is the loss of chromosomal region 9p21 and hence the inactivation of the tumour suppressor gene p16, an important regulator of the cell cycle within G1. This genetic aberration is seen in up to 80% of HNSCC (Mao et al., 1996); (Reed et al., 1996)**.** This step is believed to be followed by multiple molecular alterations that include the mutation of the tumour suppressor gene *TP53*, which was found to be mutated in over 50% of HNSCC cases (Balz et al., 2003) and was also found to be associated with tobacco and alcohol dependence, early recurrence and reduced survival after surgical treatment of HNSCC

(Mao et al., 1996). Other molecular alterations observed in patients with HNSCC include the reactivation of Telomerase, which is involved in telomere lenghthening thus protecting the acquired genetic changes taking place during tumour progression (McCaul et al., 2008); the over-expression of Cyclin D1 (CCND1), an oncogene that activates cell cycle progression (Capaccio et al., 2000); and other molecular defects which are summarised in Figure 4 with the accompanied phenotypical progression.

Figure 4: Chromosomal and molecular alterations in a hypothetical head and neck carcinogenesis model



EGF: Epidermal Growth Factor. Adapted from Argiris et al (2008)

Another event in the pathogenesis of HNSCC, which was recently explored, is the over expression of the epidermal growth factor receptor (EGFR) found in more than 95% of HNSCC and associated with poor prognosis (Kalyankrishna & Grandis, 2006). EGFR is a member of the ErbB growth factor receptor tyrosine kinase family, which is central in

HNSCC biology regulating cell proliferation, apoptosis, metastatic potential and angiogenesis (Argiris et al., 2008). Increased EGFR gene copy numbers were also found to be associated with decreased progression free survival (PFS) and decreased overall survival (OS) (Chung et al., 2007). Targeting of this receptor has been successfully exploited for therapeutic purposes and will be discussed later (Karamouzis et al., 2007).

Factors that control angiogenesis, which facilitates cancer growth and metastasis, have also been investigated in HNSCC and are believed to be crucial in the molecular pathogenesis of this and various other cancers. One of the most important of these factors is the vascular endothelial growth factor (VEGF) and its receptors. The VEGF family has been reported to be upregulated in HNSCC patients (Smith et al., 2000), with a significant prognostic value (Parikh et al., 2007). Therapeutic strategies against VEGF and its receptors are currently under evaluation in HNSCC (Ferrara, 2005), (Cohen et al., 2009).

Although HNSCC can occur sporadically, an inherited predisposition may occur as a consequence of increased mutagen sensitivity, inability to metabolize carcinogens, pro-carcinogens or repair DNA damage (Jefferies & Foulkes, 2001). Very few studies on this subject are available, possibly due to difficulties in confirming cancer diagnosis among relatives with HNSCC (Jefferies et al., 2008). However, an early large case-control study from Brazil (n=754 HNSCC patients and 1,507 controls) highlighted the importance of familial factors in the aetiology of HNSCC. The results showed that the RR for developing HNSCC if a first-degree relative had HNSCC was 3.65 (95% CI: 1.97-6.76) (Foulkes et al., 1995). In a more recent and large multicentre case-control study conducted on 956 cases aged less than 79 years, with histologically confirmed HNSCC, and 2362 controls showed that multivariate ORs were similar for a family history of oral and pharyngeal cancer (OR= 2.6) and laryngeal cancer (OR= 3.8). The OR was 3.1 for oral and pharyngeal cancer and laryngeal cancer combined. The OR was 7.1 for subjects with 2 or more first-degree relatives with oral and pharyngeal/laryngeal cancers. Compared with subjects without family history of oral or pharyngeal cancer, non-

smokers, and non or moderate drinkers, the OR was 42.6 for current smokers and heavy drinkers with a family history of the cancer (Garavello et al., 2008a).

A better understanding of the involvement of familial, molecular and genetic factors in the development of HNSCC has created optimism that the management of these patients could become more efficient, and that novel approaches to tackle this cancer could be incorporated with the current modalities in order to improve outcomes in the future.

1.3.5 Diet and lifestyle

Recent evidence supports the observation that dietary intake of both fruit and vegetables play an important role as a protective factor against the development of HNSCC**.** In a meta-analysis of 16 case-control studies, the consumption of fruit and vegetables was shown to be associated with a reduced risk of oral cancer (Pavia et al., 2006). The combined adjusted OR estimates showed that each portion of fruit consumed per day significantly reduced the risk of oral cancer by 49% (OR: 0.51; 95% CI: 0.40, 0.65). For vegetable consumption, a similar significant reduction in the overall risk of oral cancer (OR: 0.50; 95% CI: 0.38, 0.65) was reported. This study also showed that the lower risk of oral cancer associated with fruit consumption was significantly influenced by the type of fruit consumed and by the time interval of dietary recall. Indeed, a stronger protective effect was observed for citrus fruit consumption than with all other kinds of fruit consumption and for the longer time frame of dietary habit recall. By contrast, neither type nor period of consumption influenced the effect of vegetable intake. More recent large, multicentre, case-controlled studies on oral, pharyngeal and laryngeal cancer patients showed that a more diversified diet, particularly one containing a variety of vegetables and fruit, is a favourable indicator of oral, pharyngeal and laryngeal cancer risk; independently from the major recognised risk factors such as tobacco and alcohol consumption (Garavello et al., 2008b), (Garavello et al., 2009).

On the other hand, processed meat was found to be associated with an increased risk of developing HNSCC in a study that included 545 patients and 1,271 matched controls pooled from multiple case-control studies in Switzerland. The multivariate ORs for the highest quartile of intake compared with the lowest were 4.7 for oral and pharyngeal cancer, 4.5 for oesophageal cancer and 3.4 for laryngeal cancer. The authors concluded that processed meat represents a strong indicator of unfavourable diet for upper digestive tract and laryngeal cancer risk in the population studied (Levi et al., 2004). This conclusion is supported by another large case-control study on multiple cancers including upper aerodigestive tract tumours affirming the increased risk of developing cancer among patients with higher meat intake (La Vecchia, 2004).

HNSCC incidence is strongly associated with a lower socioeconomic status. For example, in Scotland from 1991-95, the incidence rates for HNSCC were twice as high for those patients in the most disadvantaged category compared with the least disadvantaged (www.cancerresearchuk.org). A more recent study, also from Scotland, showed that since 1980 the incidence of oral cancer among males has significantly increased with the rise occurring almost entirely in the most deprived areas of residence (Conway et al., 2007). The same group has also published a meta-analysis of 41 case-control studies worldwide which concluded that oral cancer risk associated with low socioeconomic status is significant and comparable with other lifestyle risk factors such as age, sex, tobacco and alcohol consumption (Conway et al., 2008).

1.3.6 Occupational: chemicals and radiation

Occupational exposure to chemicals/solvents has also been associated with an increased risk of HNSCC: chromium, radium and byproducts of leather and wood-working with sinonasal cancers (Hayes et al., 1986), (Hernberg et al., 1983) ; asbestos with laryngeal cancer (Berrino et al., 2003); coal dust with laryngeal and hypopharyngeal cancers (Shangina et al., 2006). In one recent study, the exposure time to wood dust before diagnosis of sinonasal tumours was in the majority of cases greater than 20 years, reflecting the importance of the timeframe of exposure in these cases (Fontana et al., 2008).

Medical ionising radiation e.g. radiotherapy and diagnostic radiology, can induce cancers in patients (Boice, 1981), (Berrington de Gonzalez & Darby, 2004). Radiation-induced cancers (RIC) of the head and neck have been reported. In one of the largest series of cases of RIC in the head and neck (n=65), the mean duration from radiotherapy until the diagnosis of cancer was 12.8 years in patients with a malignant primary tumor and 32.9 years in those with benign primary diseases (Miyahara et al., 1998). In another study investigating patients who underwent radiotherapy for early stage maignanat lymphoma of the head and neck (n=355) and early stage HNSCC (n=1,358), the crude incidence of RIC in the malignant lymphoma patients was 1.4% (5/355) with a 10-year probability of

0.8%, while the crude incidence of a second cancer in a previously irradiated field after an 8-year latent period in HNSCC patients was 1.8% (25/1358), with a 10-year probability of 1.6% (Amemiya et al., 2005).

Although RIC have been defined as tumours that arise from normal tissue within the irradiated field or that occurs within an irradiated field but has histopathological features different from the primary tumour , difficulties still exist in distingwishing between RIC and late recurrences as well as difficulties in determaining the latency period for RIC in head and neck tumours (Amemiya et al., 2005).

1.3.7 Extra-oesophageal Reflux Disease

The non-gastrointestinal manifestations of gastro-oesophageal reflux were categorised by the Montreal consensus as "extra-oesophageal" reflux; which means the backflow of refluxate from the stomach above the upper oesophageal sphincter and into the larynx, pharynx and respiratory tract otherwise known as Laryngopharyngeal reflux (LPR) (Vakil et al., 2006). LPR is common with between 4% and 10% of patients presenting to ENT outpatient clinics complain of reflux-related symptoms (Koufman et al., 2002). The association between LPR and the development of carcinoma remains unclear, however it is considered as a possible co-promoting factor of HNSCC (Mercante et al., 2003). A high incidence of LPR has been observed in patients with premalignant and early laryngeal cancer (Galli et al., 2002), (Lewin et al., 2003). LPR was also strongly demonstrated in patients who developed laryngeal cancer after gastric resections (Cianci et al., 2003). A recent case-control study of 96 patients and 192 controls has shown that LPR presence is associated with a significant risk of developing laryngeal cancer (Vaezi et al., 2006). On the other hand, two systematic reviews (Wilson, 2005), (Qadeer et al., 2006) and a large observational study (Nilsson et al., 2005) provided no evidence in support of the proposed association between gastroesophageal reflux disease and cancers of the larynx or pharynx.

Large multi-centre trials will hopefully reveal the exact role of “extra-oesophageal” reflux causality with HNSCC. A better understanding of the molecular basis of this disease is useful for future novel diagnostics and treatments for this common condition (Birchall et al., 2008).

1.4 Subtypes of Head and Neck cancer

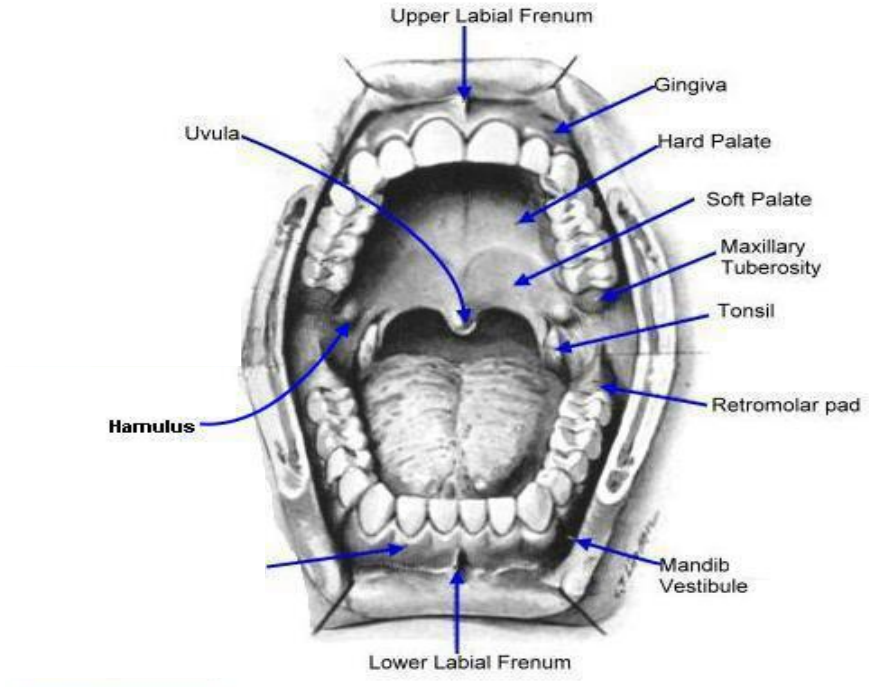
1.4.1 Anatomical subtypes

Traditionally, head and neck cancers have been grouped together for epidemiological studies and ease of categorisation. However, carcinomas of the oral cavity, pharynx and larynx differ greatly in their anatomical, biological and pathological features (Argiris & Eng, 2003).

The oral cavity extends from the lips to the oropharynx. The circumvallate papillae, anterior tonsillar pillars and the junction of the soft and hard palate form the posterior border of the oral cavity (Figure 5). Anatomical subtypes within the oral cavity include (Lee, 2003)

- Lips
- Anterior two thirds of tongue
- Floor of mouth
- Buccal mucosa
- Upper and lower alveolar ridge
- Retromolar trigone (Area behind the wisdom teeth)
- Hard palate

Figure 5: The oral cavity



(www.doctorspiller.com)

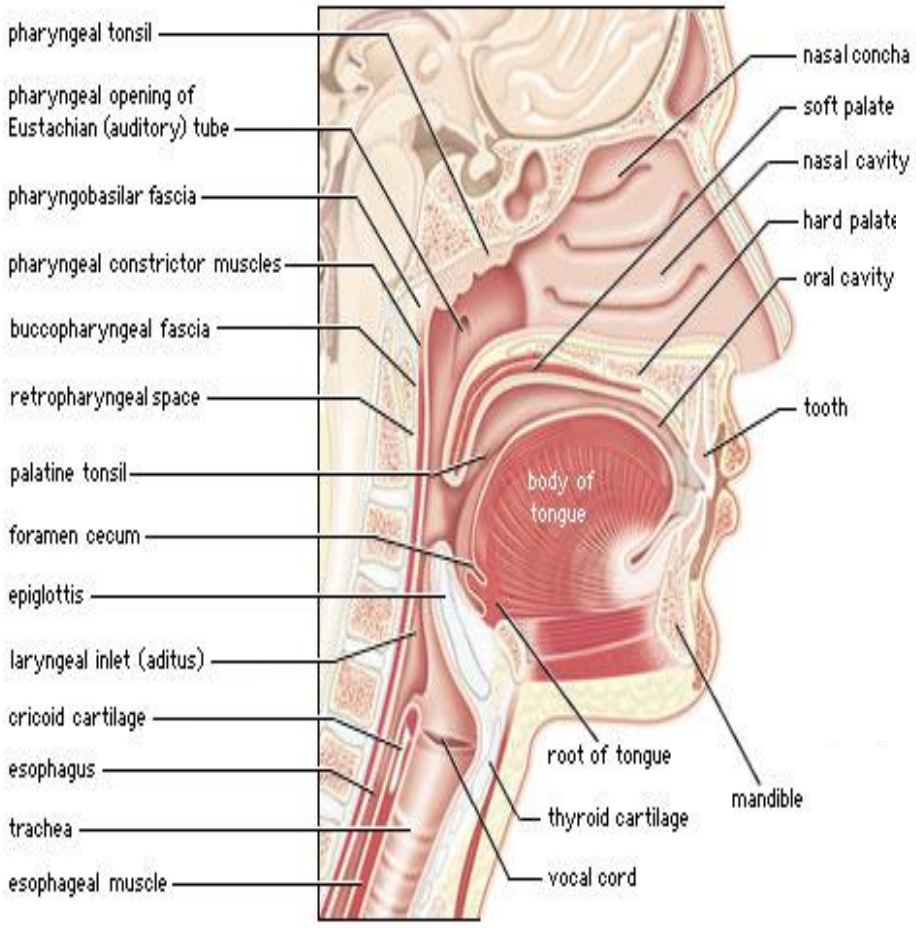
The pharynx (throat) is divided into three distinct regions oropharynx, hypopharynx and nasopharynx (Figure 6). The oropharynx begins anteriorly from the posterior one third of the tonge, tonsillar pillars and the junction between the hard and soft palate. It extends vertically to the plane of the superior surface of the hyoid bone. Anatomical subtypes within the oropharynx include (Lee, 2003)

- Base of tongue (posterior one third)
- Soft palate and uvula
- Tonsillar pillars
- Pharyngeal tonsils
- Lateral and posterior oropharyngeal walls

The hypopharynx extends vertically from the superior border of the hyoid bone to the lower border of the cricoid cartilage. It includes (Lee, 2003)

- The piriform fossae
- Lateral and posterior hypopharyngeal walls
- Postcricoid region (the pharyngo-oesophageal junction)

Figure 6: Anatomy of the pharynx and larynx



(www.britannica.com)

Each piriform fossa (sinus) extends from the pharyngoepiglottic folds to the upper end of the cervical oesophagus and is bordered by the thyroid cartilage laterally and the aryepiglottic fold, the arytenoids and the cricoid cartilage medially.

The nasopharynx is situated behind the nasal cavity and includes (Lee, 2003)

- Lateral walls (including the fossa of Rosenmuller and the torus tubarius)
- Vault (including the superior surface of soft palate)
- Posterior wall (including the adenoids)

The larynx is divided into three regions; supraglottic, glottic, and subglottic (Figure 6). The supraglottic larynx includes (Lee, 2003)

- Epiglottis
- Aryepiglottic folds, laryngeal aspect
- Arytenoids
- Ventricular band (false cords)

The glottic larynx includes both true vocal cords and the mucosa of the anterior and posterior commissures extending 1 cm below the free edge of the vocal folds.

The subglottic larynx consists of the region bounded by the vocal cords above and the inferior border of the cricoid cartilage.

Other anatomical divisions include sites where HNSCC is less likely to occur such as the nose and paranasal sinuses, salivary glands (major and minor), the ear canal and the temporal bone.

1.4.2 Histological subtypes

Squamous cell carcinoma

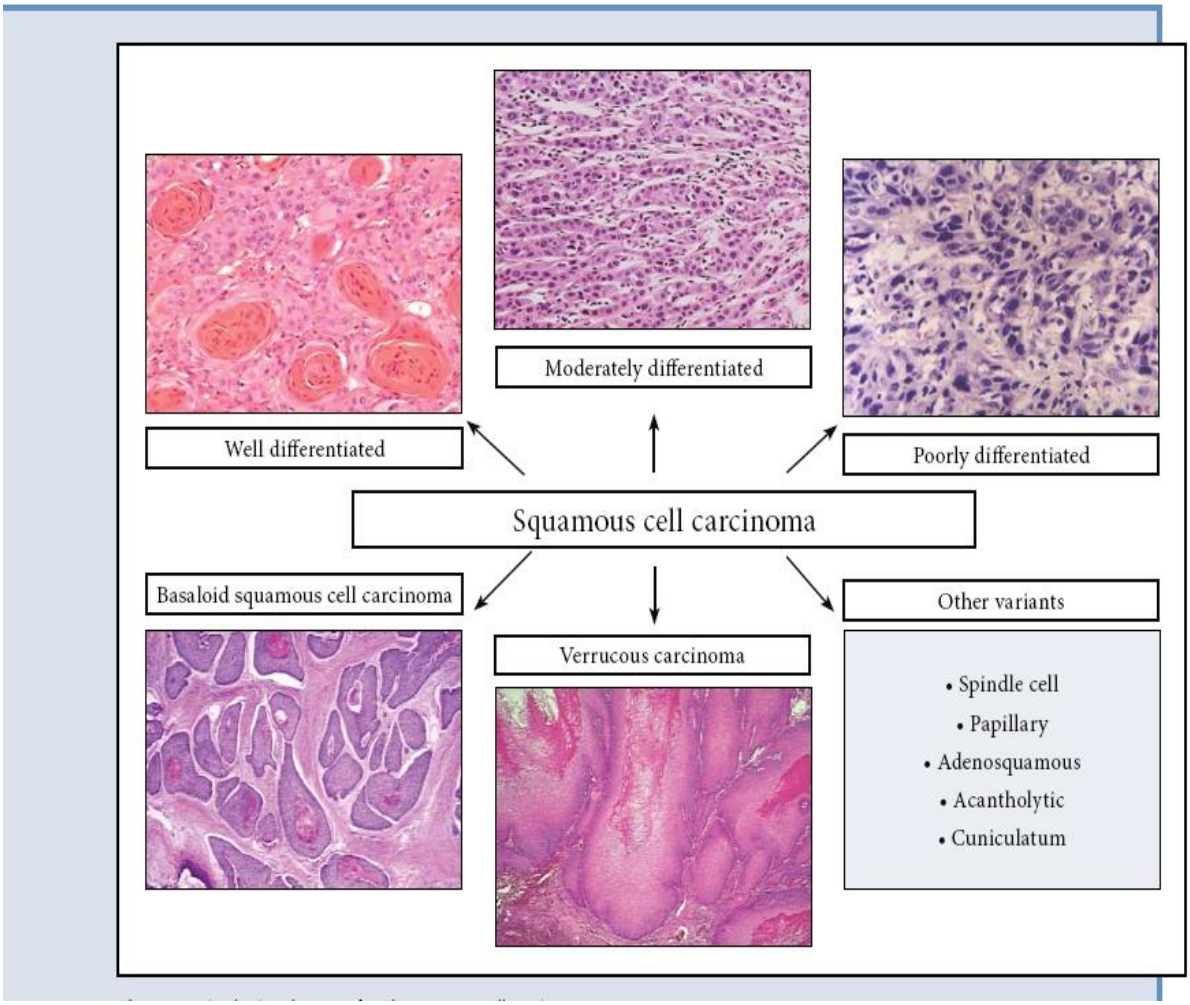
SCC represents more than 90% of the tumours arising from the epithelium of the aerodigestive tract (Vandenbrouck et al., 1987). As well as the head and neck, SCC may occur in many different organs including the skin, urinary bladder, prostate, lungs, vagina, and cervix. The hallmark of SCC is the presence of keratin or "keratin pearls" on histological evaluation. These are well-formed desmosome attachments and intracytoplasmic bundles of keratin tonofilaments (Schultz & Mantsch, 1998). SCC are classified microscopically based on a method which takes into account a subjective assessment of the degree of keratinization, cellular and nuclear pleomorphism and mitotic activity (Pereira et al., 2007). The grades of SCC include well (grade 1), moderately (grade 2) and poorly differentiated (grade 3); (Figure 7). The use of ancillary testing, especially immunohistochemical analysis, for accurately determining the diagnosis is highly recommended and vitally important for prognostic and therapeutic reasons (Stelow & Mills, 2005).

Histology reports in HNSCC include several parameters to indicate malignancy such as tumour thickness, differentiation and the level of invasion i.e. breaching the natural basement membrane whether perineural, vascular or lymphatic. Other histological parameters which are considered "unfavourable" and correlate with a more aggressive disease and poor outcome, include the presence of two or more regional nodes, extracapsular extension of nodal disease and positive margin resection (Pereira et al., 2007).

Relatively common variants of conventional SCC are verrucous and basiloid tumours which behave differently. While basiloid tumours are poorly differentiated, highly metastatic and have a poor prognosis, verrucous tumours rarely metastasise and have an excellent prognosis (Pereira et al., 2007). Although verrucous carcinoma is not known to

be invasive, it can be locally aggressive if not treated early (Orvidas et al., 1998). Other variants that are much less common to occur are shown in Figure 7.

Figure 7: Histological subtypes of squamous cell carcinoma



Adapted from Pereira et al (2007)

Non- Squamous cell carcinoma

Non-squamous cell carcinomas of the head and neck are rare neoplasms counting for less than 10% of all head and neck tumours (Licitra et al., 2004).

In the oral cavity, almost 5% of the tumours are basal cell carcinomas which occur predominantly in the upper lip. Other rare tumours include adenoid cystic carcinomas which occur in the minor salivary glands scattered in the oral cavity; sarcomas and osteosarcomas which may also arise in the soft and hard tissue of the oral cavity,

respectively (Daley & Darling, 2003). In the oropharynx, the next common malignancy after SCC is lymphoma, primarily of the palatine tonsil and counting for less than 1% of all head and neck malignancies (Mohammadianpanah et al., 2005).

In the nasopharynx, the world health organisation (WHO) classification (Sobin, 2003) has 3 types: type I which is similar to HNSCC; type II which is a transitional cell carcinoma; type III which is undifferentiated carcinoma. Type III is the most common with 75% of all NPC being types II or III.

In the nose and paranasal sinuses, a plethora of rare tumours, apart from SCC, occur. They include adenocarcinomas, melanomas, esthesioneuroblastomas, sarcomas, lymphomas, neuroendocrine carcinomas and undifferentiated sinonasal carcinomas. All these rare tumours have a poor long-term prognosis in comparison with SCC (Licitra et al., 2004).

In the larynx, neuroendocrine carcinomas, particularly the moderately differentiated subtype, are considered the most common form of non-squamous carcinoma, although still a rare entity (Mills, 2002). Sarcomas have also been reported in the larynx with a relatively good prognosis when compared with sarcomas originating from other anatomic sites (Liu et al., 2006a).

Tumours of salivary, thyroid and parathyroid glands are separate entities from the rest of HNSCC due to their varied histological subtypes and different biological behaviour (Surakanti & Agulnik, 2008), (Kebebew, 2008). The focus of this thesis is on the more common SCC of the head and neck; hence the rarer types of tumours will not be discussed any further.

1.5 Diagnosis and Staging

Diagnosis

Patients with early stage HNSCC may present with vague symptoms and minimal physical signs. Clinical presentation varies with the site involved (Table 1). Patients with oral cancers present usually with non-healing ulcers or a sore throat, while laryngeal/hypopharyngeal cancer patients present with hoarseness, dysphagia, otalgia and, in the advanced stages, with a neck mass (Marur & Forastiere, 2008). Early recognition of signs and symptoms is paramount for prompt diagnosis. No proven screening methods exist, except for visual inspection in high risk areas such as oral cavity cancers (Sankaranarayanan et al., 2005).

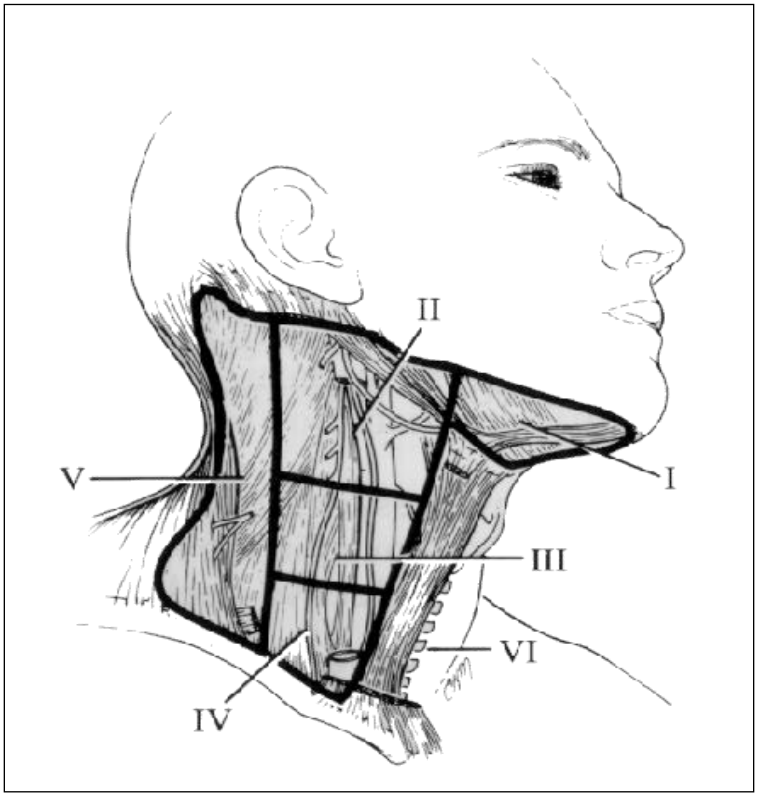
Table 1: Signs and symptoms of HNSCC

Subtype	Clinical Presentation
Oral Cavity	Ulcers, red or white patches, sores, pain, neck mass
Oropharynx	Sore throat, odynophagia, chronic dysphagia, otalgia, neck mass
Hypopharynx	Soreness, globus sensation, dysphagia, otalgia, hoarseness, neck mass
Larynx	Persistent hoarseness, shortness of breath, neck mass in supraglottic tumours, vocal cord paralysis
Nasopharynx	Otitis media unresponsive to treatment, unilateral nasal obstruction or discharge, epistaxis, cranial nerve palsies, neck mass
Sinonasal	Unilateral nasal obstruction or discharge, epistaxis, facial or dental pain, hypoesthesia, ophthalmoplagia, visual loss, neck mass

Adapted from Marur & Forastiere (2008)

The physical examination includes examining all mucosal surfaces for abnormalities, bimanual examination of the tongue base and the salivary glands, and neck palpation of lymph nodes. The location of an enlarged neck node may predict the primary site. For example oral cavity and oropharyngeal tumours tend to spread to levels I-III, laryngeal tumours to levels II and III and NPC to levels II and V (Mukherji et al., 2001). Figure 8 summarises lymph node distribution in the neck. Neck node involvement is crucial in determining prognosis as it generally reduces cure rates by 50% for a given T stage (Marur & Forastiere, 2008).

Figure 8: Levels of nodes distribution in the neck.



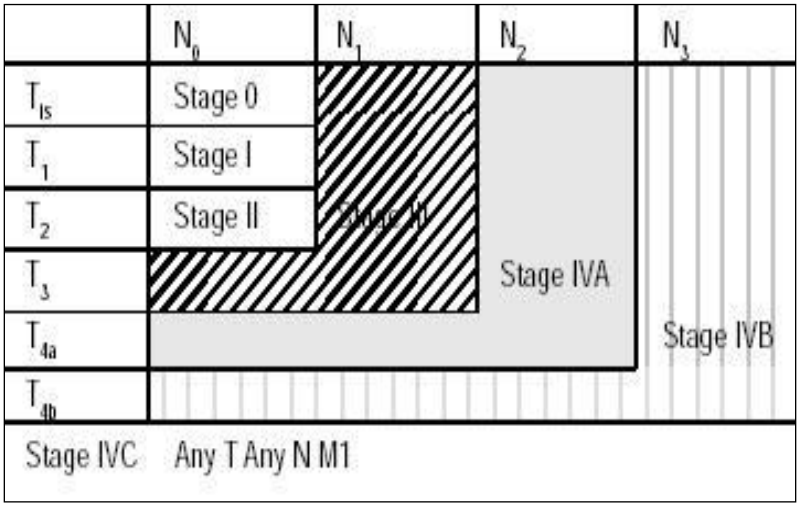
I- Submental and submandibular nodes, II- Upper jugulodigastric group, III- Middle jugular nodes, IV- Inferior jugular nodes, V- Posterior triangle group, VI- Anterior compartment group (www.bcm.edu)

Investigations involve an upper panendoscopy (nasopharyngolaryngoscopy, oesophagoscopy and bronchoscopy, as appropriate), imaging studies, i.e. Computed Tomography (CT), Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET) and biopsies of any detected lesions including fine needle aspiration cytology of neck masses. Tumours that present with neck lymphadenopathy and no apparent primary site will undergo endoscopic assessment, directed biopsies of Waldeyer's ring (nasopharynx, tonsil and base of tongue, including tonsillectomy) and PET imaging in order to identify the occult primary site (Schmalbach & Miller, 2007). However, in nearly 5% of HNSCC cases, there is an unknown primary site posing controversial management issues regarding the best treatment option for these patients (Boscolo-Rizzo et al., 2007).

Staging

Once the diagnosis has been established, the local, regional and distant extent of the disease should be determined for accurate staging. HNSCC staging is site specific. The criteria have been developed by the American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC), and undergoes regular re-evaluation and modification. The groupings are based on the T (primary tumour), N (regional node) and M (distant metastasis) method of staging (Sobin & Fleming, 1997). While the primary tumour, T, depends on the site, there is uniformity in the nodal and the overall stage grouping apart from that for nasopharyngeal and thyroid tumours. The basic principle of these staging criteria is that smaller cancers with no nodal disease have a better prognosis than a larger lesion with positive neck nodes. Appendix 1 contains the different TNM classifications for HNSCC. Stage grouping was devised to simplify the process by combining the different variables to produce an overall measure of the tumour's extent (Table 2).

Table 2: AJCC/UICC overall stage grouping for all head and neck sites*



* Excluding nasopharyngeal and thyroid tumours. Adapted from O'Sullivan & Shah (2003)

The primary benefit of such a staging method is to aid the selection of appropriate treatment regimens and advise patients about prognosis. It also facilitates communication acting as a common language between clinicians and aids research. However, drawbacks to the sole use of this system exist. The TNM classification is a poor predictor of prognosis in most of recurrent tumours due to the lack of a re-classification criterion, hence an inadequate indicator of the patient's response to treatment. It also lacks details related to the host (e.g. medical comorbidity and immune competence) and the tumour (e.g. biological and molecular markers) which can influence the overall outcome of the disease (Patel & Shah, 2005).

The most common site of distant metastasis in HNSCC is the lung, followed by mediastinal lymph nodes, liver and bones (Argiris et al., 2008). Chest imaging is performed routinely at initial assessment and may detect a second primary lung cancer or, in locally advanced HNSCC, a lung metastasis. Combined CT and PET scanning with [^{18}F] fluoro-2-deoxyglucose are increasingly being used. The combined scans are more accurate than either of them alone for detecting malignanat lesions, recurrence, nodal and/or distant metastasis and response to treatment in the head and neck (Ng et al., 2006), (Branstetter et al., 2005).

The benefits of accurately determining the stage of HNSCC is reflected in the survival rates for each stage of the disease. Early detection of tumours and therefore a lower stage of the cancer have a significant favourable effect on the overall survival for patients, while a locally advanced stage or tumours with distant metastasis have a much poorer survival (Table 3).

Table 3: Five years relative survival rates of HNSCC in the USA by stage at diagnosis 1996-2004

Site	All stages %	Local % (Stages I & II)	Regional % (Stages III & IVA/B)	Distant % (Stage IVC)
Oral cavity & Pharynx	59.7	82.2	52.7	28.4
Larynx	62.5	80.9	50.2	23.4

(www.cancer.org)

Over the last 30 years, 5 year survival rates for laryngeal cancer, of any stage, have not changed significantly (around 65%). However, figures for oral cavity and pharyngeal cancers have shown a significant improvement when all stages are combined i.e. from 53% in 1975 to 60% in 2002 (Jemal et al., 2007).

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1.6 Treatment

Several modalities to treat HNSCC exist, including surgery, radiotherapy, chemotherapy and/or a combination of these. Over the past 20 years, treatment methods have changed considerably owing to improvements in surgical and chemoradiotherapeutic techniques. Furthermore, new investigative treatments including immunotherapy and gene therapy are gaining acceptance as they progress through clinical trials (Haddad et al., 2006). Factors that influences the choice of treatment include patient factors (age and co-morbidities), tumour factors (site of primary tumour, T, N, M stage, involved margins at resection, extracapsular spread and perineural invasion). Other recent factors that are now considered include HPV and EGFR status. Goals of treatment generally consist of removal of cancer load, maintenance of quality of life, and prevention of recurrence, thus achieving the highest cure rates at the lowest cost in terms of functional and cosmetic morbidity. Treatment of head and neck tumours require a multidisciplinary approach comprising a team of head and neck surgeon, oncologist, radiologist, pathologist with support from a head and neck specialist nurse, dietician, physiotherapist and speech and language therapist. Two evidence-based documents set the standards for the management of head and neck cancer both nationally and internationally; "Improving Outcomes in Head and Neck Cancers", (www.nice.org.uk) and the "National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology, Head and Neck Cancer", (www.nccn.org).

Treatment of early stage disease

Tumour stages I and II, which represent nearly a third of all HNSCC are usually treated with either surgery or radiotherapy with the intent to cure the disease; which is achieved by over 80% of the cases (Argiris et al., 2008). Approaches to treatment differ according to the primary site of the tumour. For oral cavity tumours, the efficacy of radiotherapy is comparable with surgery (Palme et al., 2004). However, surgery is usually the preferred modality of treatment for oral cavity tumours as most of these tumours are easily accessible to the surgeon and also to avoid the late toxic effects of radiation (Mendes et

al., 2002). Another benefit of surgical management is the provision of pathological staging of the primary tumour and any occult neck disease, i.e. micrometastasis, which will guide adjuvant treatment decisions (Greenberg et al., 2003).
For early-stage oropharyngeal and hypopharyngeal cancers, recent studies have shown that radiotherapy is generally the favourable first option since it results in cure rates comparable to surgery and is usually associated with fewer side effects (Mendenhall et al., 2006a), (Mendenhall et al., 2006b), (Nakamura et al., 2006). Radiotherapy, endoscopic surgery, including laser or robotic surgery, or open surgery with voice preservation are equally effective modalities to treat early laryngeal tumours with comparable cure rates and functional outcomes (Jones et al., 2004), (Mlynarek et al., 2006).

Elective neck dissection of ipsilateral neck nodes in the clinically uninvolved neck was reported to significantly improve regional control and regional recurrence-free survival in patients with high risk of occult neck disease; however, elective neck dissection when compared with observation of the neck did not improve overall survival (Duvvuri et al., 2004). Sentinel lymph node biopsy (SLNB) to detect neck node metastasis in oral and oropharyngeal HNSCC has gained more recognition recently after being validated by a multi-institutional trial (Civantos et al., 2007). Although still not incorporated in national or international guidelines, evidence is growing for the use of SLNB in early, i.e. T1 or T2, oral and oropharyngeal SCC with no neck involvement, with a possible extension to all early-stage HNSCC in the future (Cote et al., 2007).

Treatment of locally advanced disease

Curative treatment of stages III and IV disease include using surgery, radiotherapy and chemotherapy. Traditionally, surgery and radiotherapy were the mainstay of treatment, while chemotherapy was used in palliative care (Cohen et al., 2004). Over the last two decades, advances in the surgical treatment, i.e. the use of microsurgical free tissue transfer for reconstruction of surgical defects, have improved functional outcomes after resection of a locally advanced tumour. However, the major advancement in the

treatment of these stages of the disease has been the introduction of concurrent administration of chemotherapy and radiotherapy, i.e. chemoradiotherapy (CRT).

Concurrent use of CRT in order to achieve organ preservation in resectable disease stage III or IV HNSCC was supported by a wealth of randomised control trials. The seminal work by Pignon *et al*, a meta-analysis of 63 trials including 11,000 patients with HNSCC, showed that the addition of chemotherapy to the standard locoregional treatment resulted in an absolute survival benefit of 4% at 5 years. This benefit was confined to CRT which resulted in an absolute survival improvement of 8% at 5 years (Pignon et al., 2000). An update to this meta analysis supported the results of the initial study (Pignon et al., 2007) Another meta analysis from the same group (120 randomised trials, 25,000 patient with a median follow up of 6 years) showed that the treatment benefit of CRT was maintained in both stage III and IV, each major primary site, definitive or postoperative radiotherapy, and when altered fractionation radiotherapy, especially when hyrepfractionation was used in the control group (Bourhis et al., 2007). Benefits from CRT have also been documented in the postoperative setting, where cisplatin, the standard chemotherapeutic agent used in HNSCC, was added to postoperative radiotherapy especially when high-risk pathological features such as extracapsular spread and positive resection margins are present (Bernier et al., 2005). In patients with unresectable, locally advanced HNSCC, CRT is the standard treatment, unless contraindicated due to patient co-morbidities (Adelstein et al., 2003). The use of induction (neoadjuvant) chemotherapy in locally advanced HNSCC is not yet regarded as standard treatment, however several randomised trials are in progress to establish its role in the treatment of this stage of the disease (Argiris et al., 2008).

Radiotherapy protocols for advanced HNSCC have also seen advances with encouraging outcomes in both locoregional control and lesser side effects. Conventional radiotherapy for HNSCC is usually in the form of daily single fractions of 2.0 Gy, 5 days a week, up to a total of 70 Gy for 7 weeks (Peters et al., 1993). Postoperatively, a dose up to 66 Gy improves locoregional control when extracapsular extension is present (Bernier et al., 2004). Advances include the use of altered fractionation radiotherapy, three dimensional

images and intensity-modulated radiotherapy (IMRT) (Marur & Forastiere, 2008). IMRT allows the delivery of high doses of radiation to specified clinical volumes, whilst reducing the dose and toxic effect on adjacent non-target structures such as the salivary glands. Although initial clinical results of IMRT therapy in advanced HNSCC are promising, it is still not widely used (Suzuki et al., 2006). Altered fractionation includes hyperfractionation or accelerated fractionation. Both aim for multiple fractions of radiation to be given per day to improve effectiveness and chronic toxic effects specifically at the tumour site (Bernier, 2005). A meta analysis of 15 randomised trials including 5,000 HNSCC patients (75% with locally advanced disease) showed that altered fractionation radiotherapy yielded an absolute 5-year survival benefit of 3.4% (HR 0.92, 95% CI 0.86-0.97; P=0.003) and also benefits on locoregional control in favour of altered fractionation versus conventional radiotherapy (6.4% at 5 years; $p<0.0001$); (Bourhis et al., 2006).

In general, the strategy to treat larger tumours that are locally invasive, can be summarised into: (a) surgery followed by adjuvant CRT or radiation, (b) CRT upfront, with surgery as a salvage treatment and (c) induction chemotherapy followed by CRT or other primary treatment options, i.e. surgery or radiotherapy (Seiwert & Cohen, 2005). There are no specific guidelines for "unresectable" tumours, however, invasion to the carotid artery, base of skull or prevertebral muscles are considered signs of unresectability between surgeons (Argiris et al., 2008).

More recently, cetuximab, a monoclonal antibody to EGFR, has been found to improve significantly locoregional control, progression-free survival and overall survival when combined with radiotherapy and compared with radiotherapy alone for patients with locally advanced HNSCC (Bonner et al., 2006). This multinational phase III clinical trial included 424 patients randomised to receive either radiotherapy alone or radiotherapy with weekly cetuximab. Cetuximab was the first molecularly targeted agent to be incorporated in standard practice for locally advanced HNSCC as an alternative to CRT (www.guideline.gov). The combination of cetuximab, and other agents such as EGFR-

tyrosine kinase inhibitors and angiogenesis inhibitors, with radiotherapy, chemotherapy or both are currently being explored (Karamouzis et al., 2007), (Langer, 2008).

Treatment of recurrent or metastatic disease

Patients with locally advanced disease develop locoregional recurrence in 30-40% and distant metastasis in 20-30% of cases, usually within the first 2 years of treatment (Forastiere et al., 2003). Surgery is the treatment of choice for resectable recurrent locoregional disease, usually followed by radiotherapy if it has not yet been given. If the recurrence is unresectable, CRT is the treatment of choice (Weber et al., 2003). A randomised study for patients with locoregional recurrent disease (n=130) assessed re-irradiation combined with chemotherapy compared with observation after salvage surgery has shown an improvement of progression-free survival (HR 1.6, 95% CI 1.1-2.4; p=0.01) but had no significant impact on overall survival with acceptable toxic effects in the re-irradiation group (Janot et al., 2008). However, CRT is considered as the mainstay of treatment for recurrent or metastatic HNSCC. Cisplatin plus fluorouracil have been widely accepted as a reference regimen in patients with recurrent or metastatic disease (Argiris et al., 2008). Cetuximab has also been recently investigated in patients with recurrent or metastatic HNSCC, and showed extension in survival when combined with chemotherapeutic agents and a potential role to be used alone in patients who could not tolerate chemoradiation (Vermorken et al., 2008).

Future areas under investigation in the treatment of HNSCC include the incorporation of other molecularly targeted agents, such as the EGFR-tyrosine kinase inhibitors gefitinib or erlotinib, more accurate radiation delivery methods and the the identification of biomarkers that will guide treatment decisions. The ultimate aim is not only to improve locoregional and distant control, but also to improve the overall survival of potentially curable patients with HNSCC.

1.7 Antitumour immunity and HNSCC

The role of anti-tumour immunity in relation to HNSCC has been intensely studied over the past few years. It is now becoming clear that the complex interaction between HNSCC and immune cells plays an important part in determining tumour growth and progression (Whiteside, 2008). HNSCC, in common with most other tumours, results in a suppressed immune system, with an altered serum cytokine profile and immune cells that function aberrantly.

In order for the immune system to eliminate and identify tumours, it needs to identify tumour antigens. These antigens could either be presented by tumour cells and never by normal cells and hence are called tumour-specific antigens (TSA); or they could be presented by tumour cells and normal cells and in this case are called tumour-associated antigens (TAA); (Renkvist et al., 2001). Anti-tumour responses are both innate, i.e. early, non-specific and depend on killer cells such as neutrophils and macrophages or acquired, i.e. late, antigen specific and depend on T and B lymphocytes (Hayakawa & Smyth, 2006). T-cell mediated tumour immunity is dependent on recognition of tumour antigens by the T-cell receptor upon which T cells are activated. The T-cell receptor molecule is designed to bind to molecules of the major histocompatibility complex (MHC); $CD8^+$ T cells bind to MHC class I molecules, which are expressed on all nucleated cells; whereas $CD4^+$ T cells bind to MHC class II molecules, which are expressed predominantly on cells of the immune system. As most tumour cells do not express MHC class II, $CD4^+$ T cells are dependent on presentation of tumour antigen by professional antigen presenting cells, such as dendritic cells and macrophages (Guermonprez et al., 2002). $CD4^+$ T cells function primarily by controlling other cells to perform immune responses such as $CD8^+$ T cells or B cells. Naïve CD4 T cells may differentiate into either T helper 1 (Th1), or T helper 2 (Th2) cells which differ in the cytokine repertoire they produce and therefore their function. In general, Th1 cell responses result in activation of macrophages, neutrophils and cytotoxic T cells, causing a cell-mediated response, as well as B cells producing antibodies which effectively cover extracellular pathogens for phagocytic uptake by the activated cells. A Th2 cell response primarily causes a humoral immune

response through the production of high titres of neutralising antibody, as well as causing inhibition of cell activation. In summary, Th1 and Th2 CD4 T cells act in an antagonistic manner that is significantly altered by the tumour mass (Topping et al., 2009).

1.7.1 Immunosuppression and HNSCC

HNSCC is a very good example of a solid tumour that induces severe depression of a patient's immune system. This is likely to result not just because of products secreted by the tumour but also as a by-product of many of the factors that are known to increase the risk of carcinogenesis such as alcoholism, smoking, HPV presence and poor nutrition (Whiteside, 2005). Katz highlighted the need to appreciate the general well-being of the immune system over a decade ago (Katz, 1993); however, it has only been in the past few years, particularly since Treg cells were defined and tumour vaccination studies using DC have started, that people have become seriously interested in the complex interactions of tumour and host factors.

During the last few years evidence has emerged on defects in the immune system in head and neck cancer patients and the association of HNSCC with immunosuppression. Young and colleagues analysed the mechanisms of immune suppression in HNSCC by studying tumour and lymph node tissue samples from 273 patients. They showed significantly increased levels of immune inhibitory mediators such as Transforming Growth Factor-β (TGF-β), Prostaglandin E2 (PGE2), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) and Interleukin (IL-10) secreted by the tumour. They concluded that the mechanisms of immune suppression in HNSCC are associated with an altered function of intra-tumoural $CD4^+$ T-lymphocytes and reduced cell influx of intra-tumoural $CD8^+$ and $CD4^+$ T-lymphocytes (Young et al., 1996). Furthermore, Lang and colleagues, having divided the generation of an effective anti-tumour T-cell responses into the 3 key stages of adhesion, recognition and co-stimulation, showed that the absence of B7 protein co-stimulatory molecule or the MHC antigens by tumour cells both resulted in the failure to induce activation and proliferation of T cell clones *in vitro* (Lang et al., 1999). Subsequent work has shown that immune suppression operates at a number of other, non-

mutually exclusive, points in the recognition and stimulation pathway summarised in Table 4 with a brief selection of seminal references.

Table 4: Immunosuppresive mechanisms at the host/tumour level in HNSCC

• Spontaneous apoptosis/anergy of circulating T lymphocytes (Kuss et al., 1999); (Saito et al., 2000)
• Imbalanced and decreased absolute counts of T lymphocyte subsets (Kuss et al., 2004; Kuss et al., 2005);
• Signalling defects in effector cells; e.g. a decreased zeta chain expression in the T-cell receptor signalling complex (Kuss et al., 1999); (Kuss et al., 2003)
• Predominant Th2 responses i.e. raised expression of IL-10 and/or IL-4, which inhibits Th1 cytokine secretion (Levings et al., 2002); (Rodriguez et al., 2003); (Akhurst, 2004); (Jebreel et al., 2007)
• Poor expression of co-stimulatory molecules on tumour cell surface (Wang & Chen, 2004)
• Loss of HLA class I molecules (Campoli et al., 2002)
• Production of immunosuppressive factors by tumours and the sustained activation of the NF-kappaB pathway (Whiteside, 2008)
• Over expression of regulatory T cells and dysfunctional/inhibitory DC (Zou, 2006)

Young and Whiteside have reviewed tumour-escape mechanisms in HNSCC recently (Young, 2006), (Whiteside, 2008). These reviews divide the mechanisms behind evasion of immune surveillance into two main groups. Either the tumour cells are poor

stimulators of immune cells and hence are simply not recognised, and/or they actively interfere with immune function and survival of the immune cells. Multiple ways have been observed on how HNSCC tumours "hide" from surveillance with the commonest being loss of HLA class I molecules and hence no TAA expression (Campoli et al., 2002), poor expression of co-stimulatory molecules on the tumour cell surface (Wang & Chen, 2004), the production of immunosuppressive factors by tumour cells and the presence of immune-suppressive cells such as Treg cells and subsets of Natural Killer (NK) cells (Zhang et al., 2006).

Dysfunctional DC are another principal example of how tumours can hinder an immune cell response. Briefly, DC are the most potent antigen presenting cells, and their interaction with T-cells play a major "pacemaker" role in the immune system (Dunn et al., 2005). Recent findings have shown that dysfunctional DC can be derived due to various factors secreted by tumour cells resulting in inhibition of T cell proliferation and a cytokine profile that down-regulates subsequent anti-tumour effects. Dysfunctional DC can also induce the expansion of T cells with regulatory phenotype. Such findings have been demonstrated in HNSCC (Sakakura et al., 2006) as well as in other tumour types (Gabrilovich et al., 1997), (Melichar et al., 1998). More recently, Treg cells have been shown to trigger high levels of IL10 production by DC which stimulates their expression of the negative regulator B7-H4, thus inhibiting the APC function of DC and creating an immunosuppressive phenotype (Kryczek et al., 2006).

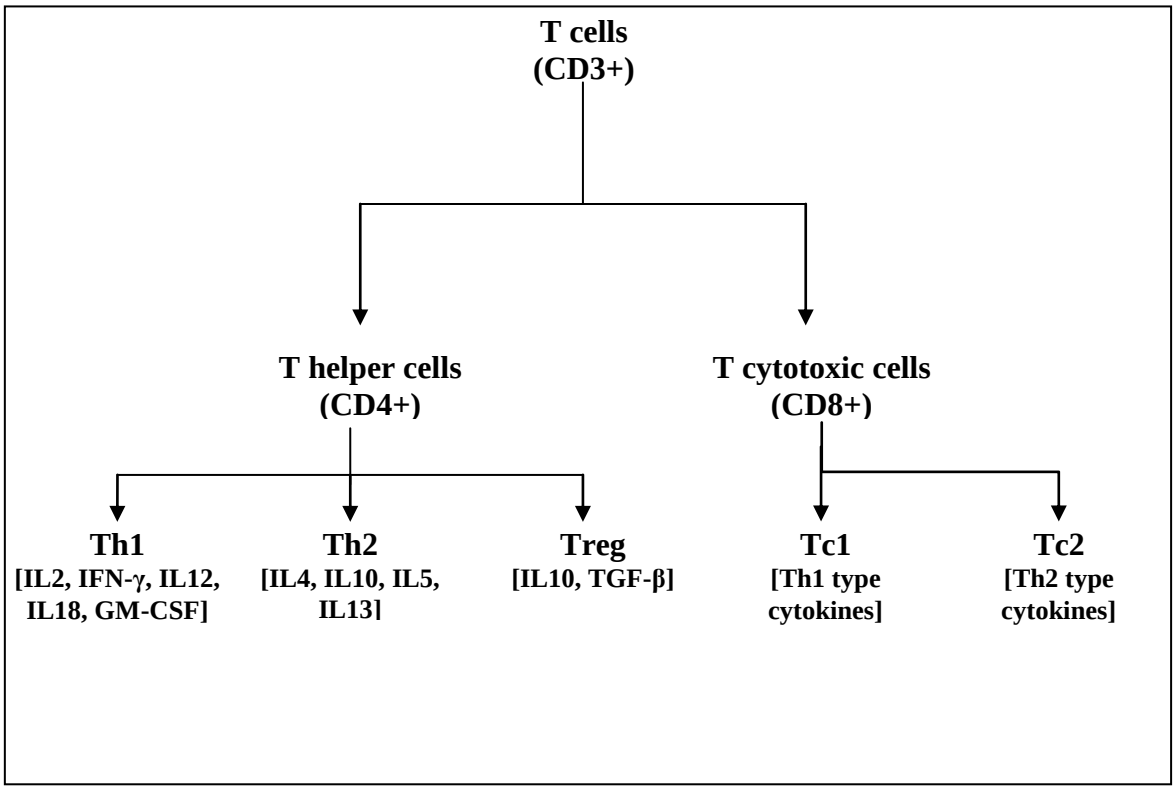
1.7.2 Cytokines

Cytokines are small molecular weight regulatory molecules that are involved in the initiation and maintenance of innate and aquired immunity. Cytokines play a major role in the host's immune response against cancer as well as a role in mediating cellular communications during the different stages of cellular growth, differentiation and regulation (Klein & Boon, 1993). Furthermore, cytokines are able to function in an antagonistic or synergistic manner by inhibiting or stimulating particular functions of other cytokines, respectively. Thus, a complex network of cytokine interactions exists in

vivo (Rakesh & Agrawal, 2005). Cytokines are divided into groups according to their structure or function. Appendix 2 shows six broad families of cytokines, defined by their structure, plus a seventh category comprising an assortment of unassigned molecules.

Various tumour types have been shown to produce cytokines in an unregulated way that will contribute to an ineffective host immune response (Sparano et al., 2004), (Inagaki et al., 2006). One such cytokine, TGF-β, which was first identified from cultured tumour tissue, hence the name, acts broadly to suppress cell mediated immunity (Pardali & Moustakas, 2007) and was found to be significantly overexpressed in HNSCC patients (Rosenthal et al., 2004). Further observations in HNSCC, as well as in many other cancers, have shown that tumour formation can significantly alter serum levels of certain Th1 and Th2 cell cytokines in the body. There appears to be an imbalance, however not exclusively, towards a Th2 cell cytokine profile (Neuner et al., 2002); (Kumar et al., 2006). Some of these Th2 cell cytokines, particularly IL10, suppress the cellular immune response, which is desirable in combating malignancies, thus permitting tumour growth (Pries & Wollenberg, 2006). The Th2 cell profile is characterised by the production of IL4, IL5, IL6, IL10, IL13 and GM-CSF while Th1 responses are characterised by the production of IFN-γ, IL2, IL12 and IL18. Another subtype of $CD4^+$ T helper cells differentiation is Treg cells, which have been shown to work through the release of IL10 and TGF-β in certain subtypes to confer their regulatory action (Bergmann et al., 2008); (Figure 9). Since Th1 and Th2 cell cytokines are believed to act antagonistically, a representative from each, i.e. IL12 and IL10, respectively, will be discussed further.

Figure 9: T cells classification showing T helper cells differentiation and their corresponding cytokines



Interleukin 10

IL10 is an 18 kDa non-glycosylated protein that is considered as an anti-inflammatory cytokine. IL10 is mainly secreted by monocytes and Th2 cells, and to a lesser degree, by B cells, macrophages, Treg cells and DC. IL10 modulates the function of several acquired immunity-related cells and is generally considered an immunosuppressive molecule although it does possess immunostimulatory properties too (Mocellin et al., 2004). IL10 was found to inhibit the transcription of the IL12 gene and thus suppressing the production of IFN-γ by Th1 cells (Wang et al., 1994), as well as regulating cellular proliferation and chemotaxis (Jinquan et al., 2000). IL10 also displays potent abilities to suppress the antigen presentation capacity of cells such as macrophages and DC (Kryczek et al., 2006). Moreover, IL10, as well as TGF-β, were found to play a major role in the generation and modulation of Treg cells and their function (Strauss et al., 2007b).

Conflicting reports regarding IL10 levels in patients with head and neck tumours exist. Several studies strongly suggested that patients with advanced HNSCC have significantly greater plasma and serum IL10 levels (Sparano et al., 2004), (Jebreel et al., 2007). IL10 has also been shown, through immunohistochemical localisation, to be significantly present in human oral and pharyngeal carcinoma cells (Chandler et al., 2002). On the other hand, other groups failed to detect IL10 levels in the serum of squamous cell carcinoma (SCC) and adenoid cystic carcinoma (ACC) patients, hence the inability to differentiate between normal controls and patients' IL10 levels (Druzgal et al., 2005), (Hoffmann et al., 2007). A multiplexed immunobead-based profiling of 60 biomarkers did not show a significant difference between serum concentrations of IL10 when patients with HNSCC were compared with a "smokers" control group, or to patients with no evidence of disease >3 years after their primary HNSCC was treated (Linkov et al., 2007). This can potentially be explained by the location of the primary tumour, which is usually included under "head and neck tumours" although each anatomical sub-site can potentially behave very differently. Other factors which might also contribute to the discrepancy in results from IL10 detection include different methods used to detect tissue, serum or plasma IL10 levels (commercial vs. in-house assays) and the relatively small number of samples studies.

IL10 levels in HNSCC patients have been used to assess the potency of novel DC vaccines which are currently under investigation (Prasad et al., 2008). Few studies have shown that the suppressive effect of IL10 is reversed by blocking its biologic action. Suppression of IL10 in PBMC cultures using neutralising antibodies has shown significant restoration of proliferative response to IL2 in one study (Avradopoulos et al., 1997) and a complete abrogation of suppression in another (Strauss et al., 2007b). However, IL10 has also been demonstrated to have potent anti-tumour activity. In animal models, Dorsey and colleagues demonstrated that systemic administration of recombinant human IL10 to animals bearing established, highly malignant, mammary tumours led to significant growth inhibition which was associated with increased numbers of $CD4^+$ cells, circulating Mig (monokine induced by IFN-γ) and IP10 (inducible protein 10 or

CXCL10) (Dorsey et al., 2002). In humans, data are limited due to the established effects of immunosuppression and associated toxicity, but patients with acute myelogenous leukaemia have shown an elevation in plasma levels of TNF-α and IL1 upon administration of IL10 (Tao et al., 2001) confirming the feasibility of using IL10 in immunotherapeutic protocols, although this might prove to be difficult until all the roles of IL10 are understood and issues about toxicity and delivery are resolved.

Interleukin 12

IL12 is a potent Th1 cytokine and plays a major role in stimulating a cell-mediated antitumour immune response, whilst inhibiting Th2 responses (Trinchieri, 2003). IL12 is a heterodimeric cytokine of 70 kDa that is mainly produced by dendritic cells, monocytes, macrophages, and B cells. IL12 stimulates the production of INF-γ and TNF-α from T and NK cells as well as the differentiation of naïve T cells into Th1 cells (Schmitt et al., 1994). IL12 also has an anti angiogenic role through the production of INF-γ that produces the chemokine IP10 which is a powerful inhibitor of tumour angiogenesis (Trinchieri, 2003), (Beadling & Slifka, 2006).

Ever since Fiorintino and colleagues showed that IL10 inhibits cytokine production by Th1 cells, including IL12, (Fiorentino et al., 1991) the two functionally distinct subsets, i.e. Th1 and Th2, have been shown to cross regulate each other (Coffman, 2006). Serum, cells and tissue studies from patients with HNSCC and other cancers have shown that levels of IL12 are suppressed and that of IL10 are increased suggesting an imbalanced *in vivo* cytokine environment that is unlikely to promote an effective cell-mediated anti-tumour response (O'Hara et al., 1998), (Smyth et al., 2003), (Jebreel et al., 2007).

The favourable role of IL12 in cancer patients has stimulated its use in immune-based therapies. Whether on its own or in conjunction with other treatments, IL12 has shown, in clinical and preclinical trials, encouraging results to support its incorporation in future therapies for HNSCC and other cancers (O'Malley et al., 2005). For example, a phase II clinical trial using intra-tumoural recombinant human IL12 (rhIL12) in 10 previously

untreated HNSCC patients led to a switch from a Th2 to a Th1 cytokine profile, induced NK cell infiltration in the tumour itself and modified the locoregional lymph node architecture (van Herpen et al., 2005). A follow up from this same study, albeit including 30 previously untreated HNSCC patients, showed that after intratumoral rhIL12 treatment for HNSCC patients, significant activation of B cells and the B cell compartment occurred, i.e. increased IFN-γ mRNA expression by B cells and a highly significant IgG subclass switch was seen in the plasma with more IgG1/ IgG4 and less IgG2, indicating a switch to Th1 phenotype. Moreover, the presence of increased tumor infiltrating B cells correlated with a better overall survival for these patients (van Herpen et al., 2008). Although little data are available for the effect of IL12 administration on patient survival and tumour progression in humans, murine and cell line studies have shown promising results.

Vaccination of a poorly immunogenic murine SCC tumour with fused dendritic-tumour cell hybrids and adjuvant IL12 demonstrated a significant decrease in tumour size and increased survival when compared with nontreated mice. The effect was not detected when either treatment was used alone (Lee et al., 2008b). Another combined treatment using IL12 gene therapy as well as external beam radiation generated potent antitumour immune responses against HNSCC and significantly increased necrosis (apoptosis) in an orthotopic murine model (Xian et al., 2005). Toxicities associated with systemic IL12 administration in humans have been reported (Hurteau et al., 2001); however, it is hoped that with the progress in combining molecular therapies with conventional modalities and the advances in nanotechnology and micro drug delivery systems that toxicity will diminish and efficacy will improve in order to reach the desired clinical outcome.

1.7.3 Regulatory T-cells

The seminal work of Sakaguchi and his team described $CD4^+$ T cells that continuously express the alpha chain of IL2 receptor (CD25) (Sakaguchi et al., 1995). These cells were shown to be important in inducing and maintaining peripheral self-tolerance, and actively preventing autoimmune diseases by the suppression of self-reactive T cells. A corollary

of these functions is that they are also thought to diminish T-cell immunity to TAA and play a major role in preventing successful vaccination and immune-based therapies (Zou, 2006).

The use of the surface activation CD25 as a specific marker for Treg cells is limited due to the fact that it is also up-regulated on activated effector T cells. However, it is possible to sub-divide $CD4^+$ T cells expression of CD25, as either low/ intermediate/ or high. The "high" group have been shown to express the Forkheadbox transcription factor P3 (FOXP3) in humans. These $CD4^+$ $CD25^{high}$ $FOXP3^+$ T-cells have a potent suppressive/regulatory capacity both *in vivo* and *in vitro* and are now commonly referred to as the naturally arising human Treg cells (Zou, 2006). In mice, the entire population of $CD4^+CD25^+$ T cells seems to confer regulatory activity, while in humans only the $CD4^+CD25^{high}$ population have a similar strong regulatory function, hence care must be taken when extrapolating between species (Baecher-Allan et al., 2005). The exact phenotype and function of Treg cells is still under intense investigation.

Sub-types, mode(s) of action and trafficking

The presence of different markers and levels of maturation of Treg cells, strongly suggest that regulatory T cells are not a homogeneous group, but that subtypes exist with distinct regulatory activities. Two major groups of Treg cells have been described; naturally occurring regulatory T cells "nTreg", and peripherally induced regulatory T cells "iTreg" which can themselves be subdivided (Table 5). When either subtype of CD4 Treg cells is stimulated by antigen, or just TCR and CD28 ligation, it can cause immune suppression, i.e. suppress autoimmune disease, tumour immunity or graft rejection (Sakaguchi, 2005). But these two main groups of Treg cells differ in their origin and mode of action, as well as their activation requirements and relative efficacy (Shevach, 2009).

Table 5: Subtypes and characteristics of $CD4^+$ Treg cells

	Natural Treg cells	Induced Treg cells	
	nTreg	**Tr1**	**Th3**
Phenotype	$CD4^+CD25^+FOXP3^+$	$CD4^+CD25^-IL10^+$	$CD4^+CD25^-TGF\text{-}\beta^+$
Origin	Thymus	Periphery	Periphery
Mechanism of suppression	Cell- Contact *in vitro*, Cell-Contact, soluble cytokines* (IL10, TGF-β), PD-1 pathway *in vivo*	IL10, TGF-β, IFN-γ	TGF-β, cell-contact*
Other characteristic markers	GITR, CTLA-4, CD127, Nrp1, LAG-3.	CTLA-4, CD122	CTLA-4
Depleting agents	CD25 mAb, denileukin diftitox, cyclophosphamide, GITR mAb, CTLA-4 mAb	IL10 mAb, TGF-β mAb, cyclophosphamide	TGF-β mAb, CTLA-4 mAb, cyclophosphamide

The current understanding of the different subtypes of Treg cells and their characteristics. * Potential mechanism of action. mAb: Monoclonal Antibody.

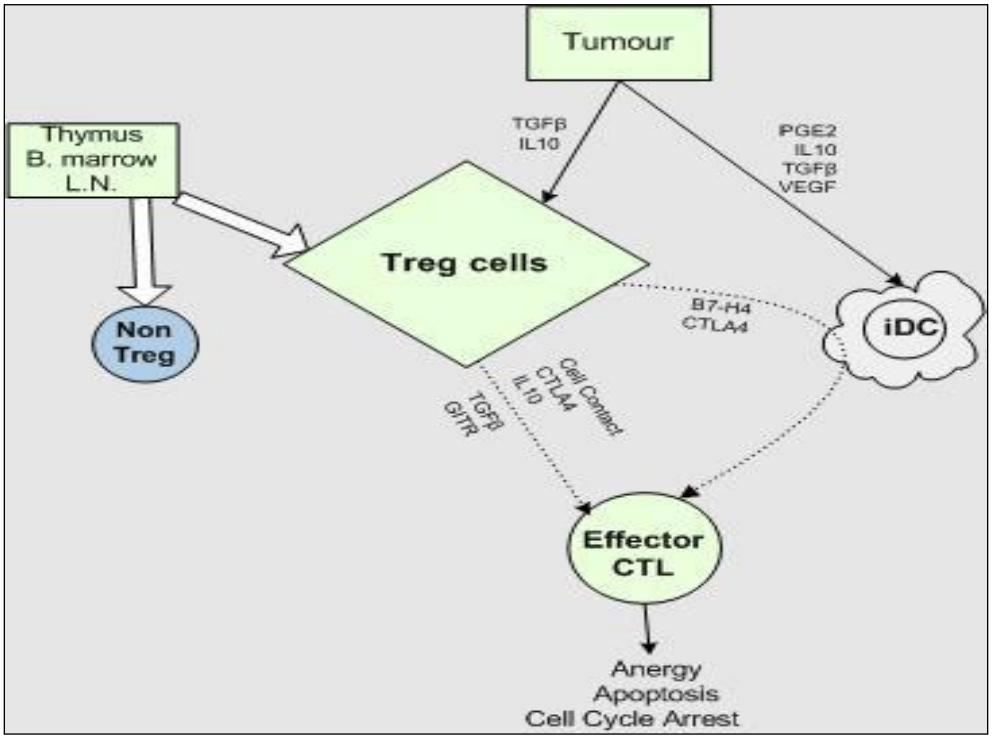
Natural Treg cells

nTreg cells are those commonly referred to in the literature as Treg cells and classically express the FOXP3 transcription factor (Hori et al., 2003), (Fontenot et al., 2003). Several other markers are also associated with nTreg, however, none of them is exclusively linked to the suppressive function of Treg cells, and they can certainly be expressed by activated $CD4^+$ cells which also express the CD25 marker, i.e. non-Treg cells. Such molecules include the glucocorticoid-induced tumour-necrosis factor (GITR) receptor (Ronchetti et al., 2004), Cytolytic T lymphocyte associated antigen 4 protein(CTLA-4) (Tang et al., 2004), low/negative expression of cell surface CD127

(Banham, 2006), (Liu et al., 2006b), Neuropilin-1 (Bruder et al., 2004), Lymphocyte Activation Gene (LAG-3) (Huang et al., 2004) and Inducable Co-Stimulator (ICOS) (Strauss et al., 2008).

nTreg cells follow the normal T cell development pathway via the thymus and, although expressing a typical alpha/beta T cell receptor (TCR), it is thought they escape deletion possibly through "leaky" clonal deletion (Schwartz, 2005). It is also not fully understood how nTreg are activated; although there is a definite need for TCR engagement, the presence of CD28 and cell-cell contact with antigen presenting cells (APC). It has also been shown that CTLA-4 expression intensifies the TGF-β signal at the point of contact, hence bringing about suppression of responding cells (Oida et al., 2006). Moreover, IL-2 expands nTreg cells and there is a dependency on IL2, both *in vitro* and *in vivo,* for activation and sustained $CD25^+$ expression (Furtado et al., 2002). It has been shown that these cells suppress the proliferation of other $CD4^+$ helper and $CD8^+$ effector T cells *in vitro* via a cell contact dependent (T-T interaction), dose dependent, and cytokine-independent mechanism (Shevach, 2000), (Gondek et al., 2005). However, *in vivo* the picture is more complex (von Boehmer, 2005). One recent study has shown that nTreg cells, through the expression of ICOS, the CD28 homolog, can induce diverse immune responses including the induction of Tr1 and Th2 cells (Strauss et al., 2008). Another observed mechanism by which nTreg cells exert their suppressive actions, is through the negative costimulator programmed death-1 (PD-1) mediated pathway after induction by NK cell-primed DC (Jinushi et al., 2007). This mechanism of suppression through APC adds to the many complex suppressive mechanisms by which nTreg can function *in vivo* (Fig 10).

Figure 10: Schematic representation of key Treg cells interactions



After their production and maturation either directly or after nonTreg activation, Treg cells are thought to be activated by the tumour itself through IL10 and TGFβ production, as well as through immature DC (i DC). Thereafter, Treg cells suppress effector cells by either direct cell contact or inhibitory molecules production. DC also play a suppressive role against effector T cells (CTL) through their immature subsets or IL10 production, having been stimulated by the tumour or by Treg cells (Dotted arrows represent the principal effects mediated by the Treg cell populations; Solid arrows represent the effects of tumour cells on Treg cells; White arrows show the origin and maturation of the Treg cell populations). L.N: Lymph Nodes.

Induced Treg cells

In comparison, iTreg cells can be divided into two further subgroups; Tr1 or $CD4^{+}IL10^{-}$ $FOXP3^{-}$ cells, and Th3 or $CD4^{+}TGF\text{-}\beta^{+}$ cells. Both subsets are generated from $CD4^{+}CD25^{-}$ precursors under the influence of cytokines and co-stimulatory milieu during the peripheral T-lymphocyte response to antigens (Battaglia et al., 2003). Their activation is better understood than nTreg cells, requiring MHC class II-bound ligands

alone (Chattopadhyay et al., 2005), unlike nTreg cells that need both TCR ligand and co-stimulation for full functional activation. iTreg exert their effect *in vitro* and *in vivo* predominantly, although not exclusively, through a cytokine-dependent mechanism, by secretion of inhibitory cytokines such as IL10 and TGF-β (Klein et al., 2003) that can also induce further iTreg differentiation (Seo et al., 2001), (Chen et al., 2005) and even nTreg differentiation (Carrier et al., 2007). Furthermore, other observations have shown that iTreg can also target and regulate innate immunity, by suppressing NK cell function *in vivo* in a cytokine dependent manner principally through TGF-β; this being demonstrated most convincingly in a mouse tumour model where the depletion of mouse T reg cells exacerbated NK cell proliferation and cytotoxicity *in vivo* (Ghiringhelli et al., 2005).

Other suggested regulatory cell subtypes include $CD8^+$ regulatory T cells. These are similar to $CD4^+$ T cells and are defined according to their specific markers or mode of action, e.g. $CD8^+CD25^+$ and $CD8^+IL10^+$ cells (Cosmi et al., 2003), (Wei et al., 2005). Indeed, $CD8^+$ Treg cells are reported to share the same phenotypic, functional features and possibly the mechanism of action of $CD4^+$ Treg cells in humans (Mahic et al., 2008); however, their role in tumour immunity is yet to be established.

The above mentioned data summarise the different subsets of Treg cells and their proposed modes of action, however the interrelation between these subtypes' remains poorly understood. Observations have suggested that nTreg cells are involved in the differentiation of iTreg *in vitro* (Dieckmann et al., 2002) and *in vivo* (Foussat et al., 2003). This process is thought to be important early in life reducing in adulthood when thymus functions regress, although the half life of nTreg cells is yet to be determined. In this context, the bone marrow is suggested as a "homing" compartment for Treg cells where they can proliferate and expand. This hypothesis (Wei et al., 2006), was based on observations regarding $CD8^+$ memory T cells (Becker et al., 2005), (Mazo et al., 2005) and the fact that more than 25% of all $CD4^+$ T cells are phenotypically and functionally Treg cells in both normal human and murine bone marrow (Zou et al., 2004).

Treg cell trafficking

Certain chemokine receptors and integrin molecules are implicated in this process; CXCL12 mediates bone marrow Treg cell trafficking (Zou et al., 2004), CCL22 facilitates Treg cell trafficking into human ovarian cancer (Curiel et al., 2004) while CCR7 and CD62L may mediate lymphoid homing of Treg cells (Lim et al., 2004), (Ochando et al., 2005). Zou summarised the non-exclusive mechanisms by which these cells concentrate in the tumour mass: i) active recruitment through chemokine release, e.g. CCL22 which binds to CC-chemokine receptor 4 expressed by Treg; ii) cytokine mediated differentiation of DC or myeloid suppressor cells into dysfunctional APC that mediate induction of a Treg phenotype and/or expansion of this cell type, and iii) direct cytokine-mediated T cell conversion into Treg cells by relatively high levels of TGF-β (Zou, 2006). The balance of these mechanisms in human tumours is not known, while the mode(s) of action of Treg cells, once accumulated at the tumour site, is similarly complex. With respect to clinical immunotherapy, identifying specific surface markers is of immediate interest since specific Treg cells can theoretically then be expanded in culture and subsequently used in immune-modulation in relevant clinical conditions.

Studies on HNSCC and other cancers

Since Sakaguchi and colleagues described Treg cells, evidence linking the activity of these cells with the anti-tumour immunity in many cancers has been emerging (Sakaguchi et al., 1995). Peripheral blood (Schaefer et al., 2005), lymph nodes (Viguier et al., 2004), tumour tissue (Curiel et al., 2004), ascites (Sasada et al., 2003) and pleural effusion (Barnett et al., 2005), are all examples of tissues in which Treg cells have been studied in patients with various tumours. Woo and colleagues were one of the first to report on Treg cells in human cancer. Their study, showed evidence of enrichment of TIL with $CD4^+$ $CD25^+$ T cells, assuming the whole group as Treg cells (Woo et al., 2001). This study was followed by a number of others confirming the presence, function and phenotypic characteristics of such cells in gastrointestinal malignancies (Sasada et al., 2003),

(Ichihara et al., 2003), breast and pancreatic tumours (Liyanage et al., 2002) and multiple epithelial malignancies (Wolf et al., 2003).

In Head and Neck cancer, Schaefer and colleagues were one of the first to study the frequency and characteristics of Treg cells in the peripheral circulation of patients with HNSCC using multicolour flow cytometry. This study used a relatively small number of patients (n=19) but nevertheless showed an increased proportion of Treg cells ($CD4^+CD25^+FOXP3^+GITR^+$) in PBMC of patients compared with normal controls. The results also showed an increased number of Treg cells in the PBMC of patients with active disease and, paradoxically, in those patients with no evidence of disease following curative therapy when compared with normal controls. The authors suggest that the latter observations support a hypothesis that the disrupted lymphocyte "homeostasis" caused in response to the tumour, demonstrated by the increased Treg cell number, does not normalise after curative treatment. Finally, the paper concluded that Treg cells are possibly responsible for the downregulation of TCR-mediated signalling in the $CD8^+$ and non-Treg $CD4^+$ T cells, thus hindering their effector and helper functions respectively (Schaefer et al., 2005). This conclusion was made after confirmation of the lower expression of zeta chain, which determines the ability of T cells to signal via TCR engagement (Whiteside, 2004), in the peripheral circulation of patients with HNSCC. The concept of an inverse correlation between Treg cells and $CD8^+$ effector T cells and their cytokine expressing cells Tc1 and Tc2 populations was further investigated by Chikamatsu and colleagues. This study similarly showed lower percentages of $CD4^+$ T cells and higher levels of $CD4^+CD25^{high}$ T cells in the peripheral blood of 42 HNSCC patients, confirming the altered immune status (Chikamatsu et al., 2007).

Further studies have shown Treg cells to be localised to tumour tissue by confirming their enrichment in TIL as compared with PBMC of HNSCC patients (Albers et al., 2005), (Strauss et al., 2007b). These reports have demonstrated that intratumoural $CD4^+CD25^{high}$ $FOXP3^+$ T cells have phenotypic and functional characteristics that are different from those of circulating $CD25^{high}$ Treg cells. Treg in TIL were shown to express IL10 and TGF-β, were $GITR^+$ and mediated a more potent suppression of effector cells

($CD3^+CD8^+$) than Treg cells from PBMC. In addition to $CD4^+CD25^{high}$ $FOXP3^+$ Treg cells, a recent study has shown TIL from HNSCC patients to contain the Tr1 subset of Treg cells i.e. $CD4^+CD25^-IL10^+TGF\text{-}\beta^+$ (Bergmann et al., 2008). This study, which included 26 HNSCC patients and 10 controls, has also demonstrated that the expression of the suppressive cytokines, i.e. IL10 and TGF-β, was significantly higher in TIL-derived than PBMC-derived Tr1 cells, and that Tr1 cells expand during tumour progression and also after cancer therapy. This last observation that Tr1 cells, and as previously reported nTreg cells as well, do not normalise after curative treatment, may have an implication towards the future role of Treg cells in disease progression, recurrence and prognosis.

Treg cell observations in HNSCC patients are largely shared by other tumours. Curiel and colleagues, in one of the largest reported human Treg studies involving a cohort of 104 ovarian cancer patients in which $CD4^+CD25^+$ T cells expressing FOXP3, GITR and CTLA-4, inhibited TAA-specific immunity *in vitro* and *in vivo,* and permitted tumour growth. The same study also demonstrated that Treg cells migrate into tumours expressing the CCR4 receptor *in vivo* and *in vitro* using the CCR4 ligand - chemokine CCL22. Furthermore, tumour Treg cells numbers correlated with poor survival and were shown to be significant predictors of death even after controlling for disease stage and surgical debulking (Curiel et al., 2004). This study was the first to report a link between Treg numbers and patient survival. Similarly, the work of Kono and colleagues in gastric and oesophageal cancer showed that an increase in specific $CD4^+CD25^{high}$ Treg cells, albeit in the peripheral circulation, correlated with tumour stage, poorer survival rates and recurrence (Kono et al., 2006). One difference between the observations by Kono and colleagues and those reported in HNSCC patients, was that Treg numbers were reduced significantly after 2 months of curative treatment; perhaps reflecting some tissue specific differences.

On the other hand, Tartour and his group (Badoual et al., 2006) showed that tumour-infiltrating $CD4^+CD69^+$ T cells positively correlated with a better prognosis in patients with HNSCC. In their study of 84 patients, infiltration by $CD4^+FOXP3^+$ T cells and

tumour size were shown to be the only two significant prognostic factors related to better locoregional control. These results are supported by conclusions from studies of lymphoma patients (Carreras et al., 2006), (Lee et al., 2008a) and, more recently, colorectal cancer patients (Salama et al., 2009) which showed that higher numbers of tumour infiltrating $CD4^+FOXP3^+$ Treg cells to be associated with better survival. The apparent confusion regarding the role of Treg in patient prognosis could be explained by the heterogeneity of $CD4^+$ T cells and indeed Treg cells or the nature of the tumour type, or some combination of the two. The heterogenecity of patients, i.e. untreated or previously treated, may as well influence data outcome. Further studies are needed to establish a clearer role for Treg cells as a prognostic marker.

Lymph node enrichment with Treg cells has also been documented in recent studies. Linehan and his group were one of the first to show that tumour-draining lymph nodes (TDLN) contain Treg cells (Liyanage et al., 2002). In line with these results, Viguier and colleagues investigated human metastatic melanoma lymph nodes and reported their enrichment with $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells with inhibitory functions; contributing to the local immunosuppressive status (Viguier et al., 2004). However, contrasting results emerged from the ovarian cancer study described previously (Curiel et al., 2004) where TDLN showed similar figures of Treg cells enrichment as lymph nodes obtained from non-cancer patients. Furthermore, the accumulation of Treg cells in TDLN, were less frequent in later stages of the disease, showing preferential recruitment of Treg cells to the tumour mass and associated ascites. The discrepancy between the above mentioned studies is not clear, however the nature of the specific tumour is most likely to be important, i.e. what factors are released directly or indirectly and hence the specific subset of Treg cell recruited. Only Albers and colleagues have reported that the levels of Treg cells in non-involved lymph nodes from tumour patients were similar to those in PBMC from HNSCC patients (Albers et al., 2005). However, in this later study, the cells measured and compared between PBMC, TIL and non-involved lymph nodes were only activated T helper cells, i.e. $CD4^+CD25^+$, which does not represent Treg cells but could include them, hence the caution with interpreting the results of this particular study.

Treg cells obviously do not act in isolation and, unsurprisingly, groups have studied associations with other key components of the immune response. The correlation between DC, the most potent APC, and Treg cells was studied in HNSCC patients. In a cohort of 45 patients, Sakakura and colleagues, using flow cytometry, first confirmed the increase in the percentage of circulating Treg cells in these patients (Sakakura et al., 2006). Secondly, they demonstrated the altered nature of DC in the same group of patients; i.e. both a lower percentage of myeloid DC and significantly lower expression of HLA-DR, a maturation marker, on the surface of the cells that were present. This immaturity of DC was also shown to be related to tumour progression as HLA-DR expression was further reduced on plasmacytoid DC with advanced disease, compared with DC taken from patients with early stage disease. Finally, a significant inverse correlation was found between DC and Treg cells, indicating their imbalance and suggesting that immature DC promote the appearance of Treg cells.

Conclusions regarding our current understanding of the involvement of Treg cells in patients with HNSCC are summarised in Table 6. However, questions regarding most aspects of Treg cell biology remain unanswered and their role in malignancy needs clarification. The merit of involving Treg cells in immunotherapeutic modalities is certainly valid, but until a better understanding of the roles that Treg cells play in anti-tumour immunity in cancer patients, such modalities of treatment will remain far from perfect. The current status of this approach is further discussed.

Table 6: Summary of current observations of Treg cells in HNSCC

Observations	Tissue & Cohort size	References
1. Increased prevelance of nTreg cells in TIL and PBMC of patients with active disease and patients with no evidence of disease when compared to controls.	Tumour, PBMC (N=24; N=24; N=35)*	(Schaefer et al., 2005); (Albers et al., 2005); (Strauss et al., 2007a)
2. Increased expansion of Tr1 cells and their inhibitory cytokines in TIL when compared to PBMC of patients with active disease and patients with no evidence of disease.	Tumour, PBMC N=26	(Bergmann et al., 2008)
2. Increased Treg cells in TIL and PBMC of nasopharyngeal carcinoma patients when compared to controls.	Tumour, PBMC N=56	(Lau et al., 2007)
3. Enrichment of regulatory T cells expressing Foxp3 within the tumour tissue compared to normal healthy and inflamed tissues.	Tumour N=?	(Cao Y, 2005)
4. Inverse correlation of Treg cells with total DC numbers.	PBMC N=45	(Sakakura et al., 2006)
5. Inverse correlation of Treg cells with total CD8+ T cells and their Tc1 and Tc2 subsets.	PBMC N=42	(Chikamatsu et al., 2007)
6. nTreg cells kill autologous $CD8^+$ T cells by Fas-mediated apoptosis	PBMC N=25	(Strauss et al., 2009)
7. Treg cells are responsible for downregulating TCR-mediated signaling in CD8+ and non Treg CD4+ T cells.	PBMC N=24	(Schaefer et al., 2005)

8. CD4+ FOXP3+ T cells positively correlate with a better locoregional control in patients with HNSCC.	Tumour N=84	(Badoual et al., 2006)

This table is a summary of selected observations from key recent studies on Treg cells in HNSCC. * The number of patients follow the references opposite respectively.

Future Implications and Immunotherapy

Soon after Sakaguchi and colleagues described Treg cells, they published a study in which $CD4^+CD25^+$ T cells were depleted in a mouse tumour model and this resulted, as anticipated, in strong induction of tumour immunity (Shimizu et al., 1999). This approach added a novel dimension to tumour immunotherapy i.e. targeting Treg cells. Methods used to achieve that, apart from depleting Treg cells, include blocking their trafficking, blocking their differentiation and signalling, and finally targeting the suppressive molecules that Treg cells use to confer their regulatory/ suppressive function or a combination of two or more of these strategies which were reviewed by (Zou, 2006).

Different approaches have been used to deplete Treg cells in mice and humans. *In vivo* treatment with a CD25- specific antibody in mouse melanoma (Shimizu et al., 1999), leukaemia (Onizuka et al., 1999) and colorectal tumour models (Casares et al., 2003), as well as treatment with denileukin diftitox, a recombinant IL-2 diphtheria toxin conjugate DAB^{389}IL-2 that selectively eliminate Treg cells in human PBMC (Dannull et al., 2005), managed to deplete Treg cells effectively and showed enhanced tumour immunity and even tumour regression in both mice and humans (Prasad et al., 2005), (Barnett et al., 2005). Chemotherapeutic drugs such as cyclophosphamide and cyclosporine have also been shown to reduce Treg cells numbers and function *in vivo* in mouse tumour models (Lutsiak et al., 2005), (Ichihara et al., 2003). One recent study involving 56 breast cancer patients showed that pathologic complete response to neoadjuvant chemotherapy is associated with the disappearance of tumor-infiltrating $FOXP3^+$ Treg cells, measured by immunohistochemistry, suggesting that chemotherapy induces an antitumour immune response combining the absence of immunosuppressive $FOXP3^+$ cells and the presence of a high number of $CD8^+$ T cells and cytotoxic cells (Ladoire et al., 2008). However, this observation is in contrast to preliminary data from HNSCC patients where Treg cells tended to expand *in vitro* and *in vivo* in the presence of chemotherapeutic agents, a point which will be discussed later (Bergmann et al., 2008).

Collectively, the above methods have been used alone or, more often, in conjunction with other conventional immunotherapeutic modalities. For example, elimination of Treg cells through a single dose of denileukin diftitox followed by vaccination with RNA-transfected DC significantly improved the stimulation of tumour-specific T cell responses in renal cell carcinoma patients (n=7) when compared with vaccination alone (n=4). No apparent clinical toxicities or evidence of autoimmunity were observed, however Treg depletion was transient (< 2 months) and the use of denileukin diftitox was restricted to a pre-vaccination setting as it has also abrogated DC-mediated activation of T cells in an *in vitro* assay (Dannull et al., 2005). Furthermore, another study combined Treg cell depletion by anti-CD25 mAb with an IL21 secreting cellular vaccine (a TS/A mammary adenocarcinoma cell genetically modified to secrete IL21; a member of the IL2 cytokine family that promotes proliferation, cytotoxic activity and IFN-γ production by murine and human $CD8^+$ effector T cells) (van Leeuwen et al., 2002). The results showed an induction of a strong anti-tumour effect caused by $CD8^+$ cells, NK cells and IFN-γ; and induced long-term protective immunity in a mouse bearing TS/A parental cell micrometastases model (Comes et al., 2006) that could not be established using a single modality alone. Another study used multimodal therapy to eliminate well established lymphoma, melanoma and colon adenocarcinoma murine tumour cell lines (Kudo-Saito et al., 2005). A viral vaccine given with a concomitant use of anti-CD25 mAb to deplete Treg cells yielded an antigen-specific immune response and improved T-cell immune response specific for a self antigen as well as those specific for a non-self antigen. However, external beam radiation was needed to eliminate the tumour as Treg depletion and the vaccine were not sufficient to stimulate the immune system to eradicate the established tumours. The study also showed the reduction of $GITR^+$ / $CTLA\text{-}4^+$ Treg cells and an increase in the percentage of activated DC; again supporting an important link between these cells as suggested previously.

Other potential strategies in targeting Treg cells can be achieved by blocking the trafficking of these cells through CCL22- specific antibody or by targeting the regulatory molecules associated with Treg cells such as FOXP3, GITR, CTLA-4, TGF-β and IL10. Treatments with GITR and CTLA-4-specific antibodies have been used in mouse models.

GITR-specific antibody induced tumour regression in mice bearing MethA- induced sarcoma and colon carcinoma (CT26) (Ko et al., 2005), while treatment with CTLA-4-specific antibody not only caused tumour regression but also increased survival in mice bearing MethA- induced sarcoma (Espenschied et al., 2003). In humans, blocking CTLA-4 *in vivo* resulted in objective tumour regression or stabilisation of the serum tumour marker CA125 in patients (n=3 out of 14, 21%) who were vaccinated with HLA-A2 restricted peptide or irradiated autologous GM-CSF-secreting tumour cells in human metastatic melanoma and metastatic ovarian carcinoma trials, respectively (Phan et al., 2003), (Hodi et al., 2003). However, this treatment resulted in a severe, but manageable, autoimmune diseases in these patients (n=6 out of 14, 43%). This established side effect of depleting Treg cells or associated molecules, also been observed in animal studies (Piccirillo & Shevach, 2001), (Stephens et al., 2001). Indeed, a variety of autoimmune diseases such as thyroiditis, insulinitis, gastritis and autoimmune diabetes have all been associated with Treg cells function and depletion (Linehan & Goedegebuure, 2005). Another set back in depleting Treg cells is that they soon recover in number unless followed by other immune or non immune therapies. In addition, the anti-CD25 mAb also depletes activated tumour-reactive $CD25^+$ effector T cells that may be counter-productive. Moreover, Treg cell elimination did not produce tumour regression in all mouse and human trials (Hodi et al., 2003), (Ko et al., 2005). One explanation for these observations is that other suppressive cells could be functioning in that particular tumour condition, such as the $CD8^+$ suppressor cells, NK-T cells and/or γδT cells (Terabe & Berzofsky, 2004). Another explanation points to the dose, methods and combinations of the different modalities used to target Treg cells, emphasising the need to establish well-controlled studies to optimise treatment (Zou, 2006).

Another, more recent, approach to tackle Treg cells was with the use of the CD25-directed immunotoxin; RFT5-SMPT-dgA. This immunotoxin was shown to mediate a transient, partial reduction in Treg cell frequency and number *in vitro*; after incubation with PBMC, and *in vivo*; after injecting 6 patients with metastatic melanoma. This study also suggested that comprehensive eradication of human Treg cells *in vivo* may require

the ability to target and eliminate $FOXP3^+$ $CD4^+$ T cells expressing both high and low levels of CD25 (Powell et al., 2008).

Almost all the data regarding the use of Treg cells in immune-based therapies are conducted in non HNSCC tumour models, which is surprising due to the prevalence of other *in vivo* studies. It is highly likely that the lessons learned from these studies could be applied in tumours of the head and neck; however, specific trials are also needed in HNSCC models to account for tumour related factors.

Stimulation of immune reactivity is a valid and tempting option for treating HNSCC; however, to achieve this goal one needs first to understand and overcome immune suppression induced by HNSCC. Multimodal therapy approach incorporating the manipulation of Treg cells, as discussed above, might prove to be beneficial. In HNSCC multiple treatment combinations, although not involving Treg cells manipulation as yet, are gaining support in clinical practice.

In summary, as our understanding of the failure of immune surveillance to control tumour progression and tumour escape mechanisms is improving, specific immunotherapy protocols are gaining wider approval. Targeting Treg cells is indeed one of the promising recent approaches to improve the efficiency of anti-cancer treatment modalities. Its use in conjunction with other traditional tumour therapy and conventional immunotherapy reflect that the complexity of the host-tumour interactions and the fact that immunodeficiency in HNSCC is multifactorial, can only be overcome by the combination of different treatment modalities.

1.8 Study Aims

1.8.1 Background

HNSCC, in common with most other tumours, results in a suppressed immune system, with an altered serum cytokine profile and immune cells that function aberrantly. It is still not clear whether these alterations simply reflect a local interaction between tumour cells and the host immune system, whether they represent local alterations causing a systematic change, or whether patients with cancer have a systematically altered immune system.

Recent observations at the molecular level in patients with HNSCC have triggered this study. One of these observations is that immune responses in cancer patients with advanced disease are biased, however not exclusively, towards a Th2 cytokine profile (Neuner et al., 2002), (Kumar et al., 2006). The other finding was that regulatory T cells (Treg cells), a heterogeneous population of cells, have been associated with suppressive activity against tumour-specific T cell responses (Zou, 2006). The exact roles of the Th1/Th2 immbalance and the prevelance of Treg cells in HNSCC patients are not fully understood; neither are their association with tumour progression, survival or prognosis. Other observations from the majority of studies on HNSCC patients have highlighted a lack of homogenecity in patients' characteristics, with a mixture of untreated and previously treated patients which can potentially influence the interpretation of the results in these studies.

In this study, it is hypothesised that the tumour in patients with HNSCC is causing a degree of immune alteration. This alteration is manifested by the skewing of the cytokine profile to produce more IL10 and less IL12, and altered immune cells in favour of producing more $CD4^{+}CD25^{high}$ "Treg cells". Hence, the hypothesis that those tumours are a major source of interleukin IL10, and are mainly responsible for the induction of Treg cells. It was decided to investigate the changes in the peripheral circulation of IL10 and IL12 levels as well as the numbers of circulating Treg cells. It is also hypothesised that

elevated levels of both IL10 and $CD4^{+}CD25^{high}$ "Treg cells" in HNSCC patients, as biomarkers of the impaired anti-tumour immune response, will correlate with tumour progression and poorer survival in such patients and will normalise as soon as the tumour load is removed.

1.8.2 Objectives of the study

i) To determine serum IL10 and IL12 levels in conjunction with the percentage of Treg cells in the peripheral blood mononuclear cells (PBMC) of a cohort of newly diagnosed HNSCC patients and to investigate the relationship of these levels with key clinicopathological parameters including tumour stage, size, lymph node presence, metastasis, recurrence and survival, after a period of follow up.

ii) To determine the effect of treatment (surgery and/or chemoradiotherapy) on serum levels of IL10 and IL12 and the percentage of circulating Treg cells by comparing pre-treatment levels with post-treatment levels of the immune parameters studied.

iii) To determine whether serum IL10, IL12 or percentage of circulating Treg cells can predict outcome in HNSCC patients and have potential as new prognostic markers for this disease.